Universitat Autònoma de Barcelona

Assessing the carcinogenic risk of water disinfection by-products. Use of in vitro models to evaluate halonitromethanes and haloacetic acids

Alícia Marsà Salvany

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Facultat de Biociències
Departament de Genètica i de Microbiologia
Grup de Mutagènesi

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Alícia Marsà Salvany

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Assessing the carcinogenic risk of water disinfection by-products. Use of *in vitro* models to evaluate halonitromethanes and haloacetic acids

Dissertation respectfully submitted by

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To Universitat Autònoma de Barcelona in partial fulfilment of the requirements for the degree of Doctor of Philosophy, as per the Doctorate Program in Genetics

Under the supervision of Dr. Ricard Marcos Dauder, Dr. Alba Hernández Bonilla and Dr. Constanza Cortés Crignola

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ABSTRACT

Disinfection by-products (DBPs) are all those chemical species that are formed during the disinfection of water. To date, more than 600 species of DBPs have been identified in chemically-disinfected waters all over the world. Although epidemiological studies show a clear link between cancer, especially bladder cancer, and DBPs exposure, none of the individual species alone has shown sufficient carcinogenic potency to account for the increased cancer risk.

To date, several different models and protocols have been used, attempting to establish the specific genotoxic and carcinogenic properties of individual DBP species. Still, most *in vitro* hazard evaluations have focused on acute exposures to relatively high concentrations of these chemicals, followed by an extrapolation to low concentrations. The lack of a more systematic methodology to assess the carcinogenic potential of DBPs, generated gaps in the literature that must be resolved. Additionally, current assessment methods led to results that do not reflect real exposure scenarios.

In this Thesis we propose the application of an *in vitro* carcinogenic risk assessment methodology based on the evaluation of a wide range of cell transformation markers, whereby we analyse the carcinogenic potential and long-term effects of two DBPs groups, HNMs and HAAs. The principal advantage of such methodology is the possibility to analyse prolonged exposures to more realistic concentrations of DBPs, thus, setting a more realistic exposure model.

Results from our first study indicate that HNMs are not capable of inducing a tumoural transformation in human pulmonary cells after an 8 weeks' exposure, neither to prompt exposed cells to induce or enhance tumoural growth. The evaluation of fibroblasts grown in contact with the CM of exposed cells, emulating the tumoural stroma, showed that in the exposure period stromal cells did not acquire the capacity to induce tumoural growth.

Evaluation of MMPs secretion determined that neither exposed cells nor stromal cells could secrete these proteinases that would facilitate tumoural migration and metastasis.

Our second study showed that although all three mono-HAAs appeared capable of inducing oxidative DNA damage after acute exposures, non-cytotoxic concentrations do not appear to be genotoxic after 8 weeks of exposure. On the contrary, cells long-term exposed to non-cytotoxic concentrations of the HAAs developed resistance to DNA-oxidising agents. *In vitro* long-term carcinogenicity assessment of HAAs proved them unable to initiate a tumorigenic transformation in urothelial cells neither in cells emulating the tumoural stroma. In addition, in any case cells were capable of inducing nor enhancing tumoural growth.

Altogether, we conclude that the use of cell transformation assays, based on prolonged exposures to low concentrations, emulating a more realistic exposure scenario, is a more consistent manner to assess the carcinogenicity of DBPs. Additionally, analyses performed in this Thesis suggest that the increased cancer incidence linked to DBPs exposure cannot be attributed to HNMs nor HAAs.

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LIST OF ABBREVIATIONS

BAA	Bromoacetic acid
BCNM	Bromochloronitromethane
BDCM	Bromodichloromethane
BDCNM	Bromodichloronitromethane
BEMX-1	(*E*)-2-chloro-3-(bromochloromethyl)-4-oxobutenoic acid
BEMX-2	(*E*)-2-chloro-3-(dibromomethyl)-4-oxobutenoic acid
BEMX-3	(*E*)-2-bromo-3-(dibromomethyl)-4-oxobutenoic acid
BIAA	Bromoiodoacetic acid
BMX-1	3-chloro-4-(bromochloromethyl)-5-hydroxy-2(5H)-furanone
BMX-2	3-chloro-4-(dibromomethyl)-5-hydroxy-2(5H)-furanone
BMX-3	3-bromo-4-(dibromomethyl)-5-hydroxy-2(5H)-furanone
BNM	Bromonitromethane
CAA	Chloroacetic acid
CDBM	Chlorodibromomethane
CM	Conditioned medium
CNIO	Centro Nacional de Investigaciones Oncológicas (Spanish National Cancer Research Centre)
CNM	Chloronitromethane
DBCM	Dibromochloromethane
DBNM	Dibromonitromethane
DBCNM	Dibromochloronitromethane
DBPs	Disinfection by-products
DBAA	Dibromoacetic acid
DCAA	Dichloroacetic acid
DCNM	Dichloronitromethane
DIAA	Diiodoacetic acid
DCF	2’, 7’– dichlorofluorescein
DCFH	2’, 7’– dichlorofluorescin
DCFH-DA	6- carboxy-2,7’-dichlorodihydro-fluorescein diacetate
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DSBs	DNA double strand breaks
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
EMX	(*E*)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid
EPA	Environmental Protection Agency
EPAA	European Partnership for Alternative Approaches to Animal Testing
EU	European Union
FBS	Foetal bovine serum
FPG	Formamidopyrimidine DNA glycosylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF	Gelbond® film
GSTT1-1	Glutathione S-transferase theta 1
HAAs	Haloacetic acids
HAA5	Sum of CAA, DCAA, TCAA, BAA, DBAA
HNMs	Halonitromethanes
IAA	Iodoacetic acid
IARC	International Agency for Research on Cancer
ICO	Institut Català d’Oncologia (Catalan Institute of Oncology)
IDCM	Iododichloromethane
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
MET	Mesenchymal to epithelial transition
MMPs	Matrix metalloproteinases
MPRs	Multidrug resistance proteins
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone
NDMA	N-nitrosodimethylamine
NEAA	Non-essential amino acids
NFE2L2 (NRF2)	Nuclear factor, erythroid 2 like 2
NOM	Natural organic matter
OECD	Organization for Economic Co-operation and Development

Ox-MX	(*Z*)-2-chloro-3-(dichloromethyl)-butenedioic acid
PBS	Phosphate-buffered saline
PNKP	Polynucleotide kinase 3'-phosphatase
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A
Red-MX	3-chloro-4-(dichloromethyl)-2(5H)-furanone
ROS	Reactive oxygen species
SEM	Standard error of the mean
TBNM	Tribromonitromethane
TCAA	Trichloroacetic acid
TCNM	Trichloronitromethane or chloropicrin
THMs	Trihalomethanes
TTHM	Total trihalomehtanes
UNICEF	United Nations International Children's Emergency Fund
UV	Ultraviolet light
WHO	World Health Organisation
XRCC3	X-ray repair cross complementing 3
ZMX	(*Z*)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid

1. INTRODUCTION

1. INTRODUCTION

1.1. Water disinfection

Throughout history, waterborne infectious diseases have been a great health concern, being one of the principal causes of death worldwide. Some of the most relevant are typhoid fever, cholera, amoebic dysentery, gastroenteritis, schistosomiasis, giardiasis, cryptosporidiosis, legionellosis and viral infections, which altogether still cause more than 2.5 million deaths per year (Unicef & WHO 2015).

First water disinfection recommendations date back to 4,000 BC, with regular disinfection using modern chemical methods dating from the beginning of the 20th century in the town of Middelkerke, Belgium. The first chemical used for disinfection was ferric chloride mixed with calcium hypochlorite, forming hypochlorous acid (White 1999). Posteriorly, the use of chemical disinfection methods raised later in the 20th century during the cholera epidemic outbreaks.

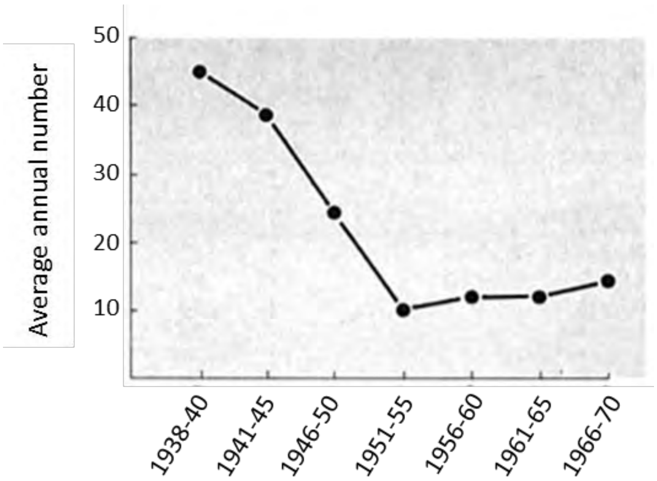

Figure 1. Average annual number of waterborne outbreaks between 1938 and 1970 in USA (Craun & McCabe 1973).

The subsequent application of water disinfection protocols was of great importance to control the number of deaths from typhoid fever, which decreased from 36 per 100,000 inhabitants in 1900 to only 8 cases in the United States by 1970. Similar outcomes happened with the outbreaks of other waterborne infectious diseases during the first half of twentieth century (Figure 1). Later on, water chemical disinfection became ubiquitous (Craun & McCabe 1973).

Despite current knowledge in water disinfection and the existence of many disinfection alternatives suiting the characteristics of diverse regions, recent data shows that even if 91 percent of the global population uses an improved drinking water source, as much as 663 million people still lack improved drinking water, and it is estimated that 2.4 billion people globally still use unimproved sanitation facilities (Figure 2).

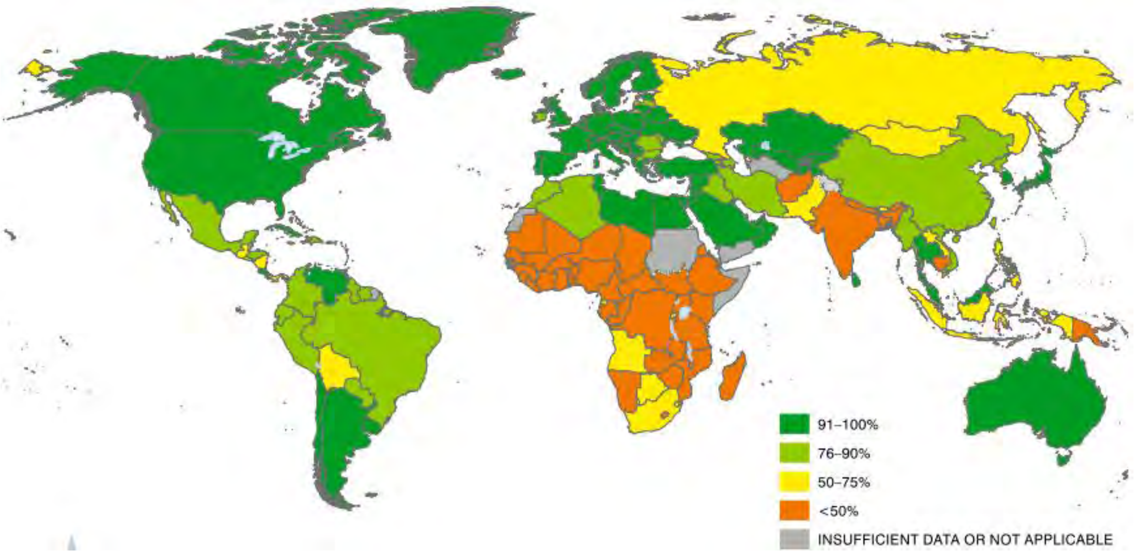

Figure 2. Proportion of the population using improved sanitation facilities in 2015 (Unicef & WHO 2015).

These data make evident that water disinfection is still a serious challenge for governments and regulatory organisms around the world.

1.1.1. Current disinfection methods

Nowadays, modern water supply systems require the use of one or, often, more water disinfectants. Habitually, there are two stages when disinfectants are added. The first one, takes place early in the disinfection process in water treatment plants, and it is known as primary disinfection. A second stage is required in most cases, where a secondary disinfectant is added to the distribution system.

There are several water disinfection methods which are regularly used according to different parameters such as the characteristics of the water of each region, along with economic and social factors. The efficiency of each method against pathogens may vary depending on the characteristics of the water source and must be monitored and controlled. Other factors to consider are, for instance the costs and risks of production and transportation. The most relevant disinfection methods currently used and their main advantages and limitations are summarised in Table 1 and briefly described as follows:

a) Chlorine

Since the first use of chemical disinfection procedures, chlorination has been the most common method to disinfect water (National Research Council 1987). Usually, chlorine is a general term that refers to several chemicals including elemental chlorine and sodium and calcium hypochlorite (Black & Veatch 2010).

Disinfection method	Advantages	Limitations
Chlorine	Simple to use and produce	Taste and odour issues Ineffective against some pathogens as *Cryptosporidium*
Ozone	Highly effective Capable of destroying organic micropollutants	Requires complex and expensive equipment Its residual is insufficient for long lasting distribution systems
Chlorine dioxide	More effective than chlorine at higher pH Less odour and taste changes	Unstable and sensible to temperature, pressure and light
Ultraviolet light	Highly effective for protozoa and bacteria	Less effective for viruses than chlorine No residual for distribution systems
Chloramines	Less odour and taste changes	Less effective

Table 1. Advantages and limitations of the main disinfection systems. Modified after US EPA water treatment manual (US Environmental Protection Agency 2011).

Chlorine is a strong oxidising agent, highly effective eliminating most microorganisms from water. Its wide use can be explained by the low costs that it represents, and the convenience of its production, transportation and use. On the other hand, chlorine is a gas at room temperature, which may generate safety problems. Another downside of the use of chlorine is the decrease of the disinfection efficacy as the pH of the water source increases, and it is not effective against some pathogens as *Cryptosporidium* (US Environmental Protection Agency 2011).

When either source of chlorine is employed, chemical reactions in the water produce hypochlorous acid, which dissociates to form hypochlorite, known as free available chlorine, and hydrogen ions, with a pK_a of 7.5:

$$Cl_2(g) + H_2O \rightarrow HOCl(aq) + H^+(aq) + Cl^-(aq)$$

$$NaOCl + H_2O \rightarrow HOCl(aq) + Na^+(aq) + OH^-(aq)$$

$$Ca(OCl)_2 + 2H_2O \rightarrow 2HOCl(aq) + Ca^{2+}(aq) + 2OH^-(aq)$$

These reactions are influenced by the water's pH, temperature and other soluble elements present in water (US Environmental Protection Agency 2011).

b) Ozone

Ozone (O_3) is a more effective disinfectant than chlorine, and its use has increased in the past years, especially in Europe, since it does not lead to any special taste or odour in water (Von Gunten 2003). Although being the most effective chemical disinfectant, currently it is mostly used in combination with other disinfectants. Downsides of its use such as its short half-life and its high reactivity make it necessary to produce it on-site, with the potential hazards and toxicity associated with its generation (Tomiyasu et al. 1985).

c) Chlorine dioxide

Chlorine dioxide (ClO_2) is a less common way of disinfection, mainly due to the large quantity that is required to treat waters with high levels of organic matter. It is highly volatile, and it must be generated on-site due to its instability and sensibility to temperature, pressure and light.

Despite the impossibility to transport it, ClO_2 is a very efficient biocide, and it is used in some cases for wastewater treatment, food processing and for secondary disinfection (US Environmental Protection Agency 2011).

d) Ultraviolet light

Ultraviolet (UV) disinfection became popular in the mid-1950s due to the improvement it provides regarding water taste and odour when compared to chlorine (Black & Veatch 2010). UV also represents lower costs than other disinfection systems, and it’s highly effective in eliminating bacteria, however, it can’t be used for the disinfection of distribution systems.

e) Chloramines

The case of chloramines is slightly different. They are formed in waters when chlorine reacts with ammonia under controlled conditions. Different types of chloramines will appear according to the ratios of chlorine to ammonia and the pH, but processes tend to optimise the formation of monochloramine. Chloramines are generally used for secondary disinfection as they are less effective than chlorine in eliminating pathogens, however, their stability is much higher. As chlorine dioxide, chloramines must be generated on-site (US Environmental Protection Agency 2011).

1.2. Disinfection by-products

Despite the huge advantages of water disinfection, the addition of chemicals as chlorine to natural or waste waters leads to the inevitable formation of disinfection by-products (DBPs). The term DBPs refers to all those chemical species that are formed during the process of water disinfection by the interaction of precursor molecules such as water disinfectants, the organic matter and inorganic ions and molecules in raw water. Such ions, as bromide, are present in raw waters naturally, or in some cases, as a consequence of human activity.

1.2.1. Discovery of DBPs

Over the years, there had been great speculation about the chemical reactions that may occur between the chlorine added to the water for disinfection purposes and the organic materials present in natural waters, but it wasn’t until 1974 when the products of these reactions were first identified, and thus, the presence in water of potentially hazardous chemicals directly linked to water disinfection (Bellar et al. 1974; Bellar & Lichtenberg 1974; Rook 1974).

The first DBPs identified were trihalomethanes (THMs). In 1974, Bellar described the presence of chloroform in Rotterdam’s water. Simultaneously, Rook discovered chloroform in drinking waters in Ohio. Soon more evidence raised, and in 1976 the US Environmental Protection Agency (EPA) published the results of a survey showing that chloroform could be found in all chlorine-disinfected waters (Kopfler et al. 1976).

Subsequently, other THMs, such as bromodichloromethane (BDCM), chlorodibromomethane (CDBM), tribromomethane (bromoform), iododichloromethane, iododibromomethane, bromochloroiodomethane and chlorodiiodomethane, were identified in drinking water (Dowty, B., Carlisle, D., and Laseter 1975). It was in the eighties when the majority of DBP groups were detected after comparing drinking water samples to raw water samples. These newly found DBP chemical groups included haloacetonitriles, haloketones, halonitromethanes and other halogenated forms. MX and furanones were identified in the late eighties-early nineties (International Agency for Research on Cancer 1991).

In the nineties, already most of the different DBPs’ groups had been identified, adding haloacetic acids (HAAs), cyanogen halides, haloaldehydes and halophenols to the previously described compounds (Singer 1994). More recently, modern techniques such as high-resolution mass spectrometry have allowed the discovery of new DBPs chemical species. Some of these newly identified species were iodoacetic acid (IAA), bromoiodoacetic acid, dibromonitromethane (DBNM), and 2,3,5-tribromopyrrole. Also, hydroxyl-halobenzoquinones and several new chlorination by-products were identified (W.

Wang et al. 2014; Pressman et al. 2010). The analysis of diverse water sources also led to the discovery of new DBP species. In fact, tribromoethenesulfonate, a novel DBP, was identified in ballast water (Gonsior et al. 2015)**.**

1.2.2. Classification of DBPs

So far, around 600 DBP species have been described (Richardson et al. 2007). DBPs consist of a very diverse mixture of chemical species, hence its classification results to some extent complex.

Here, we consider 10 major DBP groups and a heterogeneous one that includes the rest of the relevant DBPs. A summary of the most known classes of DBPs is listed in table 2, as well as the minimum and maximum concentrations of each DBP class found in swimming pools or treated waters worldwide.

Disinfection by-product	**Occurrence (µg/L)**
o **Halonitromethanes**	0.1 – 5
Chloronitromethane	
Dichloronitromethane	
Trichloronitromethane (Chloropicrin)	
Bromonitromethane	
Dibromonitromethane	
Tribromonitromethane (Bromopicrin)	
Bromochloronitromethane	
Bromodichloronitromethane	
Dibromochloronitromethane	
o **Haloacetic acids and other haloacids**	1 – 2,600
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	
o **Trihalomethanes**	0.05 - 380
Chloroform	
Bromoform	
Dibromochloromethane	
Bromodichloromethane	
Dichloroiodomethane	
Bromochloroiodomethane	
Dibromoiodomethane	
Chlorodiiodomethane	
Bromodiiodomethane	
Iodoform	
Dichloromethane	
Bromochloromethane	
Chlorodibromomethane	
Dibromomethane	
o **Oxyhalides**	
Bromate	0.2 – 25.1
Chlorate	Up to 190
Chlorite	Up to 1100
o **Halofuranones**	
MX	0.08 – 0.85
Red-MX	
Ox-MX	
EMX	
ZMX	
Mucochloric acid	
BMX-1	
BMX-2	
BMX-3	
BEMX-1	
BEMX-2	
BEMX-3	

o **Haloacetonitriles**	0.5 - 219
Chloroacetonitrile	
Dichloroacetonitrile	
Trichloroacetonitrile	
Bromoacetonitrile	
Dibromoacetonitrile	
Tribromoacetonitrile	
Bromochloroacetonitrile	
Bromodichloroacetonitrile	
Dibromochloroacetonitrile	
Iodoacetonitrile	
o **Haloketones**	1.4 – 60
Chloroacetones	
o **Haloamides**	Up to 9.4
Chloroacetamide	
Dichloroacetamide	
Trichloroacetamide	
Bromoacetamide	
Dibromoacetamide	
Tribromoacetamide	
Bromochloroacetamide	
Bromoiodoacetamide	
Bromodichloracetamide	
Dibromochloroacetamide	
Iodoacetamide	
Diiodoacetamide	
Chloroiodoacetamide	
o **Haloamines & other amines**	1 – 1180
Chloramines	
Nitrosamines (NDMA)	
Heterocyclic amines	
o **Aldehydes**	0.4 – 497
Formaldehyde	Up to 13.7
Acetaldehyde	
Chloroacetaldehyde	
Dichloroacetaldehyde	

	Bromochloroacetaldehyde
	Trichloroacetaldehyde (chloral hydrate)
	Tribromoacetaldehyde
o Other DBPs	
	Quinones
	Cyanogen halides
	Chlorophenols
	Aldoketoacids
	Carboxylic acids
	Haloacetates
	Halopyrroles
	Others

Table 2. Main DBP chemical groups and available occurrence data (Weinberg et al. 2002; Krasner et al. 2006; Richardson et al. 2007; Montesinos et al. 2011; Richardson 2011; Manasfi et al. 2016; Hang et al. 2016; Huang et al. 2017).

1.2.3. Formation of DBPs

Chlorine has historically been the most commonly used disinfectant. When it is employed, the relationship between the chlorine dose and the amount of organic carbon in water is the main factor to determine the by-products that will form. Additionally, the presence of bromide and iodide in raw waters determines the formation of brominated or iodinated compounds.

In general, the characteristics of the water source play a very important role in determining the species of DBPs that form in each situation. These also include the water pH, temperature, and the presence of organic matter and inorganic DBP precursors (Table 3). For instance, increases in water pH favour the formation of THMs, however, it leads to a decrease in the levels of HAAs, haloacetonitriles and haloketones (Singer 1994).

On the other hand, the use of different disinfectants will lead to the formation of distinct species of DBPs. Generally, the use of chlorine has been linked to the formation of THMs, HAAs, HNMs, haloacetonitriles, chloramines, chlorophenols, MX, bromate and chloral hydrate; while chlorine dioxide is associated with the formation of chlorite, chlorate and chloride; ozonation to the formation of bromate, formaldehyde, other aldehydes, hydrogen peroxides and brominated methanes;

and chloramination to the formation of dichloramines, trichloramines, cyanogen chloride and chloral hydrate (US Environmental Protection Agency 2012).

Other variables to take into consideration are the contact time and the characteristics of the distribution network. Residual chlorine in distribution systems implies a continuous formation of THMs and HAAs (US Environmental Protection Agency 2012).

Factors that determine DBPs formation	
Organic matter in water	DBP formation is proportional to the concentration of NOM Aromatic NOM increases the formation of halogenated DBPs
Ion presence in water	Bromide presence determines the formation of brominated DBPs
Water pH	A basic pH favours the formation of THMs Acidic pH can favour the formation of HAAs
Water temperature	Higher temperatures demand the use of higher disinfectant doses
Disinfectant employed	Chlorine: THMs, HAAs, HNMs, haloacetonitriles, chloramines, chlorophenols, MX, bromate and chloral hydrate Chlorine dioxide: chlorite, chlorate and chloride Ozone: bromate, formaldehyde, other aldehydes, hydrogen peroxides and brominated methanes Chloramines: dichloramines, trichloramines, cyanogen chloride and chloral hydrate
Contact time	Residual chlorine in distribution systems favours the formation of HAAs over THMs

Table 3. Summary of the factors that influence the formation of distinct DBP species (Singer 1994; Deborde & von Gunten 2008; Liu & Reckhow 2015; Li et al. 2017).

More recently, the presence of anthropogenic compounds in raw waters has led to a new scenario of DBPs formation. These compounds include pharmaceuticals, hormones, pesticides, textile dyes, UV filters, fuels, etc. Even though their levels in drinking water do not suppose a health concern, there are concerns regarding the DBPs that form during the disinfection process. For instance, analgesics as acetaminophen (paracetamol) or anxiolytics as benzodiazepines react with chlorine forming several known DBP species (Postigo & Richardson 2014; Carpinteiro et al. 2017).

1.2.4. Human exposure to DBPs

Initially, ingestion was considered the most important exposure route to DBPs. However, as many of these compounds are volatile, more recent studies suggest that inhalation is the main entry route. Studies on domestic water consumption in the US showed that most water demand was not related to ingestion, but mostly to activities, such as bathing, flushing toilets or cooking, that can lead to volatilization of DBPs and their following inhalation (Andelman 1985; McKone 1987). A posterior study carried out in Taiwan estimated that most inhalation of THMs took place while showering and cooking (Lin & Hoang 2000).

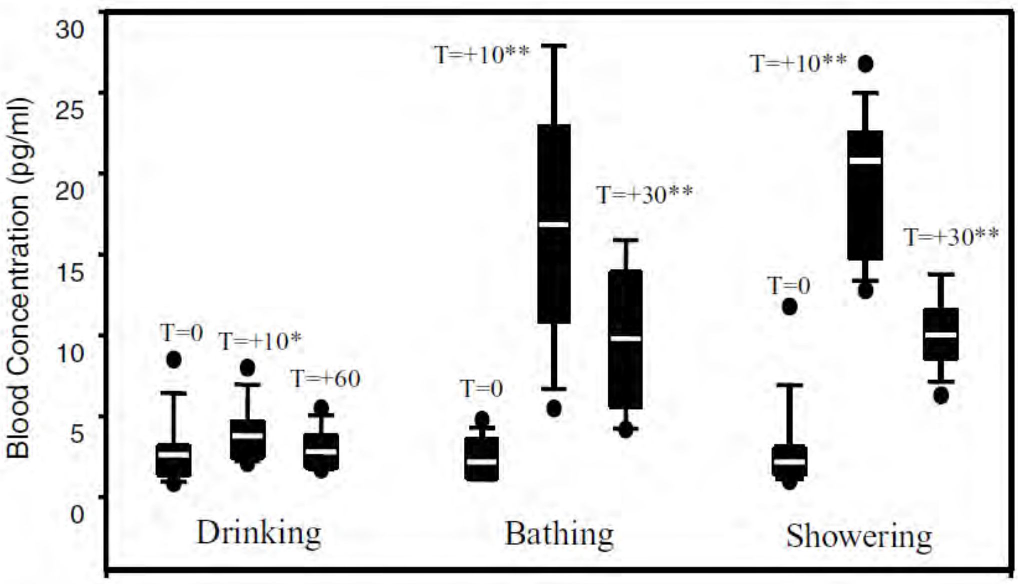

Figure 3. Bromodichloromethane levels in blood before and after drinking, bathing or showering in disinfected water (Backer et al. 2000).

Blood levels of bromodichloromethane (BDCM), dibromochloromethane (DBCM) and chloroform appeared to be the highest in individuals after 10 minutes' showers, and the lowest levels appeared after drinking treated water (Figure 3), confirming again that most DBPs intake takes place by inhalation (Backer et al. 2000).

Another important entry route for DBPs is dermal absorption. Some DBPs species, such as chloroform, can be absorbed through the skin during showering. In these cases, dermal intake can be as significant as inhalation (Brown et al. 1984; Jo et al. 1990).

Given the importance of inhalation and dermal intake, vast research has been carried out analysing DBPs' exposure by swimming in chlorinated swimming pools. Blood and lung levels of THMs have been monitored, showing significantly higher levels in chlorinated pool swimmers, with differences according to several variables such as age, sex, detoxification enzymes' polymorphisms or the intensity of the activity (Aggazzotti et al. 1993; Aggazzotti et al. 1998; Kogevinas et al. 2010; Font-Ribera et al. 2016). Parallelly, other variables taken into account when analysing exposure in swimming pools are the characteristics of the source water, being especially concerning waters obtained from seawater desalination, due to the increased levels of brominated DBPs and the increased hazard these chemicals might pose (Chowdhury 2015; Kim et al. 2015).

1.2.5. Health concerns

Despite the huge sanitary improvement that water disinfection poses, by reducing the incidence of infectious diseases and their consequently associated deaths all over the world, there are serious health concerns derived from DBP exposure.

From the moment that DBPs were discovered, there has been a rising interest to uncover whether the presence of these chemicals in drinking water can have a negative effect on human health. The main concern is the implication that, even though it is preferable to have DBPs contamination in water rather than microbial contamination, populations are exposed to DBPs for many years. For this reason, extensive and thorough research is needed.

Once the first DBPs were identified, *in vivo* and *in vitro* experiments were carried out to determine the potential health effects of these chemical species. In parallel, biomonitoring studies were conducted to assess the real effect that these chemicals could have on human populations. Taken together, these data show a clear carcinogenic potential of some DBP species, with an increased cancer incidence directly linked to DBPs exposure in the epidemiological studies (Richardson et al. 2007).

Thus, cancer became the clearest and most concerning outcome derived from DBPs exposure. Additionally, new data suggests an association between DBPs' exposure and other symptoms, such as respiratory and developmental symptoms (Kanitz et al. 1996; Fantuzzi et al. 2010). This data taken together led most governments to regulate the presence of many of these chemicals in drinking water (Richardson et al. 2007).

1.2.5.1. Toxicological evidence

Numerous *in vitro* and *in vivo* studies have analysed the genotoxic and carcinogenic potential of DBPs over the years. Data is available for most regulated compounds, but still incomplete for unregulated species. Existing information on the genotoxic and carcinogenic potential of DBPs is summarised in Table 4. As in many other fields, the evaluation of DBPs genotoxicity employed both *in vitro* and *in vivo* models, being studies *in vitro* in *Salmonella* the most predominant. As for carcinogenicity evaluation, *in vivo* models using mice and rats represent the clear majority of the studies (Richardson et al. 2007).

	Genotoxicity		**Carcinogenicity**
Regulated DBPs	*In vivo*	*In vitro*	*In vivo*
HAAs			
CAA	–	–	–
DCAA	+	+	+
TCAA	–	–	+
BAA		+	
DBAA		+	+
THMs			
Chloroform	–	–	+
Bromoform	+	+	+

BDCM	–	+	+
CDBM		+	+
Oxyhalides			
Bromate	+	+	+
Chlorite			–
Unregulated DBPs			
HNMs			
CNM		+	
DCNM		+	
TCNM		+	
BNM		+	
DBNM		+	
TBNM		+	
BCNM		+	
BDCNM		+	
DBCNM		+	
HAAs			
IAA		+	
THMs			
Iodoform		+	
Halofuranones			
MX	+	+	+
Nitrosamines			
NDMA	+	+	+
Aldehydes			
Formaldehyde	+	+	+
Acetaldehyde	+	+	+

Table 4. Summary of the available genotoxic and carcinogenic data of DBPs. Genotoxicity data includes gene mutation, chromosomal mutation and DNA damaging capacity. Modified after Richardson 2007 (Richardson et al. 2007; Zhang et al. 2015).

a) *In vitro* studies

Vast *in vitro* research has been carried out trying to determine the genotoxic and possible carcinogenic effects of DBPs since their discovery.

After the identification of chloroform as a by-product of water disinfection, the first experiments *in vitro* were carried out investigating its mechanisms of genotoxicity and carcinogenicity. Initially, most experiments used bacteria as a model organism. Between 1977 and 1995 the genotoxic potential of chloroform was tested in numerous bacterial systems with negative results in most cases (International Agency for Research on Cancer 1999).

Other early discovered THMs were tested *in vitro* as well. Contrarily to chloroform, bromoform was genotoxic in most tests in bacteria. Chlorodibromomethane and bromodichloromethane also showed a certain genotoxic capacity using the *Salmonella* Ames test (Richardson et al. 2007). THM species that were identified later were also tested using the Ames test, where dibromomethane, dichloromethane and bromochloromethane displayed genotoxic capacities (Richardson et al. 2007).

Other DBPs were also tested in bacterial systems, given the simplicity and reproducibility of these tests. The Ara test of *Salmonella typhimurium* was used to evaluate the mutagenic and lethal effects of several halogenated methanes with positive results (Roldán-Arjona & Pueyo 1993). In this study, dichloromethane, dibromomethane, iodoform and diiodomethane were clearly mutagenic, and this effect decreased in the presence of S9, while chloroform and bromoform were not mutagenic.

Some of the studies using bacteria provided significant information on the metabolism of these chemicals. In many cases, metabolic activation through addition of the S9 fraction was necessary for these species to become genotoxic. The mutagenicity of dichloromethane, BDCM, bromoform and chlorodibromomethane is mediated by a theta-class glutathione S-transferase (GSTT1-1), and comparisons between chloroform and bromoform established that the bromination of the compound confers the capability for GSTT1-1 to produce the mutagenic intermediates. This evidence demonstrates a higher carcinogenic risk in tissues expressing this gene, such as the colon, in *GSTT1-1*(+) individuals who are in contact with THMs. Furthermore, the tissue-specificity of GSTT1-1 could explain the differences on cancer incidence among tissues observed in *in vivo* studies (DeMarini et al. 1997; Pegram et al. 1997; Landi et al. 1999).

In the late eighties, the first studies using mammalian cells were developed. One of the first studies evaluated the capacity of two chloroacetones (1,1,1- and 1,1,3-

trichloroacetone) to induce chromosomal aberrations in CHO cells. Both DBPs were able to increase the number of chromosomal aberrations *in vitro* with and without S9 (Blazak et al. 1988). This study set the base for further investigations that aimed to determine the mutagenic capacity of individual DBP species in mammalian cells *in vitro*, mainly using CHO cells. Chemical species such as chloroform, iodoform, most HAAs and HNMs have exhibited genotoxic potential *in vitro* in the comet assay using rodent's cells (Richardson et al. 2007).

More recently, the mutagenicity of several HAAs was evaluated *in vitro* using CHO cells in the *HGPRT* gene mutation assay, where their mutagenic potency ranked IAA > DBAA > BAA > CAA > DCAA, while TCAA was not mutagenic (Zhang et al. 2010).

In parallel, many genotoxicity studies used human cells. In 1991 human cells were first used to analyse the genotoxicity of MX in a study similar to those using CHO cells. In this case, the DNA damaging capacity of MX was inactivated after the addition of S9, indicating that GST1-1 is not an important detoxification enzyme for this DBP (Chang et al. 1991).

From this point, extensive research has taken place analysing the genotoxic and mutagenic capacities of individual DBP species using human cells. For instance, TK6 cells have been prominently used in the evaluation of the genotoxicity of HNMs. Trichloronitromethane (TCNM) and bromonitromethane (BNM) were capable of inducing DNA damage when measured by the comet assay. However, none of the HNMs could increase the frequency of micronucleus (Liviac et al. 2009). The comet assay has also been used in human cells to assess the genotoxicity of many DBPs such as chloroform, bromoform, dichloromethane, BDCM and TCNM, with positive results (Richardson et al. 2007).

b) *In vivo* studies

In vivo studies analysing the genotoxicity and carcinogenicity of DBPs are complex, given the extensive number of DBP species. Nonetheless, extensive research has been carried out and most of the current information on the carcinogenic potential of DBPs comes from studies in animal models.

One of the first DBP species discovered, chloroform, was comprehensively investigated, not only as a DBP but also due to its many uses in the industry. Several studies evaluated its carcinogenicity as a toothpaste additive, proving chloroform's capacity to induce renal tumours in mice (Roe et al. 1979). Just two years after the discovery of DBPs and the same year the EPA showed the prevalence of chloroform in drinking water, the US National Cancer Institute published evidence of the carcinogenic potential of chloroform in rats and mice (National Toxicology Program 1976). Subsequently, chloroform was linked to the formation of hepatic tumours in rats and an increased yield of renal adenomas and adenocarcinomas in mice (Jorgenson et al. 1985; Tumasonis et al. 1987), despite the lack of genotoxicity observed in *in vitro* systems. Thus, chloroform was listed as a carcinogenic non-genotoxic DBP (International Agency for Research on Cancer 1999).

Other THMs were also carcinogenic in rodents. BDCM, bromoform and CDBM induced the formation of renal, intestinal and hepatocellular tumours in 2-year studies in rats and mice (Richardson et al. 2007). Hepatic tumours were also observed when treating mice with dichloroacetate and trichloroacetate (Bull et al. 1990).

HAAs were as well associated with carcinogenic effects through *in vivo* studies. More specifically, dichloroacetic acid (DCAA) produced hepatic tumours in mice in a dose-response manner (DeAngelo et al. 1999; Wood et al. 2015). Given the carcinogenic potential of DCAA, the US EPA and the National Institute of Environmental Health Sciences conducted a more extensive study in mice (National Toxicology Program 2007). DCAA induced squamous cell papilloma, epidermal hyperplasia and hyperkeratosis in dermal studies, pulmonary adenomas, hepatocyte cytoplasmic vacuolization in males and females, and the incidence of nephropathy was increased in males in drinking water studies. Similar outcomes appeared when dibromoacetic acid was analysed (Melnick et al. 2007).

Finally, various studies have been carried out trying to understand the carcinogenic potential of mixtures of DBPs. A mixture of potassium bromate, MX, chloroform, and BDCM was given to rats via drinking water for 10 months, leading to the formation of aberrant crypt foci in the colon, which is the putative preneoplastic lesion of kidney and colon cancer. Surprisingly, antagonistic effects were observed in the formation of renal lesions (McDorman, Chandra, et al. 2003; McDorman, Hooth, et al. 2003). These data suggest that the carcinogenicity studies with individual DBP species could overestimate the cancer risk.

1.2.5.2. Epidemiological evidence

Throughout the 1970s, several studies connected potentially hazardous chemicals found in drinking water with chemical water disinfection. Soon enough, the first studies linking exposure to these chemicals with a higher cancer incidence appeared. The first report to establish this link dates from 1976, when a statistically significant relationship between cancer mortality in Louisiana and disinfected water was found (Page et al. 1976).

In 1977 an epidemiological study conducted in Ohio showed a correlation between the use of chemically-disinfected surface waters and stomach, bladder, and total cancer rates (Kuzma et al. 1977).

Even though the first studies evaluated the appearance of all cancer types, the focus soon moved to cancer of the urinary tract, especially bladder cancer, which was essentially correlated with high levels of chloroform and other THMs in disinfected water. Cantor carried out several epidemiological studies in the US where he established a relationship between THMs and brain and colon cancers in both males and females. However, the strongest association was found between the presence of THMs in the water and bladder cancer (Cantor et al. 1978; Cantor 1982; Cantor et al. 1987).

In 1991 the International Agency for Research on Cancer (IARC) released the first monograph evaluating the potential carcinogenicity of water disinfection by-products, reviewing the uses of chlorination and carcinogenicity evidence to that date, and considering bladder cancer as the main outcome of the exposure to these chemicals (International Agency for Research on Cancer 1991).

The largest epidemiological study yet was published between 1998 and 1999, and it specifically analysed bladder, rectal and brain cancer incidence in Iowa

(Cantor et al. 1998; Hildesheim et al. 1998; Cantor et al. 1999). The study supports the association between exposure to chlorine-disinfected water and bladder and rectal cancers in both males and females, as well as brain cancer only in males. The same association was found between bladder cancer risk and estimates of DBP exposure from ingestion of drinking water, dermal absorption, and inhalation when showering, bathing, and swimming in pools in Spain (Villanueva et al. 2007).

Besides the carcinogenic potential of DBPs, which is the main health concern related to these chemicals, epidemiologic studies also found an association between DBPs and other symptoms. A raising concern has been the link between maternal DBPs exposure during pregnancy and congenital cardiac anomalies in new-borns. The first evidence of reproductive toxicity of DBPs was reviewed as early as 1986. Chloroform appeared to be teratogenic by inhalation, 2- and 2,4-chlorophenols produced slight embryo toxicity, and haloacetonitriles showed *in utero* toxicity (Smith et al. 1986). From this point, there has been vast research analysing the real effects of drinking chemically-disinfected water during pregnancy (Hwang et al. 2002).

Over the years, the quality of drinking water has been associated with congenital anomaly cases and neonatal death cases in several epidemiological studies a well as in *in vitro* studies (Shaw et al. 1990; Aschengrau et al. 1993; Bove et al. 1995; Kanitz et al. 1996), and later being directly associated with the presence of THMs (Savitz et al. 1995; Dodds & King 2001; Dodds et al. 2004; Wright et al. 2016). Similarly, new data suggests an association between prenatal exposure to certain THMs as bromoform and DNA hypomethylation in cord blood (Yang et al. 2017). However, a recent epidemiological study could not prove an association between birth outcomes and THMs exposures during pregnancy (Kogevinas et al. 2016).

Finally, there has been also an association with ocular, cutaneous and respiratory symptoms in swimming pool workers and professional swimmers, opening a debate about the potential risks from chlorinated swimming pools (Fantuzzi et al. 2010; Florentin et al. 2011). Such association is a great concern especially regarding childhood exposure, even though to date, meta-analysis studies failed to link swimming in childhood with an increased likelihood of asthma (Valeriani et al. 2017).

	THMs (µg/L)	HAAs (µg/L)	Oxyhalides (µg/L)	Others (µg/L)
WHO guidelines	Chloroform 200 Bromoform 100 BDCM 60 DBCM 100	DCAA 50 TCAA 20	Bromate 10 Chlorite 700	Trichloroacetaldehyde 10
US EPA 2006	TTHM 80	HAA5 60	Bromate 10 Chlorite 1,000	Chlorine 4000
Europe	TTHM 100	-	Bromate 10	Nitrate 50,000 Nitrite 500
Canada	TTHM 100	HAA5 80	Bromate 10 Chlorite 1,000	-
China	TTHM 100	DCAA 50 TCAA 100	Bromate 10 Chlorite 70	-
Japan	TTHM 100	CAA 20 DCAA 40 TCAA 200	Bromate 10	-
Australia	TTHM 250	CAA 150 DCAA 100 TCAA 100	Bromate 20 Chlorite 800	Nitrate 50,000 Nitrite 3,000 Formaldehyde 500 Trichloroacetaldehyde 20
South Africa [a]	Chloroform 300 Bromoform 100 BDCM 100 DBCM 60	-	-	Nitrate ≤ 11,000 Nitrite ≤ 900 Chlorine ≤ 5000

Table 5. Summary of DBPs legislation and guidelines (Karanfil et al. 2008; Werschkun et al. 2012; Ngwenya et al. 2013; Wang et al. 2015; US Environmental Protection Agency 2006). [a] The South African National Standard for Drinking Water Quality.

1.2.6. Regulation

The conclusive evidence linking DBPs to cancer incidence led the governments to develop specific regulations for these chemicals. Already in 1979, the US EPA established the total THM rule, setting the maximum allowed concentration of these DBPs in potable water to 0.1 mg/L (US Environmental Protection Agency 1979). This legislation was modified years later due to new evidence. The allowed water levels of total THMs were decreased to 0.08 mg/L in 1998, and other DBP species such as HAAs, chlorite and bromate were added to the regulated DBPs group (US Environmental Protection Agency 1998). This same year, the EU released the current legislation for DBPs, setting maximum levels for total-THMs, bromate, nitrate and nitrite (EU 1998).

The World Health Organisation (WHO) has as well proposed recommendations for the maximum level in the water of the most prevalent DBPs: THMs, HAAs, bromate, chlorite and trichloroacetaldehyde (Richardson et al. 2007). Current worldwide legislations are summarised in Table 5.

Total-THMs limit in potable waters is set at 100 µg/L in most countries (Karanfil et al. 2008). In addition, some governments have set independent maximum levels for chloroform, bromoform, BDCM and DBCM, as suggested by the WHO guidelines. Legislation on HAAs is less homogenous, with limits between 20 and 200 µg/L for individual species. The same happens with bromate, chlorite, chlorine and the rest of the regulated DBP species.

Current legislation is limited to the most known and abundant DBP species which have been proven to be genotoxic and mutagenic in bacterial models and, in some cases, in mammalian or human cells. Nevertheless, there are still hundreds of DBPs potentially mutagenic and carcinogenic that remain unregulated.

In order to develop more accurate regulations, more extensive and exhaustive research is needed. The identification of the specific DBP species associated with the increased cancer rates observed in epidemiological studies is key to protect human populations, either by improving regulations or using alternative disinfection methods not leading to the formation of those specific species.

1.3. Potentially carcinogenic DBPs

Research on the carcinogenicity of DBPs groups has not been developed uniformly among DBP classes. Some groups, such as THMs, have been widely investigated due to their high prevalence in disinfected water, while other relatively abundant groups with higher genotoxic potential have not been analysed as thoughtfully. These DBP classes include HAAs and HNMs, the chemical groups studied in this Thesis.

Important gaps exist in the carcinogenicity analysis of both groups. HNMs are highly genotoxic in several cellular systems, but its carcinogenic potential has not been determined. For HAAs, some species have been capable to induce tumours in animals, but the lack of a more thoughtful research has prevented the regulation of the potentially carcinogenic species.

1.3.1. Halonitromethanes

HNMs are small halogenated molecules with the structure X_3CNO_2, where X can be chlorine, bromine or iodine. Their formation is favored in waters disinfected with chlorine, but HNMs have also been found in waters disinfected using chloramines and ozone (Richardson et al. 2007; Hu et al. 2010; Brezonic & Arnold 2011). Their average individual concentrations in treated waters are around 3 µg/L (Manasfi et al. 2016; Hang et al. 2016).

All HNMs are cytotoxic and genotoxic *in vitro* in bacterial and mammalian cells. Their cytotoxic and DNA-damaging potential are determined by the presence of bromine or chlorine. Previous studies demonstrate that brominated HNMs are more cytotoxic and genotoxic than the chlorinated forms (Plewa, Wagner, Jazwierska, et al. 2004). Moreover, the rank order of increasing toxicity was the tri-, mono- and dihalogenated forms. A summary of HNMs genotoxicity can be found in Table 6.

Besides genotoxicity, other symptoms related to the exposure to HNMs are the induction of endoplasmic reticulum stress in human cells (Pesonen et al. 2012), as well as respiratory symptoms, congenital anomaly cases in foetuses and neonatal death cases.

HNM species	Biosystem	Genetic endpoint
Chloronitromethane	*Salmonella*	Histidine reversion + *GSTT1-1*
	Salmonella	Histidine reversion
	CHO cells	Comet assay
Dichloronitromethane	*Salmonella*	Histidine reversion
	Salmonella	Histidine reversion
	CHO cells	Comet assay
Trichloronitromethane	*Salmonella*	Fluctuation test
	Salmonella	Histidine reversion + *GSTT1-1*
	Salmonella	Histidine reversion
	E. coli	SOS chromotest
	CHO cells	Comet assay
	TK6 cells	Comet assay
	A549 cells	ROS production
Bromonitromethane	*Salmonella*	Histidine reversion
	CHO cells	Comet assay
Dibromonitromethane	*Salmonella*	Histidine reversion
	CHO cells	Comet assay
Tribromonitromethane	*Salmonella*	Histidine reversion
	CHO cells	Comet assay
Bromochloronitromethane	*Salmonella*	Histidine reversion
	CHO cells	Comet assay
Bromodichloronitromethane	*Salmonella*	Histidine reversion
	CHO cells	Comet assay
Dibromochloronitromethane	*Salmonella*	Histidine reversion
	CHO cells	Comet assay

Table 6. Genotoxic studies of halonitromethanes with positive results. Adapted from Richardson et al. 2007 (Giller et al. 1995; Schneider et al. 1999; Kundu, Richardson, Granville, et al. 2004; Kundu, Richardson, Swartz, et al. 2004; Richardson et al. 2007; Liviac et al. 2009; Liviac et al. 2011).

Among all HNMs, TCNM or chloropicrin has received the most attention given its use as pesticide, with its inclusion in several risk assessment studies (European Food Safety Authority 2011). Additionally, it is highly genotoxic in bacterial systems and mammalian cells (Richardson et al. 2007). The effects of TCNM on human cells *in vitro* have also been documented, being capable of inducing DNA damage in TK6 cells when assessed by the comet assay, and even increasing vacuolization and apoptosis in A549 cells, as well as ROS levels (Pesonen et al. 2014; Liviac et al. 2009).

BNM has also received certain attention due to the higher genotoxic potential associated to brominated DBPs. This compound is, as all HNMs, genotoxic in bacterial systems and CHO cells, however its cytotoxicity is much higher than TCNM's (Richardson et al. 2007).

On the contrary, negative results have raised from several genotoxicity studies. TCNM and BNM were reported as non clastogenic in TK6 cells (Liviac et al. 2009), and were not genotoxic in the *in vivo* wing somatic mutation and recombination test in *Drosophila* (García-Quispes et al. 2009). The same species were negative in the thymidine kinase gene mouse lymphoma assay (Liviac et al. 2011).

Few studies have been conducted analysing the carcinogenicity of HNMs. Several experiments *in vivo* using rodents attempted to link HNMs exposure to tumour formation in rats and mice, however, results were not conclusive due to the high toxicity of the evaluated concentrations (Richardson et al. 2007). The lack of conclusive results regarding HNMs carcinogenicity determines the absence of specific regulations for this class of DBPs, despite their proved mutagenicity and genotoxicity in bacterial and cellular systems. Thus, more research is needed to evaluate the real genotoxic and carcinogenic potential of HNMs in long-term exposures.

1.3.2. Haloacetic acids

Haloacetic acids is the second most abundant group of DBPs (Krasner et al. 2006). They also are small halogenated molecules, with the structure X_3COOH, where X can be chlorine, bromine or iodine (Brezonic & Arnold 2011).

HAAs appear mainly in waters disinfected by chlorine dioxide, but can also be formed by the use of chlorine, chloramines and ozone, and are present in treated waters at concentrations between 1 and 2,600 µg/L (Richardson et al. 2007; Manasfi et al. 2016). CAA, DCAA, TCAA, BAA and DBAA represent the most abundant HAA species, and the sum of its concentrations is known as HAA5.

Comparisons between different species show that iodinated HAAs are the most cytotoxic and mutagenic, followed by brominated HAAs and the least are the chlorinated forms, being the potency of the haloacetic acids higher as lower is the number of halogen atoms of the molecule (Kargalioglu et al. 2002; Richardson et al. 2008; Zhang et al. 2012).

Not all HAAs have shown mutagenic or genotoxic potential, most species have never been studied or had negative results on mutagenicity tests. Studies showing a positive association between HAAs and DNA damage are summarised in table 7. Concurrently, studies *in vivo* have shown the capacity of DCAA to produce DNA strand breaks at non-cytotoxic concentrations (Chang et al. 1992; Fuscoe et al. 1996).

HAA species	Biosystem	Genetic endpoint
Chloroacetic acid	*Salmonella*	Histidine reversion
	Salmonella	Histidine reversion + S9
	E. coli	SOS chromotest
	CHO cells-K1	*HGPRT* gene mutation assay
	CHO, FHs cells and lymphocytes	Comet assay
	L5178Y/*Tk*$^{+/-}$ cells	*Tk*$^{+/-}$
Dichloroacetic acid	*Salmonella*	Histidine reversion
	Salmonella	Histidine reversion + S9
	CHO cells	Comet assay
	L5178Y/*Tk*$^{+/-}$ cells	*Tk*$^{+/-}$
Bromoacetic acid	*Salmonella*	Histidine reversion
	Salmonella	Histidine reversion + S9
	E. coli	SOS chromotest

	CHO-K1 cells	*HGPRT* gene mutation assay
	CHO, FHs cells and lymphocytes	Comet assay
Dibromoacetic acid	*Salmonella*	Histidine reversion
	Salmonella	Histidine reversion +S9
	E. coli	SOS chromotest
	CHO-K1 cells	*HGPRT* gene mutation assay
	CHO cells	Comet assay
Tribromoacetic acid	*Salmonella*	Histidine reversion
	E. coli	SOS chromotest
	CHO cells	Comet assay
Iodoacetic acid	*Salmonella*	Histidine reversion
	CHO, FHs cells and lymphocytes	Comet assay
	CHO and TK6 cells	Chromosomal aberrations

Table 7. Genotoxic studies of haloacetic acids with positive results. Adapted from Richardson et al. 2007 (DeMarini et al. 1994; Giller et al. 1997; Harrington-Brock et al. 1998; Plewa et al. 2002; Kargalioglu et al. 2002; Plewa, Wagner, Richardson, et al. 2004; Richardson et al. 2007; Richardson et al. 2008; Zhang et al. 2010; Plewa et al. 2010; Attene-Ramos et al. 2010; Escobar-Hoyos et al. 2013).

Most research has focused on the HAAs with a single halogen substituent (mono-HAAs) given its abundance. IAA has shown to be a potent mutagen in *Salmonella* (Plewa, Wagner, Richardson, et al. 2004). CAA and BAA are also mutagenic in certain *Salmonella* strains without the need of previous metabolic activation (Kargalioglu et al. 2002).

All three species were mutagenic in the *HGPRT* gene mutation assay in CHO cells as well, and could induce DNA damage in the same cell line assessed by the comet assay (Plewa et al. 2002; Richardson et al. 2007; Zhang et al. 2010). In the same study, IAA appeared to be two times more genotoxic than BAA and 47 times more than CAA on CHO cells.

Other HAAs have also exhibited a certain mutagenic potential. DCAA and DBAA were mutagenic in bacteria and in CHO cells, and iodinated forms as DIAA and BIAA also produced DNA damage in mammalian cells (Kargalioglu et al. 2002; Zhang et al. 2010; Richardson et al. 2008). Contrarily, several studies had negative results. For instance, TBAA and TCAA were not mutagenic in bacteria, and DCAA had negative results in the comet assay in CHO cells (Plewa et al. 2002; Richardson et al. 2007; Kargalioglu et al. 2002).

As for the three mono-HAAs, despite their DNA damaging capacity, they appeared unable to increase the micronuclei frequency on TK6 cells, hence, being unable to produce fixed DNA damage (Liviac et al. 2010).

Given the vast evidence that correlates HAAs with genotoxicity in mammalian cells, several studies have focused on the mechanistic aspects of this genotoxic induction. Current data suggest the formation of oxidative damage as the cause of this phenomenon. HAAs are not capable of interacting directly with the DNA, and several studies demonstrated that mono-HAAs inactivate GAPDH in mammalian cells, reducing pyruvate and ATP production, and eventually causing mitochondrial stress and increased ROS levels, which can be translated to DNA damage when high concentrations are used (Cemeli et al. 2006; Justin A Pals et al. 2011; Dad et al. 2013).

Other evidence of oxidative damage being a key aspect on HAAs genotoxicity shows that the damage scored in human cells with the comet assay could be decreased by the addition of antioxidants (Ali et al. 2014).

In addition, gene expression and human transcriptome profile analyses showed altered expression patterns of many different functional gene groups involved in DNA repair, cell cycle regulation and apoptosis after short exposures to the mono-HAAs at relatively high concentrations (Figure 4). Two genes were downregulated by the three mono-HAAs: *XRCC3* and *PPP1R15A*, which proteins are involved in DSBs repair and cell cycle regulation, and only CAA and IAA modified the expression of *PNKP*, whose protein is related to oxidative stress (Muellner et al. 2010; Attene-Ramos et al. 2010).

These differences may indicate that different specific mechanisms take place with each specific HAA. Additionally, distinct damage repair kinetics have been observed after the exposure to the three mono-HAAs, attributed to different classes of DNA lesions or different distribution of this damage (Komaki et al. 2009).

IAA has been further investigated due to its higher genotoxic capacity, and it has been associated with an increased intracellular expression of *NRF2*, as well as of *NRF2* downstream genes (S. Wang et al. 2014). The principal role of NRF2 protein in mediating IAA-induced genotoxicity may lead to its persistent activation in long-term exposures to this HAA. The implications of such continuous activation of the pathway have been associated with inflammation and carcinogenesis (Cho et al. 2008; DeNicola et al. 2011; Ni et al. 2014). Posteriorly, CAA and BAA were also linked to the activation of *NRF2* (Procházka et al. 2015). Thus, oxidative stress becomes extremely significant in the study of HAAs carcinogenicity.

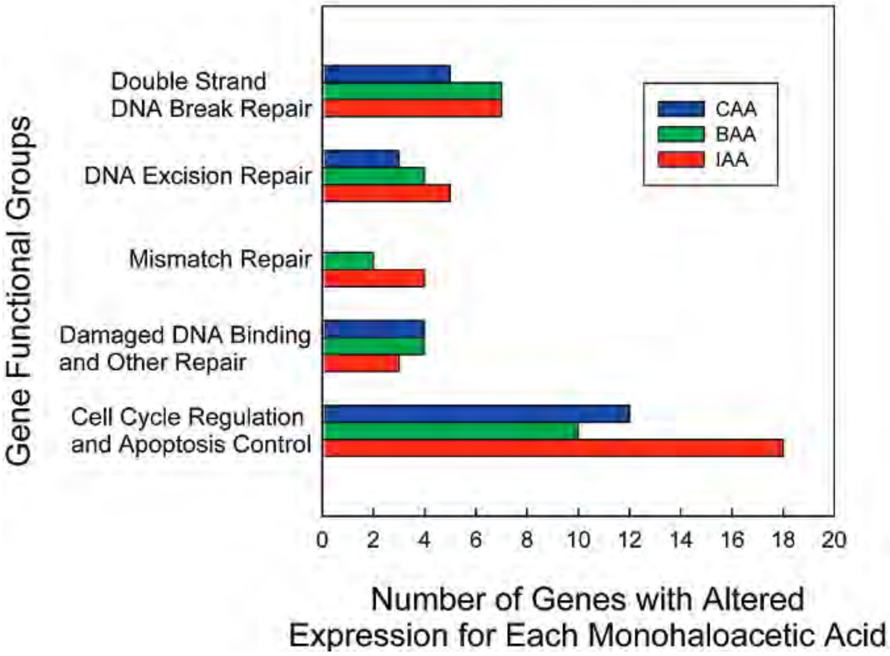

Figure 4. Alterations in gene expression within gene functional groups in human cells induced by mono-HAAs (Attene-Ramos et al. 2010).

As for the evaluation of the carcinogenic potential of HAAs, already in 1992, the US Department of Health and Human Services published a report on the carcinogenicity of CAA in rodents with negative results (US Department of Health and Human Services 1992). CAA and TCAA appeared incapable to produce liver cancer in rats (DeAngelo et al. 1997); however, more recent studies classified DCAA and TCAA as animal carcinogens (Wood et al. 2015; Von Tungeln et al. 2002). DCAA had been previously described as a mutagen and a hepatocarcinogen in rodents (Leavitt et al. 1997). Other effects previously documented include a teratogenic capacity in rats, and developmental failure in mice (Hunter et al. 1996). More recently, a non-tumorigenic fibroblast cell line exposed to IAA for 72 h was capable of forming tumours in nude mice (Wei et al. 2013).

Considering the vast evidence of the genotoxic potential of HAAs they have been included in drinking water regulations in many countries**.** There is a limit for the sum of HAA5 in the US and Canada, and individual species are regulated in other countries (US Environmental Protection Agency 2006; Karanfil et al. 2008). In spite of this, there is not a general norm, and IAA, one of the most genotoxic and possibly carcinogenic species, remains unregulated.

Hence, more research is needed to estimate the genotoxic and carcinogenic potential of individual HAAs.

1.4. Carcinogenic potential evaluation

The carcinogenic potential of DBPs has been evaluated through different methods over the years as it has been already described. However, DBPs constitute a very complex mixture of chemicals, and to identify the individual or group of species causing the increased cancer risk supposes a big challenge.

Although DBPs genotoxicity and carcinogenicity have been studied for almost 60 years, current knowledge on DBPs carcinogenicity has significant gaps. Many DBP classes have never been studied, and the analysis of DBP mixtures has just started. As other authors have previously noted, the lack of a systematic analysis of DBPs, using the same assay and conditions for all classes, caused these gaps in the literature that must be resolved (Cantor 1997; Werschkun et al. 2012). Hence, the importance of stablishing standard procedures and protocols to be followed in order to examine the carcinogenicity of the vast number of DBPs.

Over the years, the analysis of the carcinogenic potential of chemicals has employed both *in vivo* and *in vitro* models, which has allowed the analysis of diverse types and times of exposure. Limited guidelines for carcinogenicity assessment have been developed, mainly applicable to the food and pharmacological industries. The US EPA developed guidelines for the evaluation of the carcinogenic risk of chemicals based on epidemiological studies, studies using animal models *in vivo*, and the analysis of DNA lesions *in vitro* (US Environmental Protection Agency 2005).

The importance of developing and following standard procedures to analyse chemicals toxicity and carcinogenicity also led the Organization for Economic Co-operation and Development (OECD) to set specific guidelines to screen the carcinogenic potential of chemicals. The original guideline was adopted in 1981 and it has been reviewed over the years, creating a standard to follow in order to assess the carcinogenicity of chemicals such as pesticides. The goal of the studies covered by this guideline is to provide information on the possible health consequences of repeated exposures to chemicals (OECD 2009).

Protocols recommended by both organisms have been used in the analysis of DBPs carcinogenicity. Nevertheless, the lack of standard procedures supposes a serious limitation, leading to the mentioned gaps in the literature.

1.4.1. *In vivo* studies

In vivo studies are universally employed to determine chemicals carcinogenicity. *In vivo* experimentation allows the analysis of different dosage and intake pathways, and have been historically used to examine longer times of exposure. As for DBPs analysis, *in vivo* models were key to determine the carcinogenic potential of chloroform, and HAAs as DCAA.

Specific OECD guidelines exist for *in vivo* studies, especially those carried out in rodents, although guidelines for non-rodents are available as well. In this case, experimental procedures are focused on the oral intake of chemicals. Recommendations are set on the dose administration, animal model selection, and experimental procedure in general.

In vivo studies have been considered the most accurate models for carcinogenicity evaluation. However, there are certain limitations when analysing a vast number of species comprised in a group such as DBPs. For instance, the high economic costs that it supposes to test this wide range of chemicals.

On the other hand, it is necessary to take into account the detoxification pathway of each tested chemicals in order to select an animal model with the same pathways and enzymes as humans. For DBPs, the metabolic pathways that take place after their intake are still not fully known, thus the complexity to set a reliable experimental model and to eliminate the risk of obtaining false negative results.

Therefore, given the characteristics of DBPs and the fact that most species have never been analysed, *in vivo* models might not be the best option to assess the carcinogenic potential of these chemicals.

1.4.2. *In vitro* studies

In vitro studies have also been crucial to determine the mutagenic and carcinogenic potential of chemicals. Most widespread *in vitro* tests have historically used bacteria, and the Ames test has been the most predominant protocol since it was described in the seventies (Ames et al. 1973). Therein, chemicals' capacity to induce frameshift or point mutations in *Salmonella* is evaluated, with or without metabolic activation of the chemicals.

This procedure has been key to determine the genotoxic potential of countless chemicals, which has been considered a surrogate biomarker of carcinogenicity. However, the causal relationship between genotoxicity and carcinogenic potential is not always true. For instance, chloroform is a non-genotoxic carcinogen (International Agency for Research on Cancer 1999). Therefore, other analyses are required to assess carcinogenicity, in order to avoid false-negatives.

On the other hand, there is enough evidence that the cellular and molecular processes involved in *in vitro* cell transformation are similar to those occurring during *in vivo* carcinogenesis (Creton et al. 2012). Thus, the analysis of *in vitro* transformation provides a reliable system to assess the carcinogenicity of chemicals in prolonged exposures. Also, when compared to *in vivo* models, *in vitro* studies provide a faster and simpler experimental procedure to evaluate the carcinogenicity of the vast number of DBPs yet to analyse.

Within this framework, the OECD sets specific guidelines on cell transformation assays to screen the carcinogenic potential of chemicals. The OECD analyses the reliability of different models and recommends the use of the mouse cell lines BALB/c 3T3 and C3H10T1/2 for carcinogenicity analyses (Vasseur & Lasne 2012). In this case, *in vitro* treated cells, exposed to a certain potentially-transforming chemical, are injected into nude mice strains, and the formation of tumours is monitored. However, an organism within the European Commission released a series of reports recommending carcinogenicity testing *in vitro,* in an effort to reduce the use of *in vivo* models (Annys et al. 2014).

These models have been used to determine the carcinogenicity of numerous chemicals, still, there are several limitations. For instance, the models have not been tested for long-term exposures.

1.4.2.1. Analyses of cell malignant transformation

The increasing knowledge on the tumoural transformation process has allowed the application of more accurate and biologically relevant models to study carcinogenesis *in vitro.*

The process of tumoural transformation has been very well described, and the changes that take place in cells during this transformation can be easily monitored and quantified. Even though different changes may take place due to the heterogenicity of the mechanisms behind carcinogenesis, there are several processes that appear in all cases, based on events which are common to all tumoural transformation pathways (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011). Such events include an increased and chronic proliferation, an acquired capability to evade growth suppressors, resist cell death, the induction of angiogenesis, replicative immortality, and the activation of invasion and metastasis (Figure 5).

The understanding of the mechanisms behind these processes allowed the development of a series of assays to examine these universal markers, and thus assess the transformation degree of a cell line. On the other hand, the use of *in vitro* cell transformation assays supposed a huge step forward in the evaluation of carcinogenicity, providing several advantages such as the possibility to select the cellular model according to the tissue of interest, and the use of mammalian or human cells, contributing to a higher accuracy of the model.

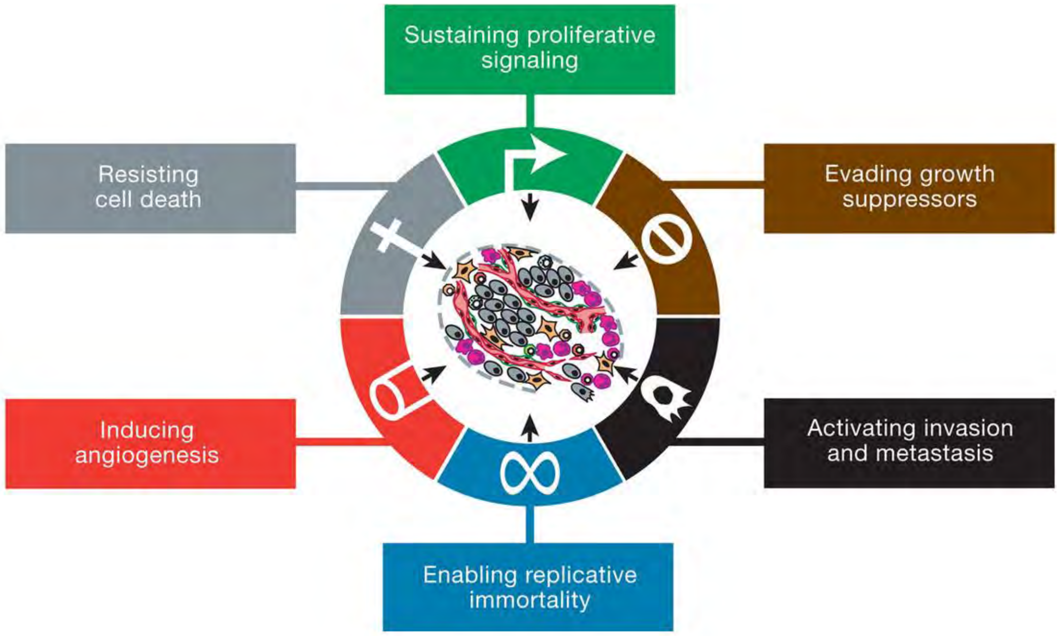
Sustaining proliferative signaling
Evading growth suppressors
Activating invasion and metastasis
Enabling replicative immortality
Inducing angiogenesis
Resisting cell death

Figure 5. Main cancer hallmarks underlying all tumorigenic processes (Hanahan & Weinberg 2011).

Already in 2007, other authors attempted to assess the carcinogenicity of several DBP species using cell transformation assays. In this case, the cell line employed was NCM460, a colon epithelial cell line, which was exposed to the DBPs for 72 h. After the exposure time, only one cell transformation marker was analysed, providing negative results (DeAngelo et al. 2007).

Subsequently, protocols to assess the carcinogenicity of chemicals using cell transformation assays have been improved, using longer exposure times, and to ensure the reliability of carcinogenicity studies based on these processes improved analysis include more than one of these markers. Over the past years, the use of such improved protocols has been validated in our group, and they have been used to evaluate the long-term carcinogenic potential of various chemicals (Annangi et al. 2015; Vales et al. 2015; Bach et al. 2016). In this Thesis, we have evaluated numerous cell transformation markers, including:

a) Cell proliferation

The deregulation in cell proliferation, in particular the ability to proliferate beyond the rates expected in the normal tissue, is one of the primary symptoms of malignant transformation. This is achieved due to a deregulation of the cell cycle, as a consequence of activating mutations in mitogens or G-protein signal transducers such as Ras, and mutations in genes involved in the G1 cell-cycle check-point. Thus, cancer cells are capable of increasing their proliferation rates (Evan & Vousden 2001).

Cell proliferation can be an easily measurable marker to monitor transformation, and it has been used in many carcinogenicity studies (Wang et al. 2011; Annangi et al. 2015; Bach et al. 2016)**.** The parameter used to monitor cell proliferation is the cellular doubling time, or the time that cells need to duplicate once.

b) Cell morphology

During cellular malignant transformation, cells undergo a process of epithelial-to-mesenchymal transition (EMT). EMT is a reversible process central in all carcinogenic transformations, but also necessary in other cellular scenarios, including embryo formation and tissue regeneration (Kalluri & Weinberg 2009).

During this process, cells progressively lose the inherent characteristics of an epithelial phenotype due to a decrease of the expression of E-cadherin and several cytoskeletal proteins. Epithelial cells lose their polarity and detach from the basal membrane while they acquire a mesenchymal phenotype (Figure 6). This way, cells are able to migrate, which allows tumour invasion and the formation of secondary tumours after the reversion to the epithelial phenotype (MET) (Tse & Kalluri 2007).

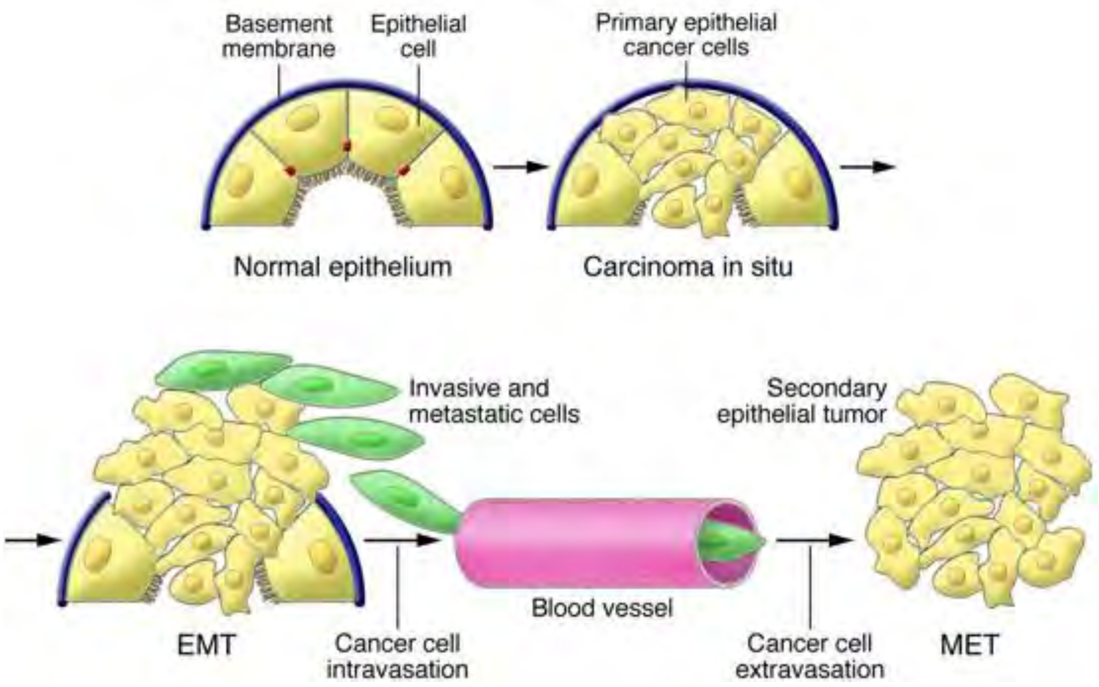
Basement membrane
Epithelial cell
Primary epithelial cancer cells
Normal epithelium
Carcinoma in situ
Invasive and metastatic cells
Secondary epithelial tumor
Blood vessel
EMT
Cancer cell intravasation
Cancer cell extravasation
MET

Figure 6. EMT contribution to cancer progression. Modified after (Kalluri & Weinberg 2009).

One of the consequences of EMT is that cells lose their intrinsic morphological characteristics and acquire a “spindle-like” morphology (Figure 7). The morphological changes during this process can be monitored and analysed during cells exposure to the carcinogens to evaluate (Bach et al. 2016).

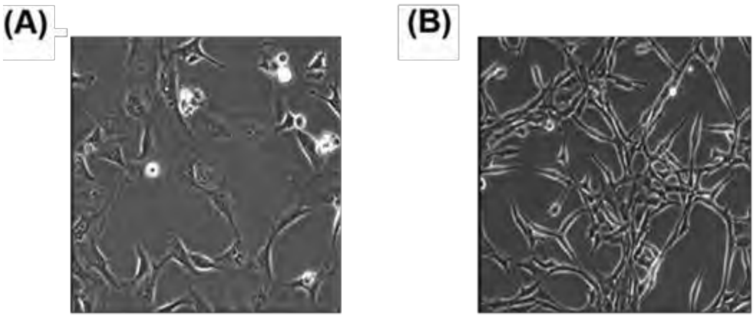

Figure 7. Non-transformed (A) and transformed (B) BEAS-2B cells exhibiting the classical “spindle-like” morphology.

c) Anchorage-independent growth capacity

Another of the characteristics that have been observed in tumours from different origins is the ability to grow independently of substrate anchorage (Borowicz et al. 2014).

Non-tumourigenic epithelial cells do not have the capacity to grow in surfes like soft-agar, as they depend on their contact to the extracellular matrix to be able to grow and divide. As the process of carcinogenic transformation takes place, cells undergoing EMT will be able to grow and divide irrespective of their surrounding environment (Kalluri & Weinberg 2009). During the transformation process, cells that acquire this feature will be able to form colonies on a semi-solid surface (Figure 8).

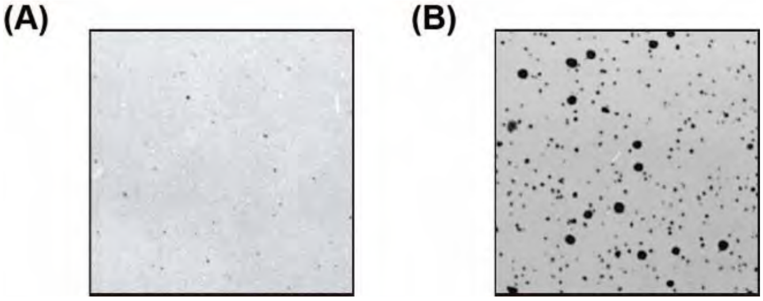

Figure 8. Non-transformed (A) and transformed (B) cell lines growth in the soft-agar assay.

Hence, the acquisition of this capability is directly linked to tumour aggressiveness and metastasis (Mori et al. 2009), and it has been used as a marker for cellular malignant transformation in many studies in the well-known soft-agar assay, being especially useful in testing carcinogens after long-term exposures (Bach et al. 2016; Vales et al. 2015; Vila et al. 2017).

d) Capacity to induce or promote tumoural growth

The interaction between cancer cells and their surrounding environment is key to understanding the process of carcinogenesis.

Cancer cells are known to secrete autocrine and paracrine signals such as growth factors, which can stimulate tumoural growth and cancer progression (Nicolson 2002; Witsch et al. 2011). Thus, an indirect manner to measure the malignancy of a cell line that has been exposed to a suspected carcinogen is to quantify its capacity to induce tumour growth. This can be achieved using a modified version of the soft agar-assay that allows the quantification of the possibly-transformed cells' secretome capability to induce cellular proliferation (Bach et al. 2016).

In this case, a tumoural cell line is grown on soft-agar in contact with the exposed cells' conditioned medium (CM), where the number of colonies that these tumoural cells form is directly influenced by the paracrine factors in the CM. This measurement can be used as marker of the cancer-inducting properties of the secretome. The size of the colonies, on the other hand, measures the secretome capacity to potentiate tumoural growth, since it indicates a faster replication rate of the cells (Bach et al. 2016).

Besides cancer cells, tumours also comprise the stroma, that includes tumour-associated fibroblasts, endothelial cells, immune-system cells, adipocytes, cytokines and other macromolecules from the extracellular matrix (Cammarota & Laukkanen 2016). Tumour-associated fibroblasts are often the most prominent cell type in the tumour stroma, and are able to directly stimulate tumour cell proliferation through the secretion of growth factors, hormones and cytokines.

Many of these factors appear to be able to induce malignant transformation of epithelial cells by themselves, emphasising the tumour-initiating capacities of the tumour stroma (Pietras & Östman 2010). This process is recreated *in vitro* using another modified version of the soft-agar assay which characterises the tumour initiation and promotion capacities of the tumour stroma, and therefore the transformation state of the epithelial cell line linked to this stroma (Condon & Bosland 1999).

e) MMPs secretion

The aforementioned changes that take place during the EMT include other processes that can influence the tumour stroma. Either tumoural cells or tumour-associated fibroblasts can secrete various proteases, including matrix metalloproteases (MMPs), that can directly degrade and remodel the extracellular matrix (Moore et al. 1993; Deryugina & Quigley 2006; Pietras & Östman 2010). This degradation is a determining factor for tumour invasion and metastasis.

MMPs are a family of zinc-dependent endopeptidases with a role in several physiological processes. 23 MMPs have been identified in humans, exhibiting distinct enzymatic capacities. The activation of MMPs is dependent on the balance between them and their inhibitors, which is altered in tumorigenesis (Kessenbrock et al. 2010).

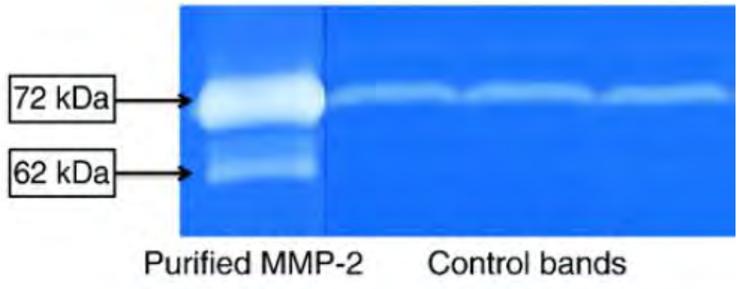

Figure 9. Zymography image of the analysis of the CM of non-transformed cells compared to purified MMP-2. Modified after (Kupai et al. 2010).

An increased secretion of MMPs can be a surrogate marker of malignant transformation, which can be measured by zymography. More specifically, the technique measures the secretion of MMP-2 and MMP-9, two proteases directly linked to the degradation of type IV collagen and gelatine, in the exposed cells' CM (Roomi et al. 2009). Such degradation can be observed and quantified in a gelatine gel (Figure 9).

1.4.2.2. Gene expression deregulation and acquisition of resistance

Gene expression deregulation takes place in the first stages of a carcinogenic process, and has been described in genes of numerous and diverse functional families (Clancy et al. 2012). One of the gene families that has mainly been analysed is the one related to DNA repair, as the deregulation of genes involved in multiple repair pathways is an early symptom of carcinogenicity. As it has been previously mentioned, gene expression changes have already been detected in cells exposed to mono-HAAs, affecting genes directly linked to DNA repair (Attene-Ramos et al. 2010).

Long-term exposures to DNA-damaging chemicals can trigger a process were cells acquire resistance to the damage caused by such chemicals. This is the most common mechanism, which becomes particularly concerning when it prompts cancer cells to acquire resistances to chemotherapy drugs (Wongvaranon et al. 2013; Terai et al. 2014). However, cases where cells subjected to long-term exposures to DNA-damaging chemicals become more sensitive to the damage have also been revealed (Bach et al. 2015).

Given the nature of DBPs exposure, human populations are in contact with these genotoxic compounds for decades, hence, it is necessary to take into consideration possible adaptation mechanisms in order to understand their mechanisms of carcinogenicity, and even the contradictory results observed in some of the *in vivo* carcinogenicity studies.

2. OBJECTIVES

2. OBJECTIVES

The lack of an efficient and fast standard methodology to analyse the carcinogenic potential of the numerous DBPs identified to date prompted us to assess new models to study the *in vitro* transformation effects of these chemicals.

Thus, the purpose of this Thesis can be divided in two main aims that include several sub-objectives.

Our first intent was to establish a more systematic manner to test the carcinogenicity and long-term effects of DBPs, based on a more realistic model of exposure. This aim was divided in two sub-objectives:

- To find a fitting cellular model to assess long-term exposures, both biologically relevant and stable, to perform such prolonged experiments.
- To assess the reliability of different cell transformation assays for the analysis of DBPs carcinogenicity.

The second was to test the effects of long-term exposures to two classes of DBPs, HNMs and HAAs, while addressing three specific sub-objectives:

- To investigate HAAs genotoxicity as an underlying mechanism for their possible carcinogenicity.
- To evaluate other possible effects of a long-term exposure to HAAs, such as the acquisition of resistance mechanisms.
- To evaluate the possible carcinogenicity derived from a long-term exposure to HNMs and HAAs analysing a series of cell transformation markers as:
    - Changes in cell morphology and proliferation.
    - Cells acquisition of anchorage-independent growth capacity.
    - Cells acquisition of the capacity to induce tumoural growth.
    - Upregulation of matrix metalloproteinases.

3. MATERIALS AND METHODS

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3.1. Evaluated DBPs

The two selected classes of DBPs, namely HNMs and HAAs, were chosen for different reasons. HNMs have exhibited a high genotoxic potential in *in vitro* studies; however, their carcinogenicity has never been evaluated. Contrarily, although the carcinogenicity of HAAs has been already investigated in *in vivo* studies, their long-term carcinogenic potential in human cells has never been evaluated.

According to that, two HNMs [bromonitromethane (BNM) and trichloronitromethane (TCNM)] and three HAAs [chloroacetic acid (CAA), bromoacetic acid (BAA) and iodoacetic acid (IAA)] were chosen for the study. Chemical formulations, CAS number and supplier of each DBP employed are indicated in Table 8 and chemical structures represented in Figure 10.

DBP species	**Chemical formulation**	**CAS number**	**Supplier**
BNM	$BrCH_2NO_2$	563-70-2	Sigma-Aldrich (St. Louis, MO, USA)
TCNM	CCl_3NO_2	76-06-2	Riedel-de-Haën (Seelze, Germany)
CAA	$C_2H_3ClO_2$	79-11-8	Acros Organics (Geel, Belgium)
BAA	$C_2H_3BrO_2$	79-08-3	Acros Organics (Geel, Belgium)
IAA	$C_2H_3IO_2$	64-69-7	Sigma-Aldrich (St. Louis, MO, USA).

Table 8. Information of the evaluated DBP species.

To ensure the reliability of the experiments, stock solutions were freshly prepared prior to every treatment in all cases, and subsequently diluted to working concentrations on cell culture medium. BNM and TCNM were weighed and diluted in distilled water to an initial stock concentration of 1 M and 100 mM, respectively.

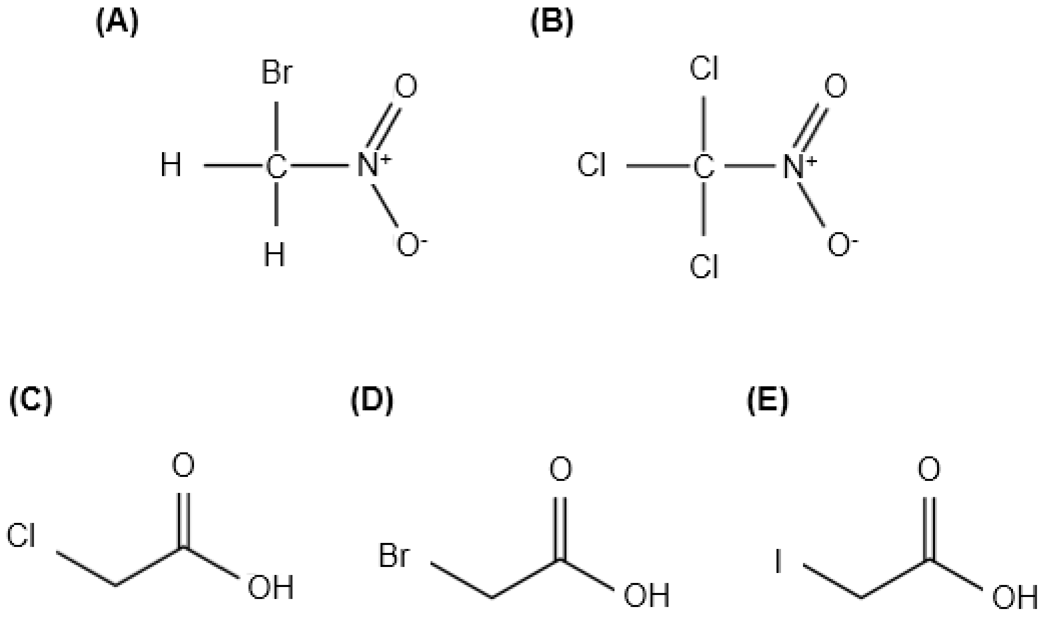


Figure 10. Chemical structures of the evaluated DBPs; (A) bromonitromethane, (B) trichloronitromethane, (C) chloroacetic acid, (D) bromoacetic acid and (E) iodoacetic acid.

For the HAAs, CAA was diluted in distilled water to an initial stock concentration of 100 mM. BAA's initial stock concentration was 50 mM, and for IAA it was 10 mM. Chemicals manipulation was conducted in a certified biological/chemical safety hood following the manufacturer's instructions.

3.2. Cell lines

Two non-tumorigenic immortalized cell lines were employed to evaluate the potential long-term carcinogenicity of HNMs and HAAs.

To evaluate HNMs transformation potential, the human bronchial epithelial cell line BEAS-2B was used. BEAS-2B cells offer an interesting study model considering that the respiratory is the principal tissue exposed to DBPs (Andelman 1985). This cell line was kindly provided by Dr. H. Norppa (Finnish Institute of Occupational Health, Finland).

The human urothelial cell line UROtsa was used in the second study. It is also a non-tumorigenic cell line, and supposes an interesting upgrade for our proposed experimental design. Bladder cancer is the main cancer type linked to DBPs exposure in epidemiological studies (Cantor 1982). Therefore, we consider that the bladder might be the main target of DBPs or the metabolites that may be causing the adverse effects. UROtsa cells were kindly provided by Dr. P. Simeonova (Health Effects Laboratory Division, USA).

For the analyses of the cellular secretome, we employed two tumoural cell lines, HCT116 and HeLa. The human colorectal carcinoma cell line HCT116 was provided by Dr. M.A. Peinado (ICO, Spain), and the human cervix epithelioid carcinoma cells HeLa, were provided by the CNIO (Spain).

To investigate the interactions between the potentially transformed BEAS-2B cells, the stroma and tumour microenvironment, we used a human lung fibroblast cell line, MRC5, bought from Coriell Institute Biorepository (USA).

All cell lines were maintained in DMEM high glucose medium (Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (FBS; PAA®, Pasching, Austria), 1% of non-essential amino acids (NEAA; PAA®) and 2.5 µg/mL plasmocin (InvivoGen, CA, USA) at 37 ºC in a humidified 5% CO_2 incubator.

3.2.1. Cell culture conditions for long-term treatments

With the objective of analysing the effects of DBPs on a more realistic setup, we carried out an 8 weeks' exposure study using concentrations of HNMs and HAAs closer to what can be found in treated waters. The conditions for long-term experiments were equal in both studies. Three separate closed vented 75-cm^2 flasks of BEAS-2B and UROtsa cells were exposed to non-cytotoxic concentrations of the two HNMs and the three HAAs.

Two concentrations were chosen for each DBP. For HNMs, the two concentrations were selected based on the cell toxicity observed after a 24 h treatment. In the case of HAAs, concentrations were based on the levels found in disinfected waters. In all cases, concentrations caused less than 10% of cytotoxicity.

The selected concentrations were:

- 1 and 5 µM for BNM.
- 5 and 25 µM for TCNM.
- 10 and 100 µM for CAA.
- 0.005 and 0.05 µM for BAA.
- 0.01 and 0.1 µM IAA.

The DBP-containing medium was changed every 48 h, and sub-confluent cells were passaged weekly. Non-exposed passage-matched cells were used as negative controls.

All *in vitro* cell transformation assays were performed after the 8 weeks of exposure, except for cell morphology and doubling times, which were evaluated throughout the whole exposure time.

3.3. Analysis of cytotoxicity and cell viability

Prior to long-term studies, cytotoxicity of the selected DBPs was evaluated in BEAS-2B and UROtsa cells to ensure that none of the concentrations selected for the long-term treatment were highly cytotoxic, and would not compromise the reliability of the experiments.

To determine the toxicity of BNM and TCNM in BEAS-2B cells, the Beckman counter method was employed using a ZTM Series coulter counter (Beckman Coulter, CA, USA).

BEAS-2B cells were plated in 6-well plates at a density of 50,000 cells per well and incubated overnight in complete DBP-free medium. The next day, medium was changed to fresh medium with concentrations ranging from 2 µM to 120 µM of BNM and from 10 µM to 1200 µM of TCNM. After a 24 h exposure, the cells were washed with PBS, and their number was assessed after trypsinization by a coulter counter. Cytotoxicity curves were derived from averaging three independent experiments, and the IC_{50} values were calculated using GraphPad prism version 5.03.

As for the second study, in order to determine UROtsa cell viability after CAA, BAA and IAA exposure, an assay that measures metabolic capacity was employed. In this case, we used resazurin, which provides a fast and highly reproducible way to measure cell viability. Resazurin is converted to the fluorescent-emitting molecule resorufin by viable cells after incubation at 37 ºC (Figure 11). Therefore, the fluorescence measured by a standard multiwell fluorimeter is proportional to the number of metabolically active, viable cells.

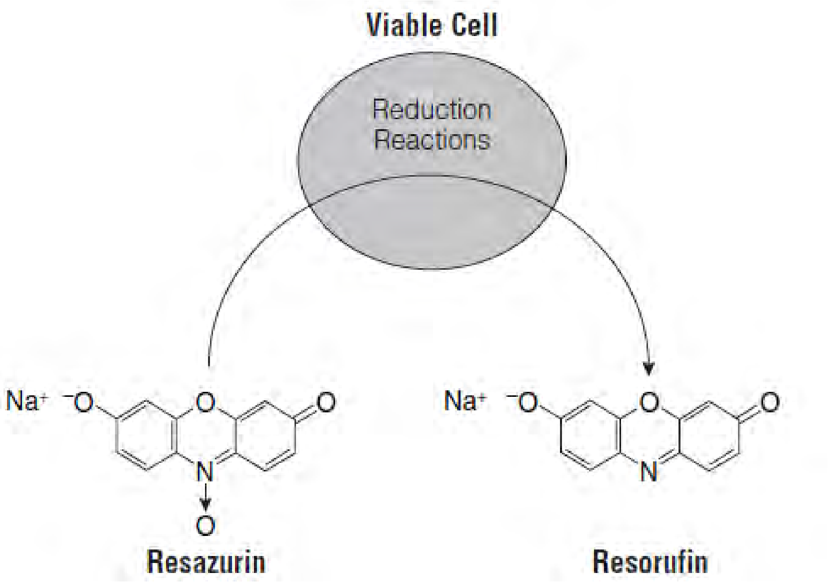


Figure 11. Conversion of resazurin to resorufin by viable cells resulting in the emission of fluorescence (Protocols & Applications Guide (Promega Corporation 2017)).

Cells were plated in opaque 96-well plates at a density of 5,000 cells per well and incubated overnight in DBP-free complete medium. The next day, medium was replaced with fresh medium with concentrations up to 10 mM of BAA, CAA or IAA. Twenty-four hours after the exposure, cells were washed with PBS and incubated in 0.44 µM of resazurin dye (Alfa Aesar, Karlsruhe, Germany) for 2 hours. Fluorescence emitted by resorufin was then measured using a microplate fluorimeter equipped with a 560 nm excitation / 590 nm emission filter set. Cytotoxicity curves derived from averaging three independent experiments, and the IC_{50} values were calculated using GraphPad prism version 7.00.

This same protocol was followed to determine whether the 8 weeks' treatment induced variations in the cells' resistance to an oxidative damage-inducing agent. UROtsa cells previously exposed for 8 weeks to the higher concentrations of the three HAAs were exposed to concentrations from 3 to 15 mM of $KBrO_3$ (Sigma-Aldrich, USA) for 24 h. Resazurin incubation and posterior data analysis were performed as described previously.

3.4. Genotoxicity: The comet assay

The capability of DBPs to produce genotoxic and oxidative DNA damage in UROtsa cells exposed to HAAs was evaluated after short-term and long-term exposures (4 h and 8 weeks, respectively) by the alkaline comet assay with and without the use of formamidopyrimidine DNA glycosylase (FPG) enzyme.

The comet assay is used to detect single- and double-strand DNA breaks in naked, supercoiled DNA. When a strand break appears, the DNA is allowed to migrate during the electrophoresis, forming a comet-like tail (Collins et al. 2008). The use of enzymes as FPG allows the detection of oxidative DNA damage, since it cuts DNA in positions where oxidized bases are detected (Azqueta et al. 2013).

For short-term exposure analysis, untreated UROtsa cells were seeded in 6-well plates at a density of 200,000 cells per well. The next day, cells were exposed to concentrations ranging from 750 to 1500 µM for CAA, 5 to 25 µM for BAA, and 2.5 to 8 µM for IAA for 4 h. Cells corresponding to positive controls were incubated 30 minutes in 200 µM MMS (Sigma-Aldrich, USA) and 5 mM $KBrO_3$.

For long-term exposure analysis, UROtsa cells and passage-matched controls were seeded in 6-well plates at a density of 200,000 cells per well, with DBP-containing medium when necessary. Positive controls were incubated as previously described.

Finally, to evaluate a possible resistance to oxidative damage after the long treatments, long-term exposed cells were incubated with 2.5 mM $KBrO_3$ for 30 minutes.

At the end of the treatments, cells were washed twice with PBS and collected by tripsinization. Following, a 1:10 mixture was prepared containing the cells and 0.75% agarose at 37 ºC. 7 µL drops of this mixture were placed onto the Gelbond® film (GF, 10.5 x 7.5 cm), with 3 replicate drops from each treatment.

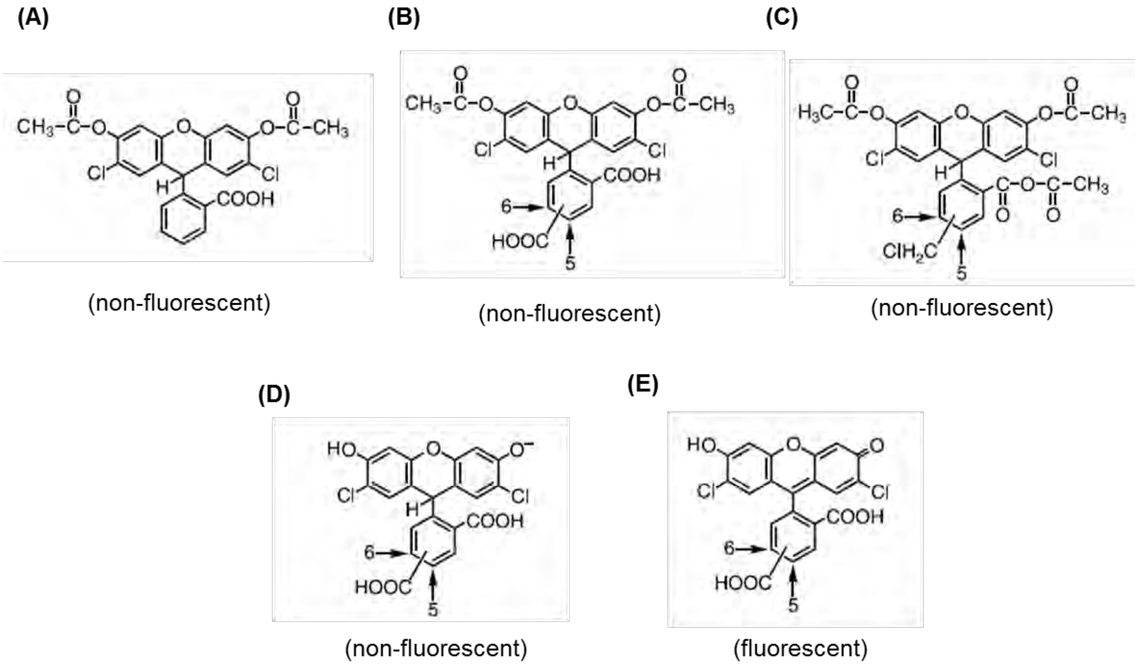
Two identical GFs were processed simultaneously for each experiment. Both films were lysed overnight by immersion in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 M Tris base, 1% Triton X-100, 1% lauroyl sarcosinate, 10% DMSO, pH 10) at 4 °C. Following the lysis, one film was incubated for 30 min at 37 °C in enzyme buffer containing FPG, and the other one in enzyme buffer alone.

Both films were washed with electrophoresis buffer (0.3 M NaOH and 1 mM Na_2EDTA, pH 13.2), and placed into a horizontal gel electrophoresis tank. Both films were incubated 35 min in the same buffer to allow DNA unwinding. Then, electrophoresis was performed at 0.8 V/cm and 300 mA for 20 min at 4 °C. After the electrophoresis, both films were rinsed with cold PBS for 15 min, fixed in absolute ethanol for 2 h and air-dried overnight at room temperature.

Prior to observation, GFs were stained for 20 min with 1/10,000 diluted SYBR Gold (Molecular Probes). Once mounted on an acrylic slide and covered with an appropriate coverslip, comet tails were measured using the Komet 5.5 Image analysis system (Kinetic Imaging Ltd., Liverpool, UK). 100 cells were scored for each treatment, and the percentage of DNA in tail was the parameter used to measure the DNA damage. Final results were obtained averaging the results of three independent experiments.

3.5. Intracellular generation of ROS

Intracellular ROS was measured by plate fluorescence, using the 6- carboxy-2,7’-dichlorodihydro-fluorescein diacetate (DCFH-DA) assay. DCFH-DA is a fluorescent probe that allows the detection of several oxygen-derived reactive molecules like H_2O_2, $ONOO^-$, lipid hydroperoxides and $O_2^{\cdot -}$. Once inside the cell, DCFH-DA is cleaved to 2’, 7’– dichlorofluorescin (DCFH). This compound is then oxidized in the presence of intracellular ROS, producing 2’, 7’– dichlorofluorescein (DCF), a fluorescent molecule figure 12.



(non-fluorescent) (non-fluorescent) (non-fluorescent)

(non-fluorescent) (fluorescent)

Figure 12. Structures of (A) DCFH-DA, (B) carboxy-DCFH-DA, (C) chloromethyl-DCFH-DA, (D) deacetylated DCFH, and (E) the deacetylated, oxidized product, DCF. Modified after www.thermofisher.com (Thermo Fisher Scientific Inc. 2017).

To detect intracellular ROS, UROtsa cells were seeded at a density of 5,000 per well in opaque 96-well plates, and incubated overnight. The next day, cells were pre-incubated with DCFH-DA in DMEM medium for 30 min T 37 ºC.

Cells were then washed twice in PBS to eliminate all remaining DCFH-DA, and were treated with the selected concentrations of the HAAs, followed by incubation at 37 ºC. Positive controls were treated with 10 mM $KBrO_3$ and 100 mM H_2O_2 (Sigma-Aldrich, USA).

Plate fluorescence was measured after 30 min, 1 h, 2 h and 4 h of exposure. ROS levels are displayed as the percentage of the untreated control mean fluorescence.

3.6. *In vitro* cell transformation assays

Cell malignant transformation was evaluated in both BEAS-2B and UROtsa cells after an 8 weeks' exposure to the selected DBPs via the analysis of a wide range of cancer phenotypical markers (Figure 13).

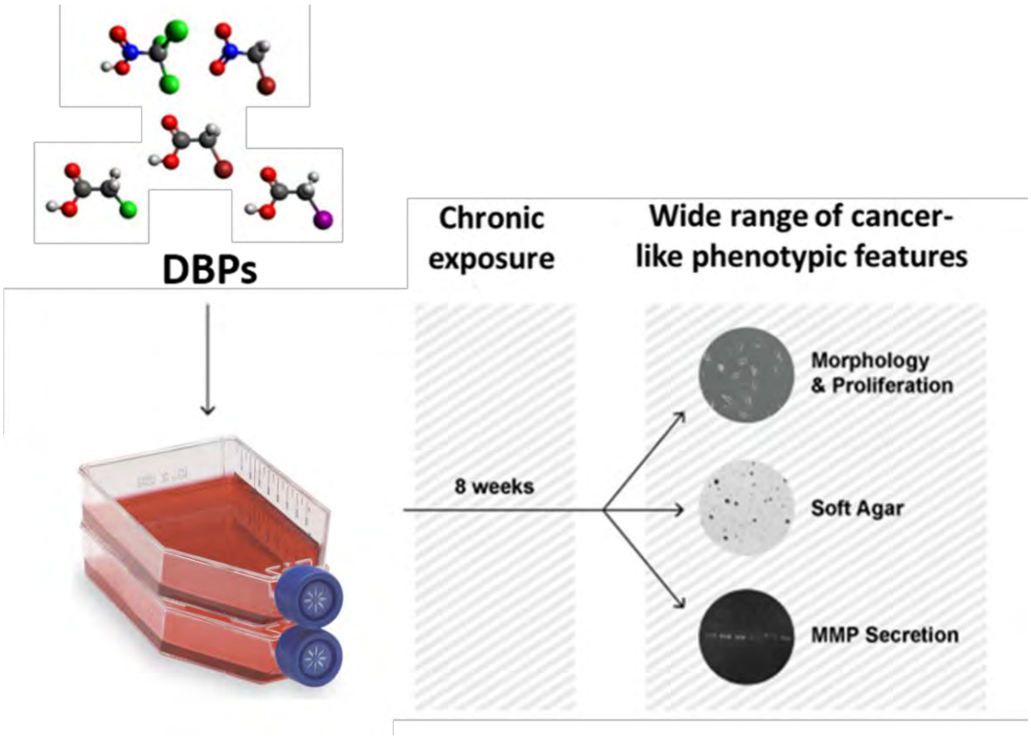

DBPs
Chronic exposure
Wide range of cancer-like phenotypic features
8 weeks
Morphology & Proliferation
Soft Agar
MMP Secretion

Figure 13. Experimental design for *in vitro* long-term carcinogenicity analyses.

3.6.1. Cell proliferation

Cellular proliferation was evaluated throughout the whole exposure period to analyse whether the exposure to DBPs can induce changes in the cell cycle. BEAS-2B and UROtsa cells were plated in 6-well plates at a density of 50,000 and 100,000 cells respectively, once a week during the eight weeks of treatment.

Cells were collected by trypsinization and counted at 24 h intervals by the Beckman counter method. Proliferation was defined as the time necessary for doubling the cell population, and it was calculated per the equations referred in http://www.doubling-time.com/compute.php (Roth 2006).

3.6.2. Cell morphology

Changes in cell morphology were monitored throughout the 8 weeks' exposure period in a qualitative manner. Morphological changes, expected due to the acquisition of a tumoural phenotype, include characteristics of an epithelial to mesenchymal transition (EMT). More specifically, the most visible change is that cells undergoing EMT show a very clear “spindle-like” morphology (Tse & Kalluri 2007).

BEAS-2B and UROtsa long-term exposed cells and unexposed passage-matched controls were plated in 6-well plates at a density of 50,000 and 100,000 cells, respectively, and pictures were taken 24, 48 and 72 hours after seeding by inverted microscopy. Exposed cells morphology was always compared to passage-matched untreated controls.

3.6.3. Anchorage independent growth capacity

The colony formation capacity of BEAS-2B and UROtsa cells was assessed after long-term exposures to HNMs and HAAs to determine if a long-term exposure to these DBPs promotes a carcinogenic transformation. In all cases, the number and size of colonies formed in treated cultures is compared to passage-matched controls.

In both studies, cells were collected and individualised using a 30 µm filter. 10,000 cells were suspended in a 1:1:1 mixture containing 1X DMEM (supplemented with 10% FBS, 1% NEAA, 1% L-Glu 200 mM and 2.5 µg/mL Plasmocin), 2x DMEM (with 2X supplements) and 1.2% Bacto Agar (DIFCO, MD, USA). The mixture was then plated in 6-well plates over a 0.6% base agar (in supplemented 2X DMEM) and incubated at 37 ºC for 21 days.

After this time, cell colonies were stained overnight with 1 mg/mL of (2-p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT, Sigma, MO). Plates were scanned and colony counting was performed using OpenCFU open-source Software.

A modified version of the assay was performed using 48 and 72-h conditioned medium (CM) from long-term exposed BEAS-2B and UROtsa cells and passage-matched controls, to assess their capacity to promote the malignant growth of tumour cells via their secretome.

In this case, a tumoural cell line is used to evaluate the capability of the long-term exposed cells CM to promote and induce the growth of the tumoural cell line. For the first study, the tumour-inducing capability of BEAS-2B’s secretome was assessed using HCT116 cells. On the second study, HeLa cells were used to assess the tumorigenic capability of UROtsa cells' secretome.

A total of 10,000 individualised HCT116 or HeLa cells were resuspended in the CM of the corresponding cells, and mixed in a 1:1:1 ratio with supplemented 2x DMEM and 1.2% bacto agar. The subsequent steps of the protocol were performed as described above. Plates were incubated at 37 ºC for 15 days and stained as previously described.

It has also been described that transformed cells promote tumoural growth in an indirect way, stimulating stroma cells to secrete factors that will promote their proliferation (Bach et al. 2016). Thus, to further analyse the cancer-like phenotype in long-term exposed BEAS-2B, their 48 h CM was used to incubate lung fibroblast MRC5 cells. Then, the 48 h CM of stimulated MRC5 stroma cells was collected and used for the soft-agar assay with HCT116 cells as indicated above.

HCT116 and HeLa cells seeded in 1xDMEM supplemented with 10, 20 and 30% FBS were used as a positive control for cell growth and dose-dependent growth response.

3.6.4. MMPs secretion

In our first study, we evaluated the activity of secreted matrix metalloproteinases 2 and 9 of long-term exposed BEAS-2B and passage-matched controls by zymography. A standard zymography resolves cells CM under denaturing conditions in precast gels containing its gelatine substrate by SDS-PAGE. Following renaturing, developing and staining steps allow the observation of protease activity of the MMPs found in the CM, that appear as the degradation of the gelatine substrate.

To quantify the secretion of MMPs in long-term exposed BEAS-2B, cells were cultured in basal DMEM medium (without serum or supplements) for 72 h to obtain a CM. Then the zymography was performed following the manufacturer's instructions (Bio-Rad, Hercules, CA).

The area of protease activity was measured densitometrically using the ImageJ analysis program.

To analyse whether long-term exposed BEAS-2B could prompt the secretion of these enzymes, MRC5 fibroblasts were grown in 48-h CM of long-term exposed BEAS-2B and passage-matched controls. Subsequently, the 48-h CM of stimulated MRC5 stroma cells was collected and used for zymography as indicated above. Medium containing 10% FBS was used as positive control in all cases.

3.7. Statistical analysis

For each assay, three independent experiments were performed in triplicates and the mean and SEM were calculated.

One-way analysis of variance followed by Dunnett’s multiple comparison test was performed to compare treated with untreated time-matched controls. In all cases, a two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 7.00.

4. RESULTS

4. RESULTS

The results obtained during the elaboration of this Thesis can be divided into two different studies, each one aiming to assess the tumorigenic potential of two classes of DBPs: HNMs and HAAs.

4.1. First study: *In vitro* studies on the tumorigenic potential of the halonitromethanes trichloronitromethane and bromonitromethane

In this study, we aimed to fill-in the existent gaps in the literature determining the carcinogenic potential of two HNM species (BNM and TCNM). Thus, a more systematic model of exposure is proposed wherein a human pulmonary cell line, BEAS-2B, is exposed to subtoxic concentrations of two different HNMs for an extended period of time, considering that the respiratory tract is one of the main entry routes of DBPs into the human body.

4.1.1. Short-term analyses: Cytotoxicity of BNM and TCNM.

Prior to the long-exposure treatment, HNMs cytotoxicity on BEAS-2B cells was evaluated to ensure the selection of low cytotoxicity concentrations for long-term experiments. Hence, a wide range of concentrations of each HNM were tested. Short-term (24 h) cell viability was assessed with concentrations that ranged from 2 µM to 120 µM for BNM and from 10 µM to 1200 µM for TCNM.

BNM and TCNM displayed significant differences in terms of toxicity (Figure 14). Thus, BNM was more cytotoxic than TCNM, being the IC_{50} values 32 ± 10 µM and 111 ± 17 µM, respectively. Both HNMs were clearly cytotoxic at 50 µM, with a mean viability of 32.4 ± 7.2% and 64.2 ± 7.4% for BNM and TCNM, respectively, when compared to untreated cells.

Given these results, two different concentrations of each HNM inducing less than a 10% decrease in viability were chosen to carry out the subsequent long-term experiments. The selected concentrations were 1 and 5 µM for BNM and 5 and 25 µM for TCNM.

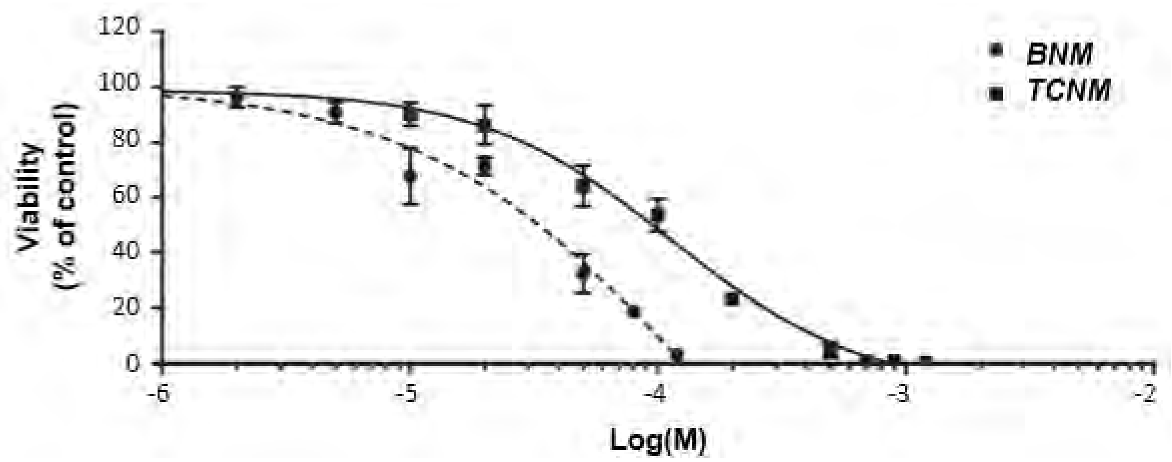


Figure 14. BEAS-2B cell viability curves after 24 h of exposure to increasing concentrations of BNM and TCNM.

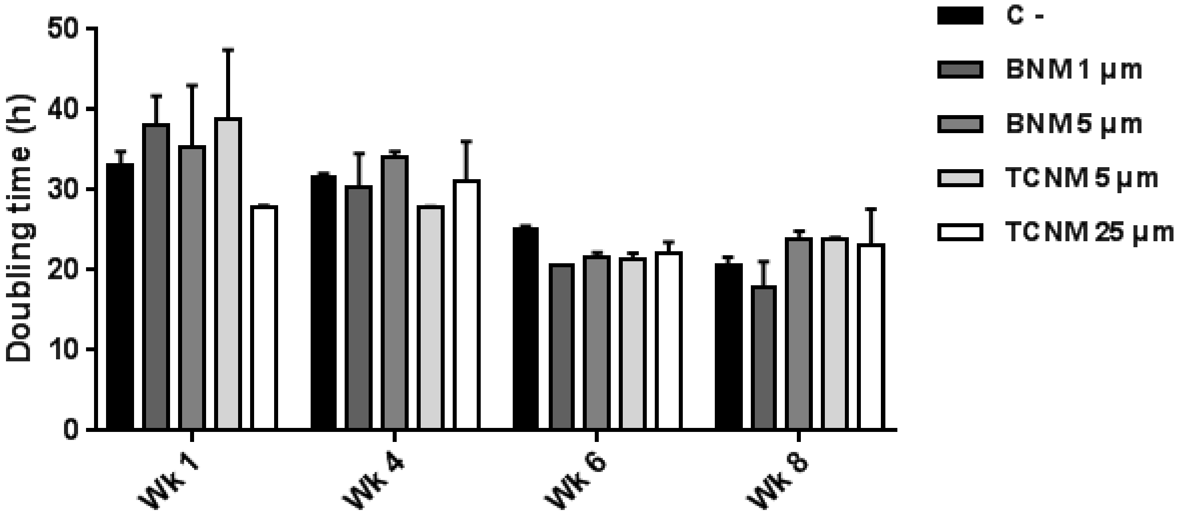
4.1.2. Long- term carcinogenicity analysis

Previous *in vitro* studies analysing the carcinogenic potential of most DBPs species evaluated only short exposure times. The only long-term studies available employed *in vivo* models, with the difficulties that those models suppose in terms of extrapolation of the results to human populations given the unawareness of DBPs metabolisation, in addition to the high costs in terms of time and money. According to that, our proposal of study focuses on long-term exposures and sub-toxic concentrations.

With the objective of analysing the tumorigenic potential of HNMs in a model that reflects better human exposure, BEAS-2B cells were grown in contact with the two selected HNM species, BNM and TCNM, for eight weeks. After the exposure period, several markers of cell transformation were analysed, including cell proliferation and morphology, anchorage-independent growth capacity and secretion of MMPs, in order to determine whether or not BEAS-2B cells acquired a transformed phenotype.

4.1.2.1. Cell proliferation

Cell proliferation was one of the selected markers to assess whether a carcinogenic transformation was taking place in cells exposed to HNMs, as cell cycle deregulation is one of the most common and clear symptoms of cell malignant transformation.



	Control		BNM 1 µm		BNM 5 µm	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Wk 1	33,15	1,17	38,24	2,44	35,42	5,36
Wk 4	31,50	0,41	30,43	2,92	34,12	0,49
Wk 6	25,22	0,22	20,69	0,35	21,62	0,43
Wk 8	20,54	0,79	17,81	2,34	23,84	0,76

	TCNM 5 µm		TCNM 25 µm	
	Mean	*SEM*	*Mean*	*SEM*
Wk 1	38,84	6,09	27,82	0,21
Wk 4	27,77	0,15	31,05	3,58
Wk 6	21,44	0,51	22,07	1,02
Wk 8	23,92	0,14	23,07	3,25

Figure 15. Doubling times of long-term exposed BEAS-2B and unexposed passage-matched controls measured at week 1, 4, 6 and 8 of exposure.

Cellular doubling times measured throughout the exposure period indicated a clear increase in cellular proliferation in all cases, including the untreated controls (Figure 15). Doubling time was drastically reduced between the first and sixth week of treatment, and a more stable cell proliferation could be observed between weeks six and eight. In the negative controls, the average doubling time in week one was 33.15 ± 1.17 h. It showed a small decrease to 31.5 ± 0.41 h in week four, radically decreasing to 25.22 ± 0.22 h in week six, and finally got to 20.54 ± 0.79 h in week eight. A similar decrease in the proliferation rate was observed on all treated cell samples, showing no significant differences between the non-exposed controls and the cells exposed to HNMs.

Thus, the obtained indicated that neither BNM nor TCNM treatment increased the proliferation rates significantly when compared to time-matched controls, and the changes observed in cell proliferation over time might be a result of cell culture and manipulation.

4.1.2.2. Cell morphology

Changes in cell morphology were monitored as well throughout the whole exposure period, considering the detection of any changes in cell morphology as another indicator that cells may be under a process of carcinogenic transformation.

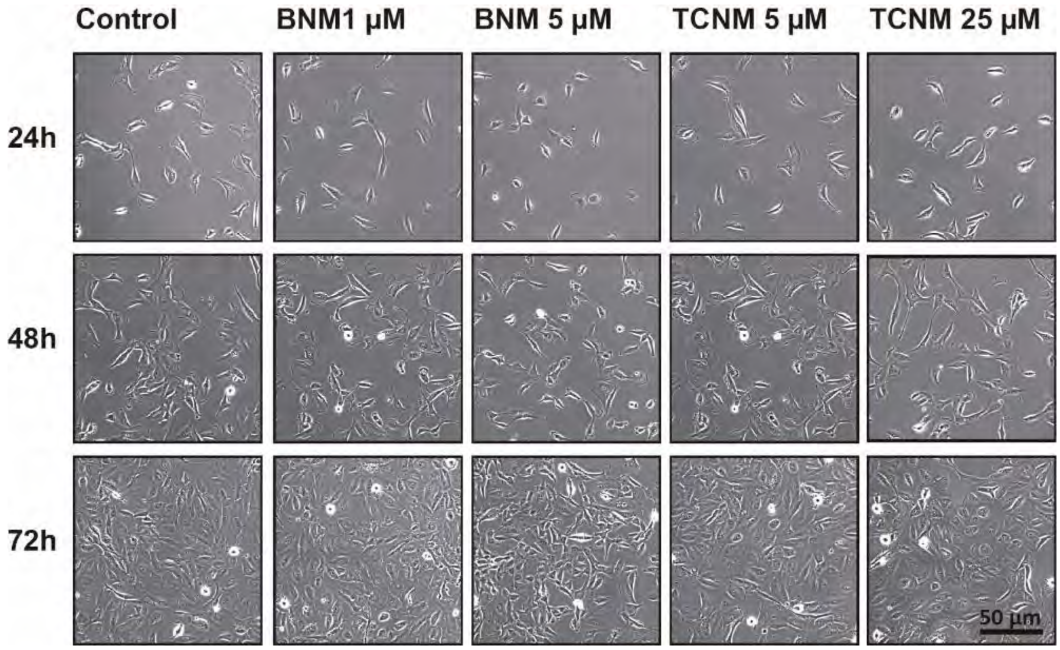


Figure 16. Cell morphology of BEAS-2B cells after 8 weeks of BNM and TCNM long-term exposure. Representative pictures of cell morphology taken 24, 48 and 72 h after seeding.

A prolongated exposure to HNMs did not prompt any noticeable changes in cell morphology, and no differences were observed throughout the whole exposure time when the cells grown in the HNMs-containing medium were compared to passage-matched untreated controls (Figure 16). In no case cells acquired the “spindle-like” morphology that would indicate that a EMT is taking place.

4.1.2.3. Anchorage independent growth capacity

BEAS-2B cells are known to form numerous big colonies in soft-agar after being exposed to a carcinogen (Son et al. 2012; Vales et al. 2015; Vales et al. 2016), in contrast to the smaller colonies that can be detected in non-transformed BEAS-2B. The capability of these cells to grow anchorage-independently was assessed in a soft-agar colony formation assay after the 8 weeks of exposure.

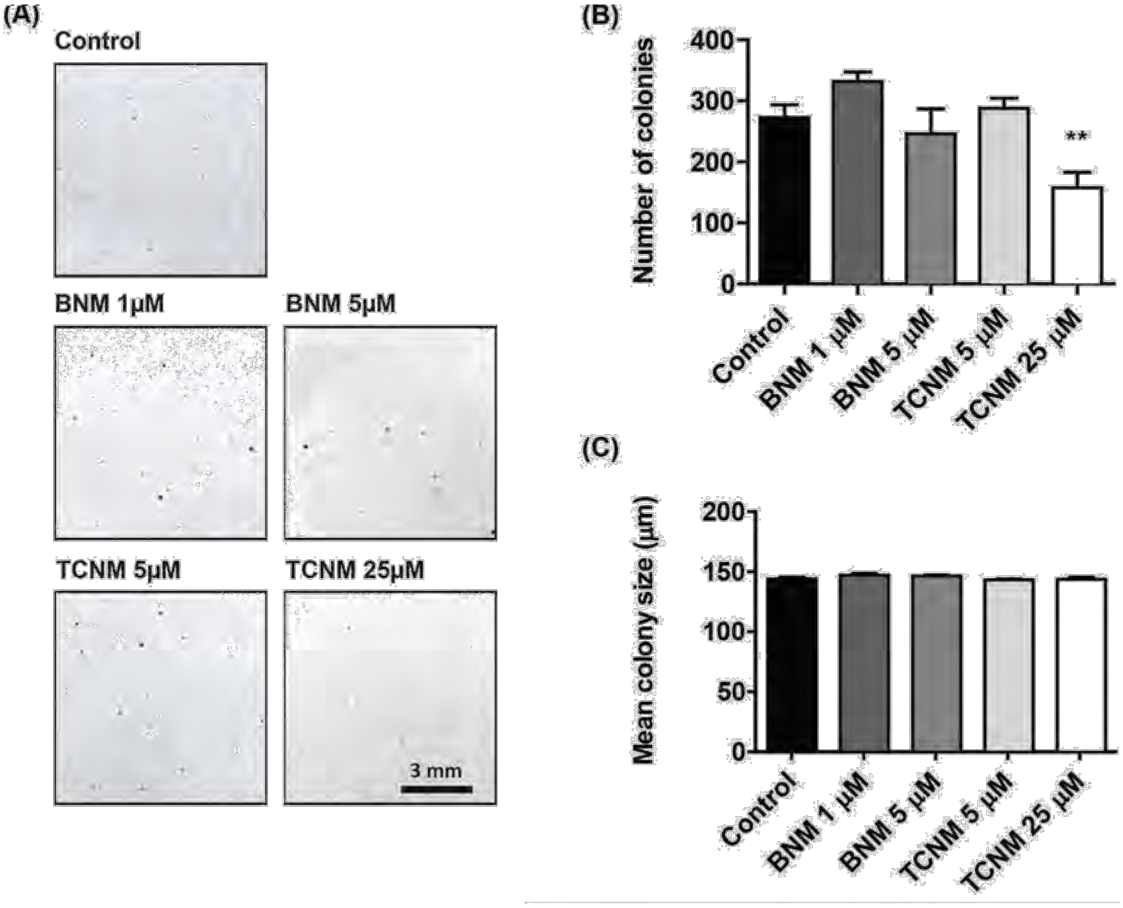


Figure 17. Anchorage-independent growth capacity of BEAS-2B cells after 8 weeks of BNM and TCNM long-term exposure. A) Representative images of BEAS-2B colonies in soft-agar. B) Mean number of colonies per well C) Mean colony size per treatment.

In all cases, BEAS-2B cells formed relatively small-sized colonies (Figure 17A). Colonies formed by the long-term exposed cells were compared to the untreated controls in all cases, since all cultured cell lines presented a certain degree of anchorage independent growth capacity.

No relevant variations in colony number appeared among treatments (Figure 17B). Control samples formed a mean of 273 ± 21 colonies, while cells long-term exposed to displayed 332 ± 16 and 246 ± 40 colonies for its lowest and highest concentrations, respectively. On the other hand, cells long-term exposed to 5 µM TCNM formed an average of 288 ± 16, while the number of colonies formed by cells exposed to 25 µM TCNM was significantly lower, 157 ± 25 colonies. This decrease in the number of colonies might be due to a certain degree of cytotoxicity of TCNM, which was not evident after the 8 weeks of exposure but becomes relevant following the subsequent 21 days of growth on soft-agar.

When colony sizes were considered (Figure 17C), the mean size was similar in all cases, including the negative control. The mean colony diameter in the negative control cells was of 142.65 ± 1.51 µm, against 145.68 ± 1.58 and 145.06 ± 0.77 µm in cells exposed to 1 and 5 µM BNM respectively, and 141.84 ± 0.74 and 142.24 ± 1.81 µm in cells exposed to 5 and 25 µM TCNM respectively.

4.1.2.4. Analysis of the long-term HNM-exposed cells secretome

Previous results suggest that HNMs long-term exposed cells did not acquire a transformed phenotype. However, the exposure might induce other changes that can eventually lead to a carcinogenic outcome.

The interplay between the different cell populations and their environment has a big role on determining the formation and invasion capacity of a tumour. Cells undergoing a malignant transformation secrete paracrine signals that enhance the tumorigenic potential of nearby cells (del Pozo Martin et al. 2015), being another interesting feature that can be analysed to establish the malignancy of a certain cell line. Consequently, the analysis of the secretome of exposed cells has been included in this study.

4.1.2.4.1.HNM-exposed cells’ capacity to induce tumoural growth

To evaluate the capability of long-term exposed BEAS-2B cells to promote tumour growth, the tumoural cell line HCT116 was grown in soft-agar in contact with the CM of the long-term exposed cells. Subsequently, colonies formed by HCT116 cells in the soft-agar assay were counted and measured.

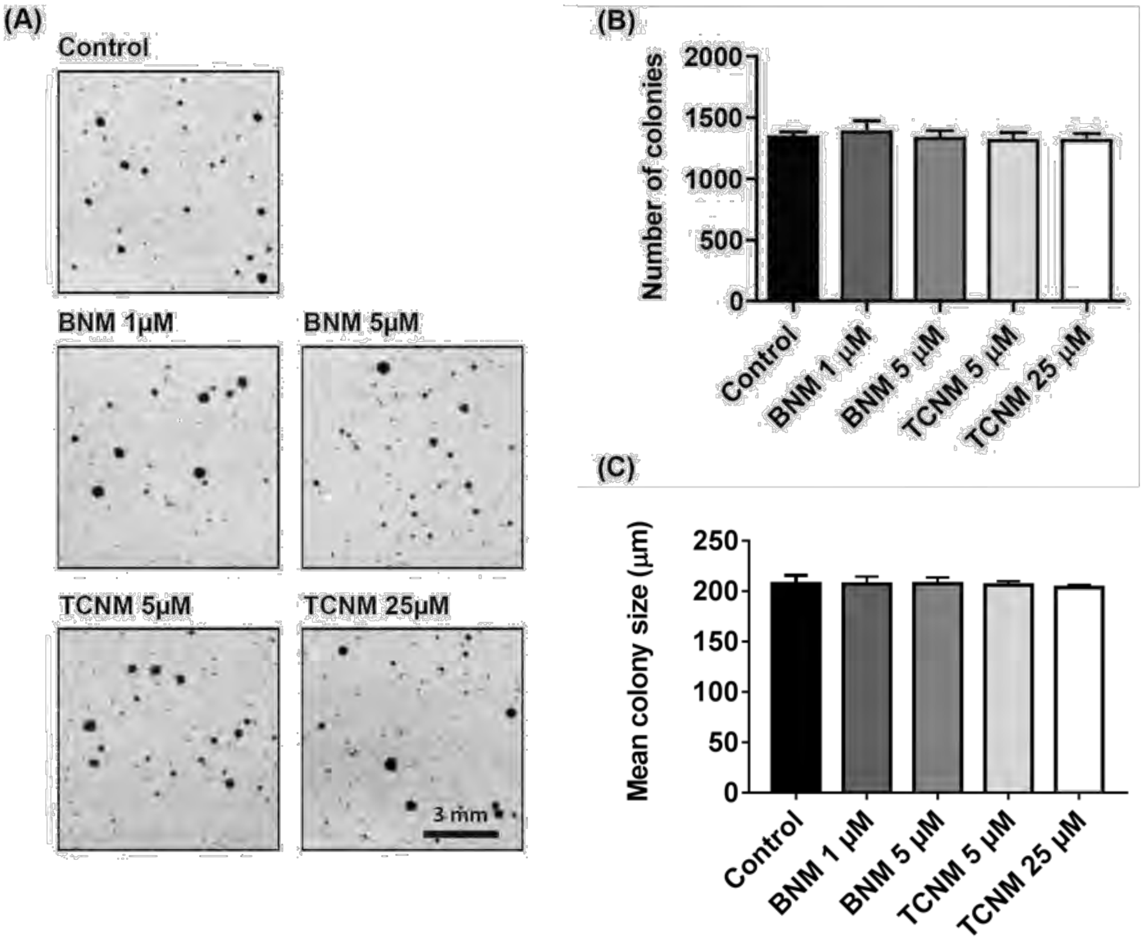


Figure 18. Promotion of HCT116 anchorage-independent cell growth by the secretome of BEAS-2B after 8 weeks of BNM and TCNM long-term exposure. A) Representative images of HCT116 colonies in soft-agar. B) Mean number of HCT116 colonies per plate. C) Mean colony sizes per treatment.

As expected given their tumoural nature, HCT116 cells formed a higher number of colonies appreciably bigger than BEAS-2B cells (Figure 18A). Nevertheless, o differences in colony number nor size were observed as a consequence of HNMs treatments of BEAS-2B cells.

Cells grown in contact with untreated BEAS-2B CM displayed an average of 1,338 ± 44 colonies. Similar mean values were observed in HCT116 cells grown in contact with the CM of the HNMs-exposed BEAS-2B, showing an average of 1,382 ± 91 and 1,329 ± 62 for 1 and 5 µM BNM, and 1,309 ± 68 and 1,309 ± 61 colonies for the CM of 5 and 25 µM TCNM (Figure 18B).

Colony size was also considered in this case, as an indicator of the exposed-cells secretome to enhance tumoural growth. No significant differences appeared in this parameter either (Figure 18C). The average size of HCT116 cells exposed to untreated cells CM was 216.00 ± 1.17 µm, whereas HCT116 colonies grown in HNM-exposed BEAS-2B CM showed mean values of 214.70 ± 2.84 and 213.90 ± 4.34 µm for 1 and 5 µM BNM, and 210.00 ± 2.74 and 206.53 ± 5.70 µm for 5 and 25 µM TCNM, respectively.

4.1.2.4.2.HNM-exposed cells’ capacity to influence the stromal cells’ secretome

To assess long-term exposed cells’ influence on stroma cells, the tumour-promoting capacities of stroma cells grown in contact with long-term exposed BEAS-2B secretome was analysed. This parameter can help to determine whether BEAS-2B are undergoing a process of carcinogenic transformation.

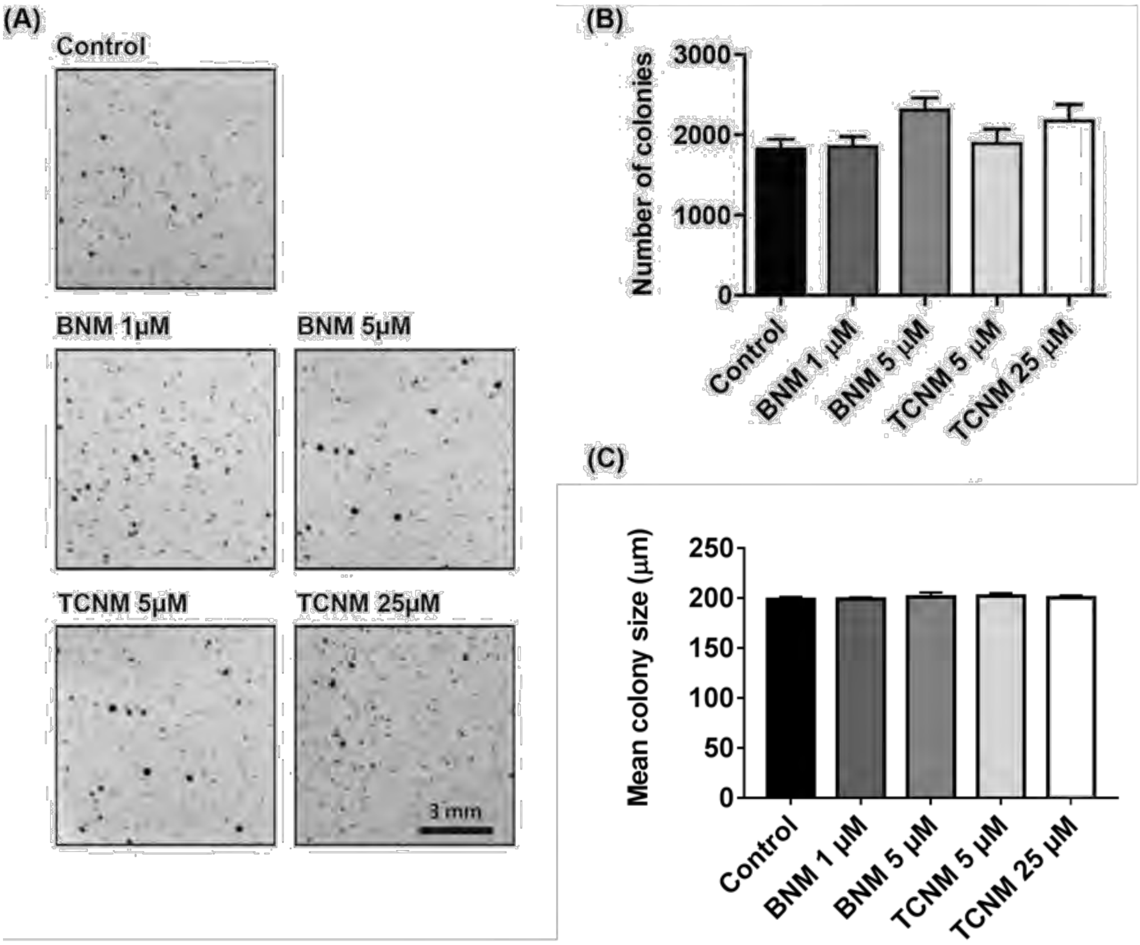


Figure 19. Promotion of HCT116 anchorage-independent cell growth by the secretome of stromal MRC-5 cells grown in HNMs-long-term CM. A) Representative images of HCT116 colonies in soft-agar. B) Mean number of HCT116 colonies per well. C) Mean colony sizes per treatment.

Thus, MRC5 lung stroma fibroblasts were grown in contact with HNM long-term-exposed BEAS-2B CM. Subsequently, HCT116 cells were plated in contact with MRC5's secretome in a soft-agar assay.

HCT116 cells formed a considerable number of colonies (Figure 19A). No significant differences appeared among treatments. HCT116 cells grown in CM of control MRC5 presented an average number of colonies of 1,822 ± 119, similar to the number of colonies formed by HCT116 cells grown in the CM of stromal cells treated with the CM of exposed BEAS-2B, which was of 1,848 ± 127, 2,306 ± 149, 1,889 ± 178, and 2,171 ± 204 for 1 µM BNM, 5 µM BNM, 5 µM TCNM and 25 µM TCNM, respectively (Figure 19B).

When mean colony sizes were analysed, the results consistently showed no HNM-associated effect. Hence, HCT116 cells grown in CM of control MRC5 cells showed a mean colony size of 198.61 ± 2.15 µm, whereas colony sizes of HCT116 cells grown in CM of exposed MRC5 cells were 199.25 ± 1.66, 201.06 ± 4.74, 202.19 ± 2.83 and 200.56 ± 2.24 µm for 1 µM BNM, 5 µM BNM, 5 µM TCNM and 25 µM TCNM, respectively (Figure 19C).

4.1.2.4.3.Secretion of MMPs

Finally, the content of extracellular proteinases such as matrix metalloproteinases (MMPs) in exposed cells CM was analysed by zymography, where an increased MMP activity would be indicative of cell malignant transformation.

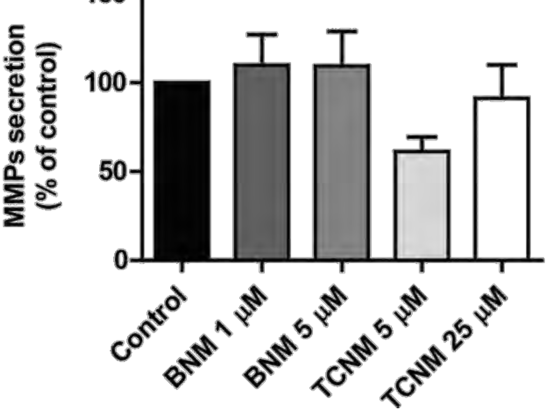


Figure 20. Activity of MMP 2+9 secreted by BEAS-2B after 8 weeks of BNM and TCNM long-term exposure.

BEAS-2B cells long-term exposed to the HNMs did not show an increased secretion of MMP 2+9 (Figure 20). A decrease was observed in the levels of MMPs secreted by cells exposed to 5 µm TCNM, but it was not considered biologically-relevant.

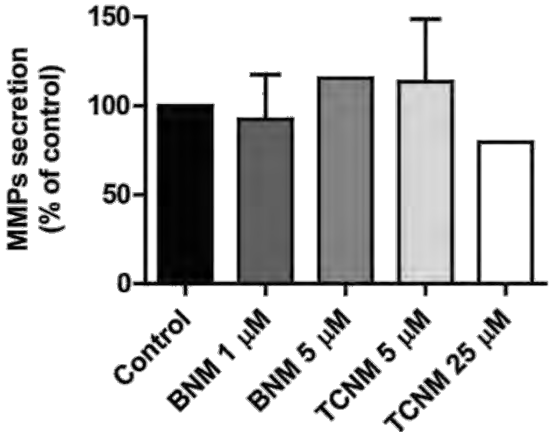


Figure 21. Activity of MMP 2+9 secreted by stromal MRC5 cells exposed to CM of long-term exposed BEAS-2B cells.

The secretion of MMPs is mainly conducted by stroma cells as a response to stimuli received from tumoural cells, hence the interest in analysing the secretion of MMP 2+9 in the MRC5 cells grown in CM of long-term-exposed BEAS-2B cells.

No biologically-relevant differences were observed in any case (Figure 21), being the long-term exposed cells unable to increase the secretion of MMPs 2 and 9 of stromal cells.

4.2. Second study: Hazard assessment of haloacetic acids in human urothelial (UROtsa) cells

Given the high abundance of haloacetic acids in disinfected waters (up to 2,600 µg/L) its genotoxic capacities have been widely investigated. Similarly to HNMs, there are few *in vitro* studies directly measuring the carcinogenic potential of these chemicals. In addition, most of the *in vitro* research conducted with these DBPs species used short times of exposure and tested relatively high concentrations.

The aim of this study was to analyse the possible role of HAAs in the increased bladder-cancer incidence linked to DBPs exposure. In this case, we focused on analysing the effects of a prolonged exposure to HAAs at concentrations found in treated waters worldwide, and thus, setting a more realistic model of exposure.

4.2.1. Short-term analyses

4.2.1.1. Evaluation of the cytotoxicity of the mono-HAAs.

Preceding the long-treatments, short-term (24 h) cell viability after HAAs exposure was assessed. Cytotoxicity of the three mono-HAAs in UROtsa cells was evaluated using a wide range of concentrations.

All three HAAs displayed cell toxicity after 24 h of exposure, however, appreciable differences appeared in UROtsa cells' sensitivity to each DBP (Figure 22).

IAA showed the strongest cytotoxic effect, with an IC_{50} value of 2.4 ± 0.2 µM. BAA presented an IC_{50} of 6.7 ± 0.4 µM. Interestingly, CAA cytotoxicity was more than 100 times lower than the brominated and iodinated acids, with an IC_{50} of 794 ± 61 µM, indicating that there's a correlation between cytotoxicity and the relative atomic mass of the halogen atom present in the molecule.

Subtoxic concentrations similar to what can be found in treated waters were chosen to perform the long-term experiments. The selected concentrations were 10 and 100 µM for CAA, 0.005 and 0.05 µM for BAA, and 0.01 and 0.1 µM for IAA.

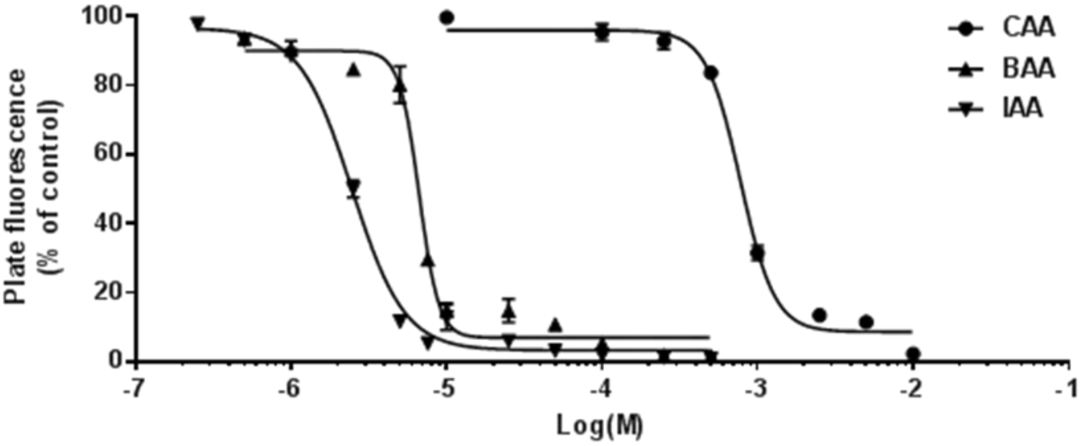


Figure 22. UROtsa cell viability curves after 24 hr of exposure to various concentrations of CAA, BAA and IAA.

4.2.1.2. Genotoxicity

Also prior to long-term treatments, the genotoxic potential of the three HAAs was evaluated in UROtsa cells by the comet assay, with and without FPG to assess these DBPs capacity to induce DNA damage, and at the same time its capacity to induce oxidative DNA damage.

No relevant increases in the levels of DNA damage was observed in cells exposed to non-cytotoxic concentrations of all three HAAs for 4 h when compared to the negative control cells. Although the percentage of tail DNA was under 5% in all treatments, the positive control (MMS) produced wide levels of DNA breaks, indicating that the assay was well conducted. Thus, results support the incapability of the selected DBPs to produce direct DNA damage under the tested conditions (Figure 23A,C,E).

Conversely, a significant increase in the levels of oxidative damage was observed for CAA at concentrations higher than 1 mM and in all tested concentrations of BAA and IAA (Figure 23B,D,F), with a percentage of DNA in tail of 16.44 in the negative control and percentages ranging from 21.70 to 39.65 in DBPs-exposed cells. Both BAA and IAA were capable of inducing oxidative DNA damage in a dose-dependent manner, but this dose-dependency was not observed for CAA.

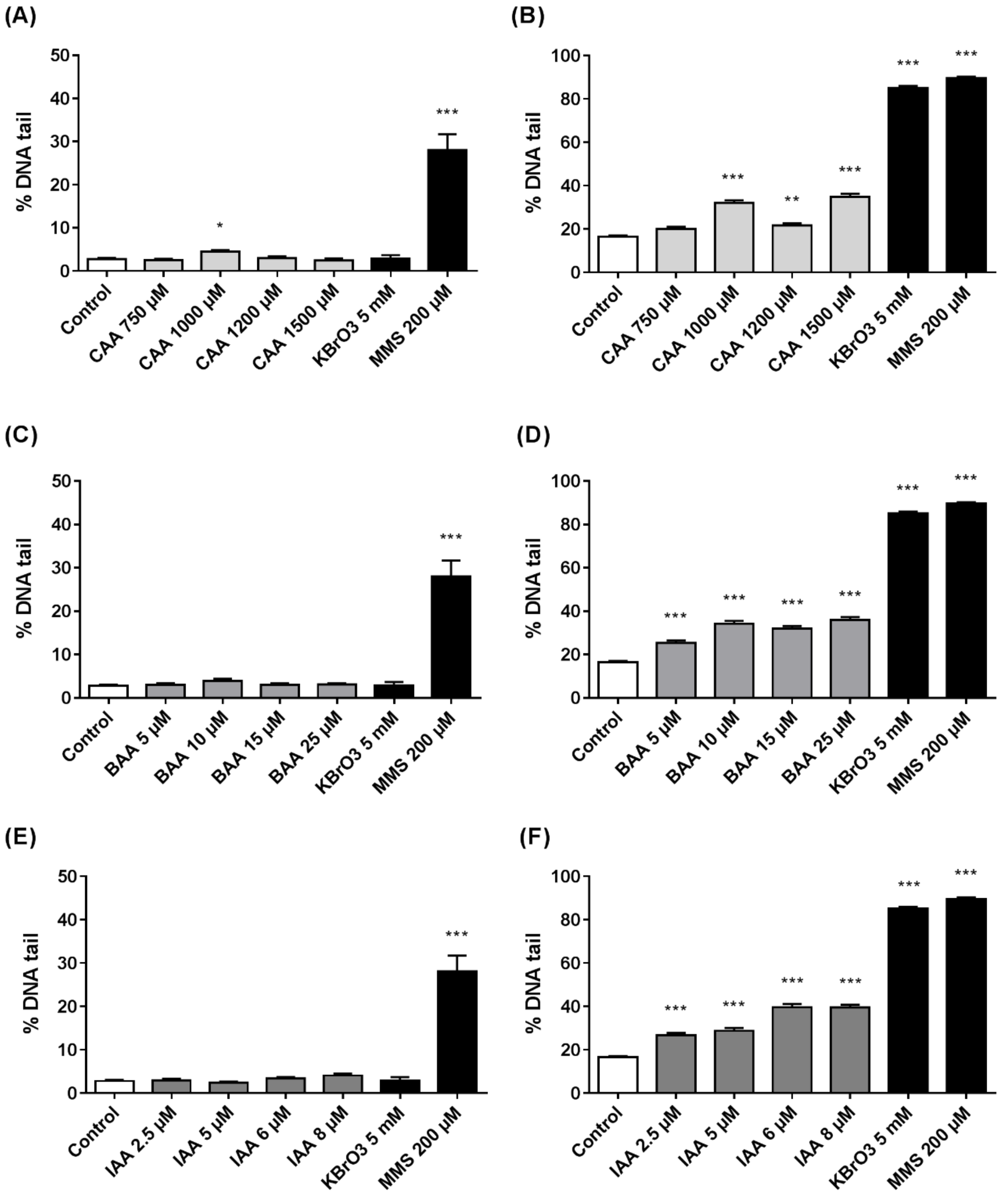


Figure 23. Genotoxic (A, C, E) and oxidative (B, D, F) DNA damage in UROtsa cells after 4 hours' exposure to non-cytotoxic concentrations of CAA, BAA and IAA.

This induction of DNA oxidative damage could have mutagenic effects if not repaired promptly, even though DNA breakage was not observed at the analysed concentrations.

4.2.1.3. Intracellular generation of ROS

Previous data suggests that HAAs mode of action is based on the induction of oxidative damage; thus, their ability to induce oxidative stress was evaluated via the measurement of intracellular ROS.

ROS formation in cells exposed to HAAs was measured in a time-course experiment, with measures taken after 30 minutes, 1, 2 and 4 hours of exposure.

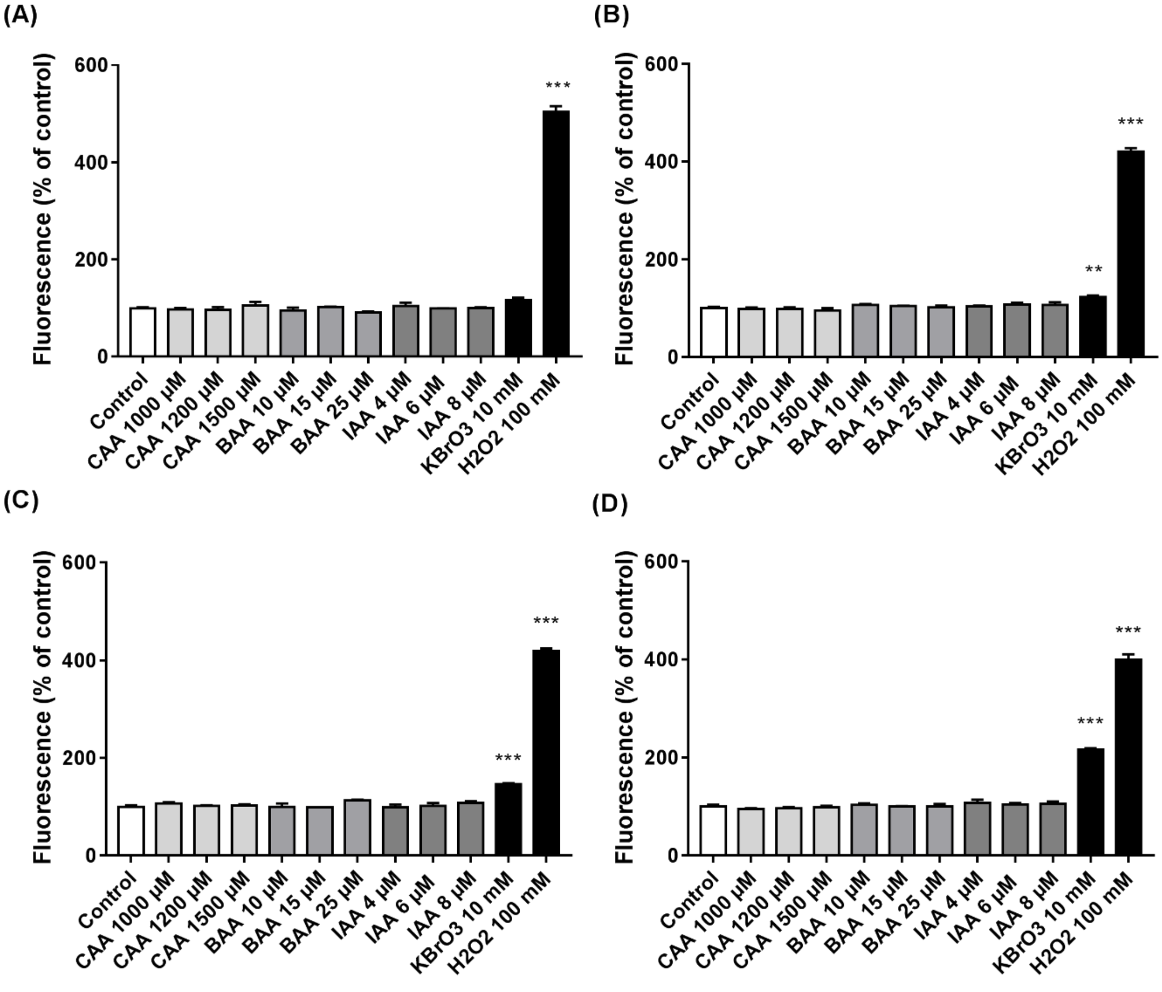


Figure 24. Intracellular ROS in UROtsa cells after 30 min (A), 1 h (B), 2 h (C), and 4 h (D) incubation with increasing concentrations of the mono-HAAs.

No significant differences appeared when comparing DBPs-exposed cells to negative controls (Figure 24). Two distinct positive controls were used. Cells exposed to $KBrO_3$ exhibited increased intracellular ROS levels that were significant after 1 h of exposure, and increasing over time, up to 216.26 % of the control after 4 h of exposure.

Intracellular ROS measured in cells exposed to H_2O_2 was significantly higher than in negative controls at all time points, and decreasing over time. The percentages of ROS relative to control in this case were of 504.08, 420.98, 419.15 and 399.22, with 30 minutes, 1, 2 and 4 hours of exposure respectively.

4.2.2. Long-term genotoxicity analysis

HAAs long-term DNA damaging capacity was assessed growing UROtsa cells in contact with two different non-cytotoxic concentrations of CAA (10 and 100 µM), BAA (0.005 and 0.05 µM) and IAA (0.01 and 0.1 µM) for 8 weeks. These concentrations reflect the range of concentrations found in disinfected waters worldwide for each of the tested HAAs. Genotoxicity was again analysed at the end of the exposure period.

DNA damage was assessed using the comet assay, with and without FPG. Results show that, similarly to acute treatments, long-term exposure to HAAs doesn’t change the level of DNA damage compared to the untreated controls (Figure 25A). The percentage of DNA in tail, averaging 2.82%, was not altered by any of the treatments.

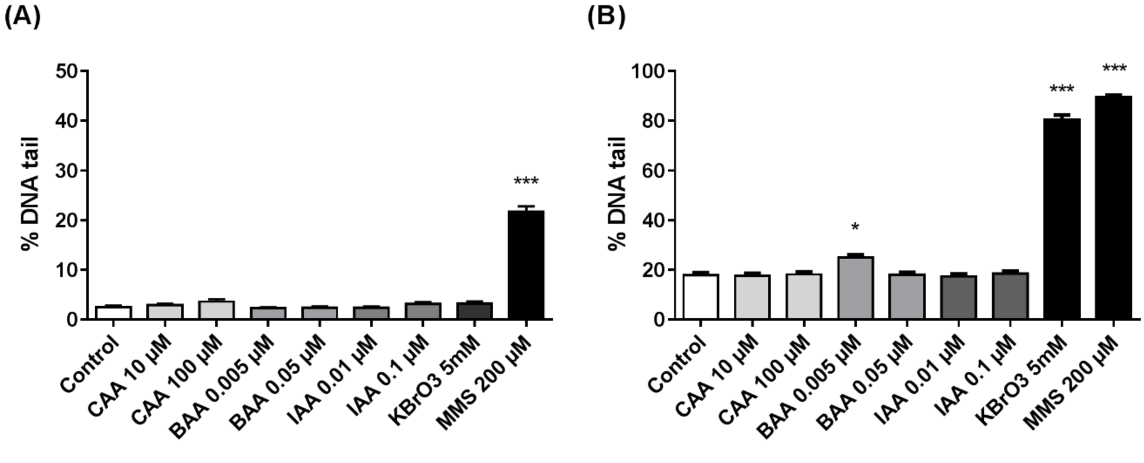


Figure 25. Genotoxic (A) and oxidative (B) DNA damage in UROtsa cells after a long-term exposure to CAA, BAA and IAA.

On the other hand, and in contrast to what was observed in short-term experiments, no oxidative damage appeared after 8 weeks of exposure to the HAAs (Figure 25B). The percentages of DNA in tail were 3.64%, 2.04% and 3.14% for the highest concentrations of CAA, BAA and IAA, respectively, which are not significantly different to those observed in the untreated controls.

Taken together, our results show that, even though HAAs induce oxidative damage in UROtsa cells when subjected to an acute exposure, long-term exposures do not, suggesting that assays based on acute exposures might overestimate the real mutagenic potential of HAAs.

4.2.2.1. Acquisition of resistance

To further analyse the effects that a prolonged exposure to HAAs can have on human cells we evaluated whether long-term exposed UROtsa cells acquired any resistances to oxidising agents, considering that such phenomenon could explain the apparent contradictory data obtained in the comet assay.

4.2.2.1.1.Cytotoxicity analysis

Cell viability was assessed in long-term exposed cells after a short-term exposure to $KBrO_3$, as a model oxidising agent, to determine whether a long-term exposure to low concentrations of the HAAs promotes a resistance to oxidising agents.

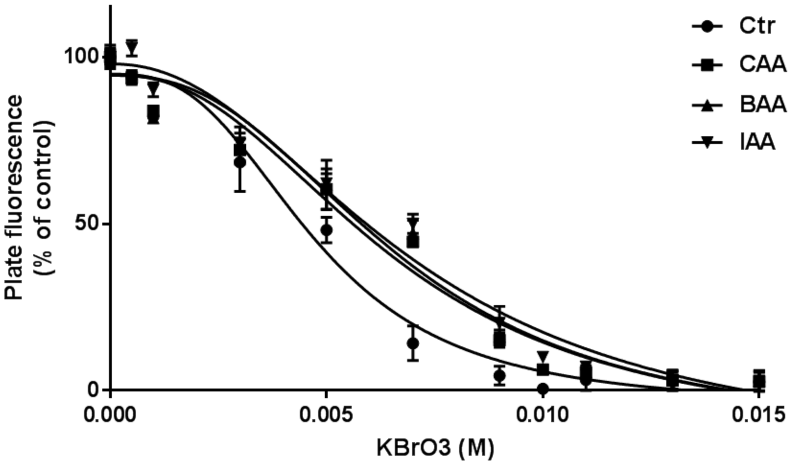


Figure 26. Cell viability curves of long-term exposed cells after 24 hr of exposure to increasing concentrations of KBrO3.

Significant differences appeared in terms of cytotoxicity between non-exposed and exposed cells. The IC_{50} in the untreated control was 4.76 ± 0.17 mM, whereas the IC_{50} of cells that were exposed for 8 weeks to low concentrations of CAA, BAA and IAA was 6.66 ± 0.38 mM, 6.68 ± 0.39 mM, and 6.99 ± 0.47 mM, respectively (Figure 26).

Surprisingly, cells that were exposed to each HAA displayed a very similar behaviour, with very similar IC_{50}.

4.2.2.1.2.Genotoxicity analysis

To confirm that HAAs long-term exposure prompts a phenomenon of resistance acquisition, the behaviour of long-term exposed cells was evaluated in a genotoxicity assay using $KBrO_3$.

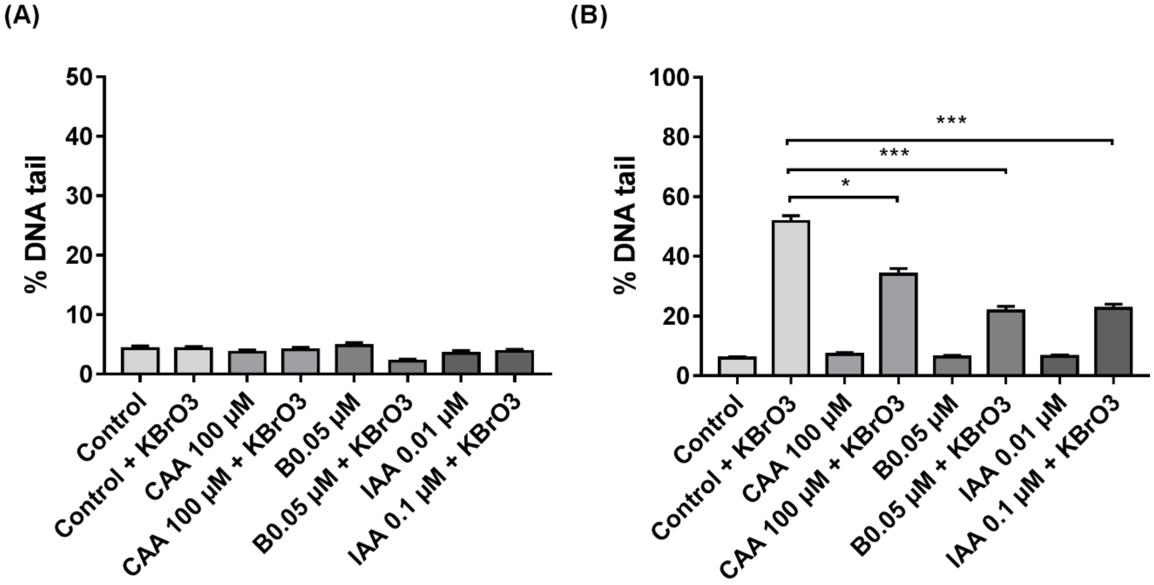


Figure 27. Genotoxic (A) and oxidative (B) DNA damage in long-term exposed UROtsa cells after 30 minutes' exposure to 2.5 mM KBrO3.

There were no changes in the levels of genotoxic DNA damage between treated and untreated cells (Figure 27A).

On the contrary, the FPG-modified version of the alkaline comet assay showed significant differences on the levels of oxidative DNA damage between cells that had been long-term exposed to the three HAAs and the passage-matched untreated controls after $KBrO_3$ exposure (Figure 27B). Lower levels of DNA oxidative damage were observed in long-term exposed cells after 30 minutes of exposure to $KBrO_3$. The percentage of tail DNA was of 51.80 ± 1.79 in untreated cells, whereas in cells long-term exposed to CAA, BAA and IAA was of 34.07 ± 1.85, 21.75 ± 1.55, 22.62 ± 1.46 respectively, confirming that long-term treated UROtsa cells developed some resistance to oxidative damage.

4.2.3. Long-term carcinogenicity analysis

Long-term carcinogenicity of HAAs was as well evaluated throughout and at the end of the exposure period, when several markers of tumoural transformation were analysed.

4.2.3.1. Cell proliferation

Cell proliferation was monitored during the whole exposure period. Final results analysed at the end of the 8-weeks exposure period showed no significant differences between long-term exposed cells and unexposed passage-matched controls (Figure 28).

Doubling time in untreated cells after the eight weeks of exposure was 17.54 ± 0.71 hours. In cells exposed to CAA it was of 18.44 ± 0.14 and 24.27 ± 2.14 hours; in cells exposed to BAA was of 20.67± 1.25 and 17.26 ± 0.40 hours; and, in cells exposed to IAA was of 22.27 ± 2.56 and 18.43 ± 2.40 hours.

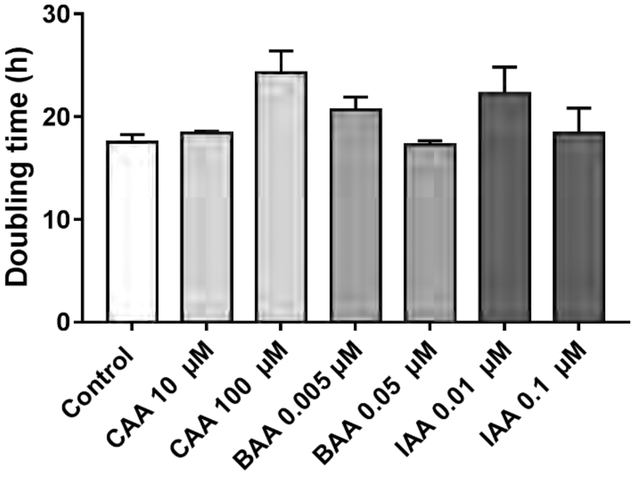


Figure 28. UROtsa cells proliferation rates after 8 weeks of CAA, BAA and IAA exposure.

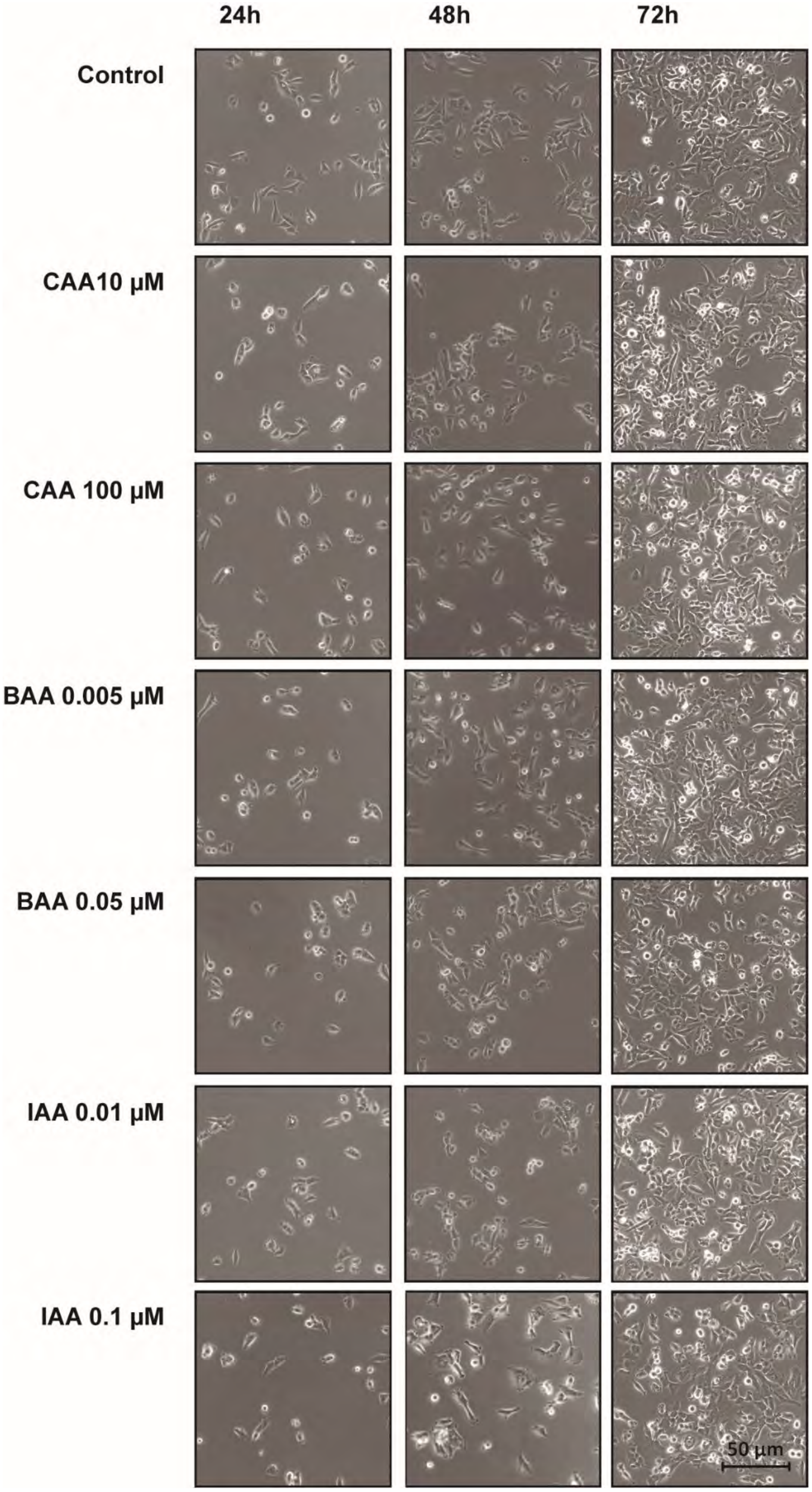

24h
48h
72h
Control
CAA10 µM
CAA 100 µM
BAA 0.005 µM
BAA 0.05 µM
IAA 0.01 µM
IAA 0.1 µM
50 µm

Figure 29. UROtsa cells morphology after 8 weeks of CAA, BAA and IAA exposure. Pictures taken 24, 48 and 72 h after seeding.

4.2.3.2. Cell morphology

Long-term exposed UROtsa cells morphology was monitored throughout the whole exposure period. Figure 29 shows cells morphology after the 8 weeks of exposure 24, 48 and 72 hours after seeding. Even though morphology changes between 24 and 72 hours after seeding can be observer in all cases due to the cells' confluence, no noticeable changes were observed between the different treatments and the untreated control in any of the analysed timepoints, suggesting that mono-HAAs-treated cells are not under an EMT process.

4.2.3.3. Anchorage independent growth capacity

UROtsa cells form a considerable number of small colonies in soft-agar, therefore any increase in their colony-formation capacity is a clear indicator of tumorigenic transformation. Thus, UROtsa cells' colony-forming capability was assessed after the 8 weeks' exposition to mono-HAAs and in a time-matched untreated control.

In all cases UROtsa cells spontaneously formed a great number of small colonies (Figure 30A). Nevertheless, no significant changes were apparent as a consequence of the HAAs exposure. Untreated cells formed a mean of 1827 ± 352 colonies, while cells long-term exposed to CAA formed 1974 ± 245 and 1848 ± 202 colonies for its lowest and highest concentrations, respectively. On the other hand, cells exposed to BAA formed an average of 1904 ± 223 colonies and 1873 ± 151 colonies for each concentration. Finally, IAA exposed cells were able to grow 1932 ± 158 and 2090 ± 201 colonies for 0,1 µM and 0,01 µM, respectively (Figure 30B).

When colony sizes were taken into account, all the experimental conditions presented similar mean sizes (Figure 30C). The average colony size for untreated controls was 158.67 ± 3.84 µm, colony sizes of cells exposed to CAA were 147.93 ± 1.86 and 159.34 ± 2.08 µm, 154.74 ± 4.19 and 155.06 ± 1.51 µm for BAA, and 147.90 ± 1.62 and 146.59 ± 0.76 µm for IAA 0,1 and 0,01 µM, respectively.

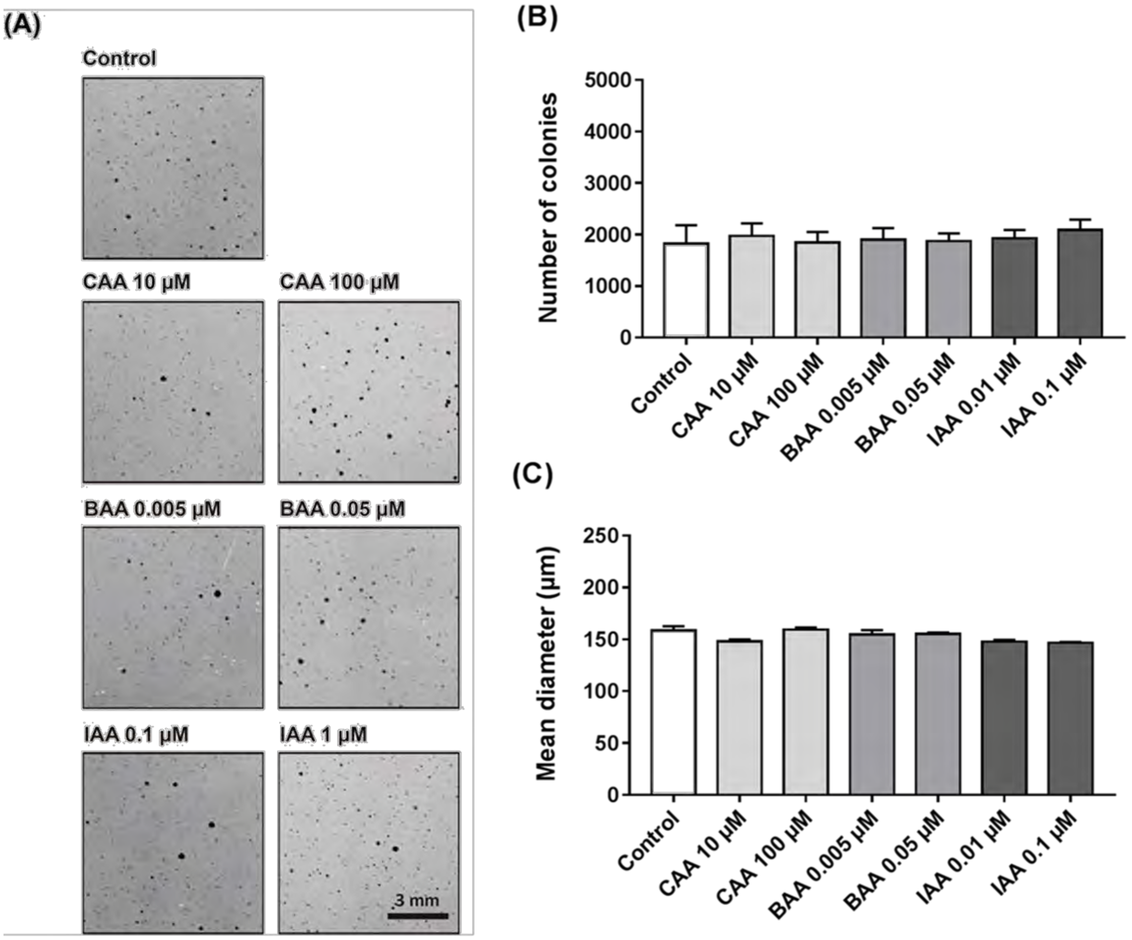


Figure 30. Anchorage-independent growth capacity of UROtsa cells after 8 weeks of the HAAs long-term exposure. B) Mean number of colonies per well. C) Mean colony size per treatment.

4.2.3.4. Analysis of the long-term exposed cells' secretome

The analysis of long-term exposed cells' secretome was as well included in this study to assess all possible changes taking place after a prolonged exposure to HAAs.

To evaluate UROtsa cells capacity to induce and promote tumour growth, HeLa cell line was grown in soft-agar for two weeks in contact with the CM obtained from long-term exposed UROtsa cells. As usual, the number and size of the colonies formed was evaluated. HeLa cells are known to spontaneously form big colonies in soft-agar (Junjie et al. 2013), and provide a good model to easily detect induction and enhance of the tumoural growth.

Colony number was similar in all cases. Average colony number formed by HeLa cells grown in the CM of untreated controls was of 3755 ± 322. Cells grown in contact with the CM of exposed cells formed 3873 ± 272 and 3897 ± 271 for CAA, 3762 ± 230 and 4112 ± 493 colonies for BAA, and 3269 ± 451 and 3150 ± 42 for IAA (Figure 31A,B).

Colony size was also measured to obtain information on the cells capacity to promote tumoural growth. The average colony size of HeLa cells exposed to control CM was 196.50 ± 2.63 µm, whereas HeLa colonies grown in the CM of CAA-exposed UROtsa showed mean values of 208.18 ± 5.89 and 201.97 ± 5.91 µm respectively. HeLa grown in the CM of BAA-exposed cells had average colony sizes of 205.67 ± 6.37 and 192.15 ± 5.31 µm, and for IAA those were 191.05 ± 3.14 and 202.34 ± 10.57 µm (Figure 31C).

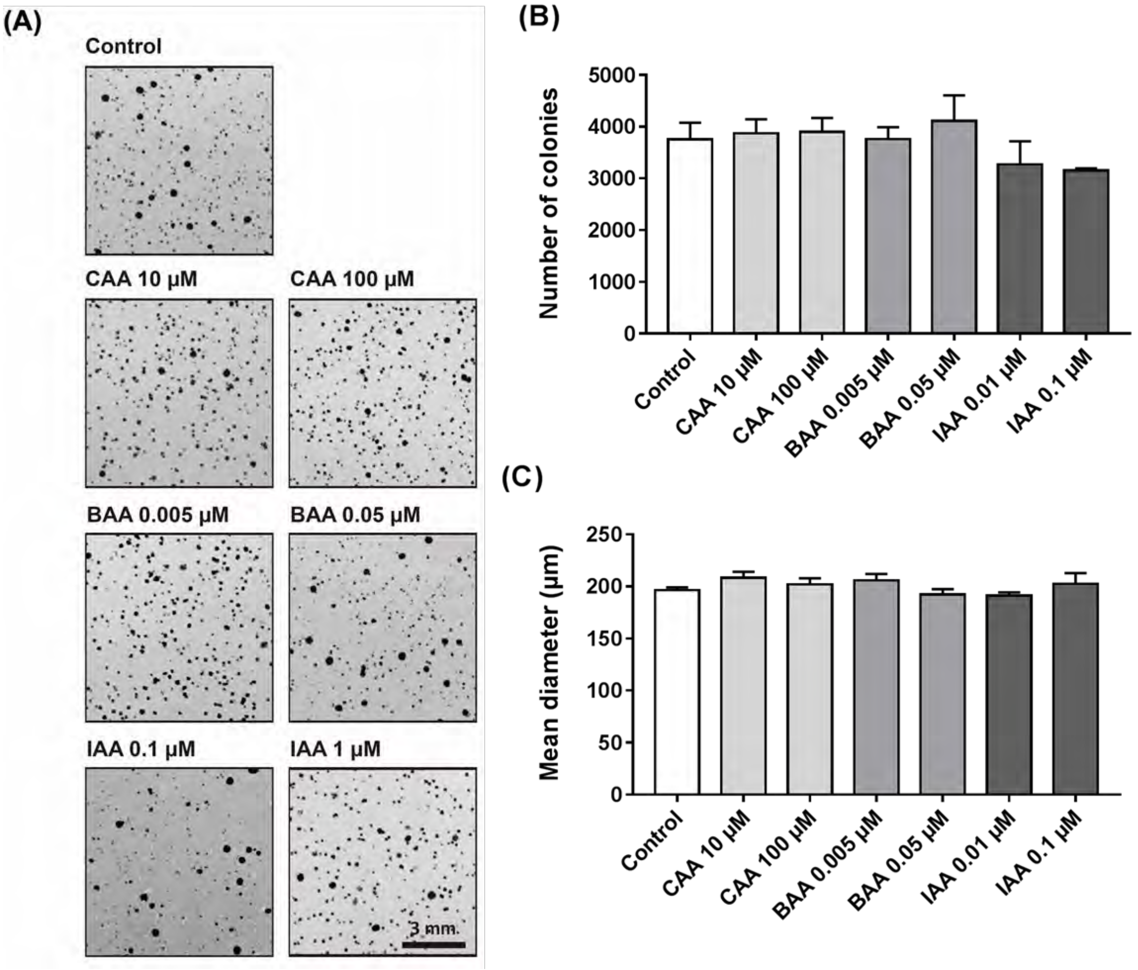


Figure 31. Promotion of HeLa anchorage-independent cell growth by the secretome of UROtsa after 8 weeks of HAAs long-term exposure. B) Mean number of HeLa colonies per plate. C) HeLa mean colony diameter.

5. DISCUSSION

5. DISCUSSION

The prevalence of disinfection by-products in potable waters has been a health concern since their discovery in the seventies. From that moment, more than 600 species have been identified, representing all kinds of structures and chemical groups, with new species being discovered every year. Over the past forty years DBPs have been widely studied, with research focusing on their formation, presence in waters, their entry routes into the human body, their cytotoxicity and genotoxicity, and their direct link with the incidence of several diseases.

Such intensive research led to the identification of cancer as the most prevalent and concerning outcome of DBPs exposure (Page et al. 1976; Cantor 1982; Richardson et al. 2007; Villanueva et al. 2007), even though, in the last twenty years epidemiological evidence suggests that DBPs might also contribute to respiratory and developmental toxicities (Kanitz et al. 1996; Fantuzzi et al. 2010).

Regardless of the evidence directly linking DBPs to certain types of cancer, the specific species behind the observed increased cancer incidence have not yet been determined. Many DBPs have been classified as genotoxic, and a few have exhibited carcinogenic properties in mammalian cells, but to date none of the individual species alone has shown sufficient carcinogenic potency to account for the observed cancer risk (Richardson et al. 2007). Moreover, evidence of carcinogenic DBPs without a genotoxic capacity (International Agency for Research on Cancer 1999) determines the necessity of a more direct carcinogenicity evaluation. The lack of a more systematic methodology to assess the carcinogenic potential of DBPs generated severe gaps regarding the specific carcinogenicity of each DBP class.

The presence of DBPs in treated waters worldwide has led governments and institutions to set specific legislations to regulate these chemicals based on experimental evidence of their putative hazard (Richardson et al. 2007; Manasfi et al. 2016; Hang et al. 2016). Nevertheless, the aforementioned problems could miscalculate the actual hazard of each DBP, therefore having dangerous consequences regarding the lack of regulation of these chemicals.

In order to develop better risk estimations and more accurate regulations to protect human populations, a more standardised carcinogenicity analysis is required. Historically, recommendations for carcinogenicity assessment of chemicals focused on both *in vivo* and *in vitro* models. However, as DBPs carcinogenic analysis in *in vivo* studies have serious limitations in terms of costs and the knowledge of DBPs detoxification, *in vitro* studies seem more suited to analyse the vast number of species that still have not been investigated, as well as DBPs' mixtures. Moreover, several organisms, including the European Partnership for Alternative Approaches to Animal Testing (EPAA), defend the improvement of hazard evaluation and risk assessment protocols implementing the principles of the 3Rs (replacement, refinement and reduction in animal studies) (Annys et al. 2014). In this regard, recommendations include previous OECD proposals that point to two cellular models to study the carcinogenic potential of chemicals, the mouse cell lines BALB/c 3T3 and C3H10T1/2 (Vasseur & Lasne 2012). Still, these models have not been used to test long-term exposures. Additionally, the main interest in carcinogenicity evaluation would be to set up a human cellular model that would be more representative of the actual human exposure.

Also, as human populations are exposed to these chemicals for many decades, the use of models representing long-term exposures becomes especially interesting. For these reasons, this Thesis proposes a more elaborated experimental design that uses human cell lines and a prolonged exposure to biologically relevant concentrations, analysing numerous cell transformation markers, thus providing a more realistic model of low-dose, long-term exposures. Such model has been validated in our group over the past years, and used to evaluate the long-term carcinogenic potential of various chemicals (Annangi et al. 2015; Vales et al. 2015; Bach et al. 2016), demonstrating the importance of the analysis of such more realistic exposure scenarios.

One of the advantages of this *in vitro* model is its cell line independency, thus, the cellular model can be selected according to the chemical to analyse. In this Thesis, cell line selection for each study was based on the current knowledge of DBPs exposure. For the first study, we decided to analyse the effect of DBPs on BEAS-2B, an immortalized cell line derived from normal bronchial epithelium, as the respiratory tract is considered the first tissue in contact with DBPs and the main route of exposure to these chemicals, and several studies indicate that domestic water consumption, including bathing, showering and cooking represent the highest percentages of DBPs exposure through inhalation (Andelman 1985; Lin & Hoang 2000). On the other hand, the urinary tract represents one of the main endpoints of the effects of DBPs in the human body, considering that bladder cancer is the main cancer type associated to DBPs exposure in humans (Cantor 1982; Villanueva et al. 2007), so we also assessed the carcinogenic potential of DBPs on UROtsa cells, a urothelium cell line derived from a healthy donor.

Both BEAS-2B and UROtsa cell lines have been previously validated in long-term carcinogenicity experiments analysing other potentially carcinogenic chemicals such as nanoparticles and arsenic (Eblin et al. 2007; Eblin et al. 2008; Wang et al. 2011; Garcia-Canton et al. 2013; Vales et al. 2015; Choo et al. 2016).

In this framework, we selected two DBP classes for our studies: HNMs and HAAs.

A total of five DBP species were analysed in the context of this Thesis, bromonitromethane (BNM), trichloronitromethane (TCNM), chloroacetic acid (CAA), bromoacetic acid (BAA) and iodoacetic acid (IAA). BNM and TCNM have been described as potent DNA-damaging agents but had never been evaluated as possible carcinogens in a system measuring carcinogenicity in a direct manner (Richardson et al. 2007). BAA and CAA are genotoxic (Kargalioglu et al. 2002) and have been regulated in several countries; however, their carcinogenic potential remained unspecified (Zhang et al. 2010). Finally, IAA has shown carcinogenic potential in mammalian cells, but the lack of evidence in more diverse cellular models has prevented its classification as human carcinogen (Wei et al. 2013). To our knowledge, the studies included in this Thesis are the first evaluating the carcinogenic potential of these species after a long-term exposure scenario.

5.1. DBPs cytotoxicity and genotoxicity

Previous studies reveal significant differences in the cytotoxicity of DBPs, which may depend on several factors such as their inorganic precursor or the halogenated groups present in the molecule (Plewa, Wagner, Jazwierska, et al. 2004). There is no standard protocol for the measurement of DBPs cytotoxicity in human cells, therefore all existing methods are considered an approximation and are used according to the advantages that each method offers for a determined experimental design.

In the studies included in this Thesis, the cytotoxic potential of HNMs was measured in a direct manner, counting the cells with a cell counter after 24 h of treatment. Alternatively, for the analysis of HAAs, an indirect measure was employed. In this case the parameter measured was cells metabolic activity after 24 h treatments, what provides information on the relative number of metabolically active cells. In this case, this method offered practical advantages, permitting a high-throughput screening.

In both cases, short-term cytotoxicity analysis revealed differences in the cytotoxic potential among DBPs species. For HNMs, the brominated compound BNM was more cytotoxic than the chlorinated TCNM. BNM exhibited an IC_{50} of 32 ± 10 µM while it was of 111 ± 17 µM for TCNM. This same rank order of toxicity has also been observed in other mammalian cells, where brominated nitromethanes were more cytotoxic than their chlorinated analogues (Plewa, Wagner, Jazwierska, et al. 2004). In this case, HNMs cytotoxicity was measured after 72 h of exposure, where BNM showed an intermediate cytotoxic capacity and TCNM was the less cytotoxic of the 9 HNMs analysed, with an IC_{50} of 7.06 and 536 µM for BNM and TCNM respectively. This same cytotoxicity rank was not found in bacterial systems, which might indicate that a certain metabolisation is needed for this compounds to acquire its cytotoxic potential (Plewa, Wagner, Jazwierska, et al. 2004).

Similarly, significant differences appeared between the different HAAs species. The brominated species, BAA, was more cytotoxic than the chlorinated form, with an IC_{50} of 6.7 ± 0.4 µM and 794 ± 61 µM respectively. Interestingly, the iodinated form, IAA, showed the strongest cytotoxic effect, with and IC_{50} value of 2.4 ± 0.2 µM. Cytotoxicity of these HAAs assessed in other cellular models has similar outcomes, and the same rank order has been observed in CHO cells (Plewa et al. 2002; Plewa, Wagner, Richardson, et al. 2004), whose cytotoxicity analyses after 72 h of exposure revealed an IC_{50} of 9.56, 848 and 2.95 µM for BAA, CAA and IAA respectively. This results confirm that there's a correlation between cytotoxicity and the relative atomic mass of the halogen atom present in the molecule, as other authors previously stated (Kargalioglu et al. 2002; Richardson et al. 2008; Zhang et al. 2012).

In general, cytotoxicity analyses provide a solid ground for the selection of concentrations to investigate other biological endpoints and particularly to study long-term exposure conditions. Due to the lack of previous experiments analysing the carcinogenic potential of HNMs *in vitro*, the selection of concentrations for this study was primarily based on the cytotoxicity observed in BEAS-2B cells. To increase the reliability of our study, and to make it more relatable to real exposures to HNMs, the priority was to analyse concentrations with low (less than 10%) cytotoxicity, excluding from the study long-term effects due to cytotoxicity. Therefore, the selected concentrations were 1 and 5 µM for BNM and 5 and 25 µM for TCNM.

On the contrary, information in the literature on the genotoxicity and possible carcinogenicity of HAAs led us to select concentrations that match with those found in disinfected waters around the world. Hence, the selected concentrations were 10 and 100 µM for CAA, 0.005 and 0.05 µM for BAA, and 0.01 and 0.1 µM for IAA, none of which caused cytotoxicity levels over 10%.

The genotoxic potential of chemicals is closely related to their cytotoxicity, and it has been used as a surrogate marker of carcinogenicity for many years. As for the selected DBPs, both HNMs and HAAs have exhibited genotoxic effects *in vitro* in rodent's and human cells (Plewa et al. 2002; Plewa, Wagner, Jazwierska, et al. 2004; Richardson et al. 2007). Thus, given the direct association of HAAs with DNA oxidative damage, in our second study, we evaluated the DNA-damaging capacities of HAAs in UROtsa cells both after short- and long-term exposures.

Surprisingly, none of the mono-HAAs showed any DNA damaging capacity in the comet assay after a 4 h exposure when concentrations, up to 1500 µM CAA, 25 µM BAA and 8 µM IAA were used.

Previously reported genotoxic concentrations for CAA were around 300 µM, 13 µM for BAA, and over 5 µM for IAA in CHO cells (Plewa et al. 2000; Plewa et al. 2002; Plewa, Wagner, Richardson, et al. 2004). Genotoxic activity in other human cells such as small intestine epithelial cells has also been measured, with the mono-HAAs generating a 20% comet tail with 1040, 23,8 and 5.9 µM for CAA, BAA and IAA respectively (Attene-Ramos et al. 2010), while in human lymphocytes lowest significant concentrations were 730, 8 and 45 µM for CAA, BAA and IAA respectively (Escobar-Hoyos et al. 2013). Discrepancies between this data and results from our study can be attributable to the distinct metabolic activity of each cell line. Even though HAAs do not seem to require metabolic activation to damage the DNA, their genotoxic potential has proven variable in distinct bacterial strains, with or without the addition of S9 (Richardson et al. 2007).

Previous studies suggest that oxidative damage might be responsible for the cytotoxicity and genotoxicity associated with these compounds. Elevated levels of 8-hydroxydeoxyguanosine, a marker of oxidative stress-derived DNA damage, have been found in mice treated with HAAs (Larson & Bull 1992; Austin et al. 1995; Parrish et al. 1996; Lodovici et al. 2000). Moreover, it has been demonstrated that mono-HAAs are capable of inactivating GAPDH in mammalian cells, eventually causing mitochondrial stress and increased ROS levels (Cemeli et al. 2006; Justin A Pals et al. 2011; Dad et al. 2013). Herein, all three mono-HAAs were capable of inducing oxidative DNA damage after only 4 h of incubation measured by comet assay with the addition of FPG.

Contrarily, the measurement of intracellular ROS revealed that none of the mono-HAAs was capable of increasing intracellular ROS levels measured between 30 minutes and 4 hours of exposure. The levels of intracellular ROS represent an indirect measure of DNA damage, for this reason results are not directly comparable to the comet assay results. Such lack of observable ROS induction might be indicative that ROS production happens before this period of time. Previous data analysing GAPDH function after HAAs exposure showed that the maximum function decrease takes place after 10 to 20 minutes of exposure (J. A. Pals et al. 2011). Our observations would support the hypothesis that HAAs first inhibit GAPDH, what causes an inhibition of the glycolysis and the posterior

ROS increase leading to genotoxic damage. Such inhibitions would take place within the first 30 minutes of exposure, with oxidative damage still observable after 4 h of exposure in the comet assay. Another possibility that cannot be ruled out is that the specific ROS species produced by mono-HAAs cannot be detected by DCFH-DA, which only detects H_2O_2, $ONOO^-$, lipid hydroperoxides and $O_2^{\cdot -}$.

On the other hand, $KBrO_3$ interacts directly with DNA, and the slow increase in the levels of ROS can be an indirect consequence of this damage, while H_2O_2, being a ROS specie itself that easily permeates cell membranes (Birben et al. 2012), can be detected inside the cells throughout the whole experiment.

The evaluation of genotoxic DNA damage in cells long-term exposed to the HAAs led to similar results to those obtained after short-term exposures. Consistently with short-term experiments, an increase in the levels of DNA damage was not observed in any of the tested concentrations. There were no differences among treatments either when oxidative damage was taken into consideration. Hence, a prolonged exposure to the three mono-HAAs could not induce accumulative oxidative DNA damage.

Taken together, the evaluation of HAAs genotoxicity performed in this Thesis suggest that the oxidative damage related to short-term HAAs exposure is not time cumulative and long-term effects could not be detected in our cell system.

Nonetheless, it is well-known that long-term exposure to certain DNA-damaging chemicals can trigger the activation of a high variety of mechanisms in cells. Changes in gene expression determined by the exposure to HAAs have already been analysed in previous studies. As it has been mentioned, the mechanism for HAAs genotoxicity and carcinogenicity have been widely investigated and it has been proven that these DBP family is capable of altering the expression of numerous genes. The main functional groups altered by HAAs exposure are involved in double strand DNA break repair, DNA excision repair, mismatch repair, other DNA repair pathways, cell cycle regulation and apoptosis control (Attene-Ramos et al. 2010).

On the other hand, HAAs exposure has been linked to the generation of ROS and oxidative DNA damage, and thus, to the deregulation of genes involved in repairing this kind of damage. Previous research indicates that mono-HAAs induce an upregulation of *NRF2* and that this gene presumably becomes permanently activated in prolonged exposures to these chemicals (S. Wang et al. 2014; Procházka et al. 2015), implying that such overactivation may determine the onset of a carcinogenesis process (DeNicola et al. 2011; Ni et al. 2014).

In some cases, prolonged exposures to DNA damaging agents induce an adaptive response where changes in gene expression determine the acquisition of resistance to agents causing the DNA damage by that same initial mechanism. Such response to DNA damage has been observed in prolonged exposures to other chemicals such as agents employed for chemotherapy or arsenic (Pi et al. 2005; Sun et al. 2011; Wongvaranon et al. 2013).

Thus, besides the potential carcinogenic process, deregulation in damage-response genes triggered by HAAs exposure could initiate a process were cells acquire resistance to genotoxic or oxidising agents. To prove this hypothesis, we subjected long-term HAA-exposed cells to a series of experiments assessing their resistance to oxidative DNA damage. The first evaluation revealed a reduction of the cytotoxicity of $KBrO_3$ on cells that had been in contact with HAAs for eight weeks. The IC_{50} of these cells were significantly higher when compared to untreated time-matched control cells.

Interestingly, cells exposed to each mono-HAA exhibited the same behaviour when facing the oxidative insult, supporting the hypothesis posed in previous studies that suggest that the same mechanisms could be taking place in response to CAA, BAA and IAA exposure. Thus, the increased resistance to oxidising agents observed after the long-term exposure to DBPs could be attributed to a deregulation of genes involved in DNA repair, especially the repair of oxidative DNA damage, in the detoxification of such agents or in multidrug resistance proteins (MPRs).

To further test this hypothesis, long-term exposed UROtsa cells were subjected to a comet assay after being treated with $KBrO_3$, with and without the addition of FPG. DNA damage was in this case measured after 30 minutes of exposure to the oxidising agent. Results corroborate what was found on the cytotoxicity test, since long-term exposed cells exhibited lower levels of oxidative DNA damage than controls after the exposure to $KBrO_3$.

This data suggests that the DNA-damage mechanisms observed in short-term experiments have further implications in long-term exposures to these chemicals. Hence, further research will be needed to determine whether the adaptive mechanisms observed in this Thesis might be caused by an upregulation of *NRF2* and/or its downstream genes.

The confirmation of this hypothesis would imply that prolonged exposures to environmentally-relevant HAAs concentrations have consequences beyond direct carcinogenicity that must be also analysed.

5.2. Long-term carcinogenicity studies

5.2.1. Direct tumoural transformation

DBPs presence in drinking waters all over the world determines a continuous and prolonged exposure to these chemicals with extensive evidence linking this exposure to an increased cancer incidence in epidemiologic studies (Page et al. 1976; Kuzma et al. 1977; Cantor 1982; Hildesheim et al. 1998; Cantor et al. 1998; Cantor et al. 1999; Villanueva et al. 2007).

Given these particular exposure conditions, the assessment of the potential carcinogenicity of DBPs becomes more relevant when long-term exposures are analysed, especially if low and more biologically-relevant concentrations are employed.

No conclusive data has been published discussing the long-term carcinogenic capacity of HNMs, and the evaluation of the long-term carcinogenicity of HAAs, principally analysed by *in vivo* studies, has led to contradictory results (Richardson et al. 2007). Several species have shown opposite carcinogenic potentials when analysed in different studies and animal models. For instance, TCAA was non-carcinogenic in a study in rats, but was still classified as an animal carcinogen due to further studies in mice (DeAngelo et al. 1997; Von Tungeln et al. 2002). Regarding the mono-HAAs, one *in vitro* study analysed the carcinogenicity of IAA, pointing to a mild carcinogenic effect derived from exposures to relatively high concentrations of this HAA (Wei et al. 2013).

This lack of a more standardized analysis of the carcinogenicity of these compounds, as would be the use of the same battery of assays for all DBPs' classes, prompted us to propose the use of a battery of *in vitro* cell transformation assays to determine the carcinogenicity of HNMs and HAAs, creating an experimental design that allows the use of human cells and environmentally-relevant concentrations.

The evaluation of DBPs carcinogenic potential using *in vitro* cell transformation assays is still rare, while the evaluation of genotoxicity as a surrogate biomarker of carcinogenicity is more extended. Over the past years, our group has developed different standard protocols to assess the carcinogenicity of chemicals, using *in vitro* cell transformation assays that evaluate the degree of transformation of a non-tumoural cell line after the exposure to a possible carcinogen (Bach et al. 2016; Vales et al. 2015; Annangi et al. 2015).

In this Thesis, we propose the use of this approach to assess the long-term carcinogenic transformation capacities of HNMs and HAAs, evaluating a series of cell transformation markers *in vitro*.

To date, numerous cell transformation markers have been characterized in the process of cellular malignant transformation *in vitro*. Such markers include cell proliferative capacity, cell morphology, anchorage-independent growth capacity, the capacity to induce tumoural growth, and the secretion of proteins modifying the tumour stroma as MMPs. The observation of these markers serves as a clear indicator that a tumoural transformation process is taking place.

Alterations in the cell cycle are one of the clearest hallmarks of cancer, being an increased cell proliferation rate one of the first signs of a tumoural transformation (Evan & Vousden 2001). In our studies, the exposure to HNMs and HAAs did not determine an increased cell proliferation, as doubling times did not decrease when compared to the time-matched controls. In both studies, the lack of differences in cell proliferation between the cells grown in the DBP-containing medium and untreated, time-matched controls also indicated that the selected concentrations had no cytotoxic effects during the eight weeks of treatment, validating their use in the long-term exposure analysis.

Interestingly, major differences appeared over time in BEAS-2B cells long-term exposed to the HNMs and the time-matched unexposed cells. Doubling times decreased significantly over time, going from 33.15 ± 1.17 hours in week one to 20.54 ± 0.79 hours in week eight in the negative control, which can be indicative of changes in cell biology that take place during cell culture and manipulation. Such intense changes were not observed in UROtsa cells long-term exposed to HAAs (data not shown).

Another of the determining characteristics of a tumoural transformation is the EMT process, which is the main responsible for tumour invasion and metastasis (Tse & Kalluri 2007; Morata-Tarifa et al. 2016). EMT consists of a series of changes that determine the loss of their intrinsic epithelial characteristics while they acquire a mesenchymal phenotype.

During EMT, cell morphology is altered due to changes in the expression of many genes related to membrane and cytoskeletal proteins, thus the analysis of cell morphology provides valuable information on cells tumorigenic transformation. The morphology of both BEAS-2B and UROtsa cells long-term exposed to DBPs was monitored throughout the whole exposure period, but no relevant differences appeared between treated and untreated cells.

Another way to monitor whether cells are undergoing a EMT process is by the analysis of their anchorage-independent growth capacity. Only tumoural cell lines are capable of growing in a surface as soft-agar, thus, the lack of differences between exposed cells and unexposed controls observed in our studies also suggest that cells are not undergoing EMT. As for HNMs, a more detailed analysis of the results reveals that, contrarily to what was observed in doubling time analyses, the cells treated with the highest concentration of TCNM presented some degree of cytotoxicity. This difference could be due to the nature of the soft-agar assay, since colony formation needs an additional incubation time of 21 days.

5.2.2. Tumour induction and enhancement of tumoural growth

Even though previous results suggest that a HNMs and HAAs did not induce direct tumorigenic transformation of the tested cell lines, the capacity of long-term exposed cells to induce tumoural growth could not be excluded. It is well known that cells secrete paracrine signals that can stimulate cancer progression (Witsch et al. 2011). Thus, a prolonged exposure to HNMs and HAAs could result on an alteration of the cells' secretome, which could restructure their surroundings and induce and promote tumoural growth, directly or indirectly.

The modified soft-agar assay has been described as a method to evaluate the cells' secretome capability to induce and enhance tumoural growth, and indirectly, to measure how long-term exposures can influence cancer progression (Bach et al. 2016). In our studies, exposed cells secretome was tested using two different tumoural cell lines, HCT116 and HeLa, looking for the ideal conditions to observe tumoural growth. HCT116 appeared to grow optimally when in contact with BEAS-2B cells' CM, however they did not react the same way when grown with UROtsa cells' CM (data not shown). Thus, HeLa cells were used to evaluate the tumoural induction capacities of UROtsa cells.

In any case, neither HNMs nor HAAs exposure could enhance the capacity of exposed cells' secretome to induce tumoural growth, as measured by the number of colonies grown by a tumoural cell line exposed to the CM of the DBP-exposed cells. The average number of colonies formed by each cell line was around 1,300 colonies for HCT116 cells and around 3,700 for HeLa cells.

The evaluation of long-term DBP-exposed cells' capacity to enhance cancer progression was extrapolated from the size of the colonies formed by each tumoural cell line in soft-agar. An increase in colony size was not observed in either study. The average size of HCT116 colonies was 212.23 µm while mean colony size in HeLa cells was 199.69 µm with no differences among treatments in any sample.

Hence, the indirect soft-agar assays performed in our studies suggest that cells long-term exposed to HNMs and HAAs cannot induce or enhance tumoural growth via their secretome.

In the context of EMT, fibroblasts of the tumour stroma play a prominent role inducing and facilitating tumoural growth and metastasis. In terms of the evaluation of carcinogenicity, it is important to evaluate the actions induced by the tumour stroma when a tumoural transformation takes place, the tumour stroma has a significant role in cancer progression (Pietras & Östman 2010).

To mimic this situation *in vitro*, the CM of long-term exposed cells is used to stimulate fibroblasts, as it would happen *in vivo*. Then tumoural cells are grown in soft-agar in contact with the CM of such fibroblasts (Condon & Bosland 1999). This experimental design was used to evaluate whether such indirect mechanism of carcinogenicity could be triggered by a prolonged exposure to HNMs. However, the number of colonies formed by the tumoural cell line was not influenced by HNMs treatments. This experiment indicated that a prolonged exposure to the HNMs could not prompt exposed cells to secrete factors that would induce stroma cells to secrete tumour-inducing paracrine signals. Parallelly, the analysis of colony sizes indicates the capacity of the tumour stroma to enhance cancer progression (Condon & Bosland 1999). Our experiments show that HCT116 cells formed colonies with an average size of 200,2 µm, with no significant differences among treatments and the untreated, time-matched control. Hence, a prolonged exposure to HNMs cannot induce or enhance tumoural growth directly nor indirectly.

Within the context of EMT, both cells undergoing the tumoural transformation and tumour associated fibroblasts are able to secrete proteases to remodel the extracellular matrix, a process that becomes decisive in tumour invasion and metastasis (Pietras & Östman 2010). The aforementioned changes taking place during the EMT include other processes that can influence the tumour microenvironment. Either tumoural cells or tumour-associated fibroblasts can secrete various proteases, including matrix metalloproteases (MMPs) that can directly remodel the extracellular matrix (Pietras & Östman 2010), thus becoming a determining factor for tumour invasion and metastasis. Increased levels of these enzymes in the cellular secretome indicate that a tumorigenic process is taking place. Consequently, the analysis of MMPs secretion is widely used in *in vitro* carcinogenicity studies (Vila et al. 2017). In this Thesis, MMP2 and MMP9 activity was measured in BEAS-2B cells after they were long-term exposed to

HNMs. MMPs secretion did not increase significantly in exposed cells when compared to time-matched controls. A reduction in MMPs levels was observed in the secretome of cells long-term exposed to 5 µM TCNM, which was considered non-biologically relevant in this case. These results, together with the incapability of long-term exposed cells' secretome to promote tumoural growth in the soft-agar assay, indicate that a prolonged exposure to HNMs is not capable to prompt non-tumoural stroma cells to secrete factors that induce tumorigenic transformation and invasion.

Parallelly, MRC5 fibroblasts stimulated with the CM of long-term exposed cells were subjected to the same analysis in order to determine if long-term DBP-exposed BEAS-2Bcells could induce stromal cells to increase the production of these proteases, what would facilitate cancer progression. The CM of MRC5 fibroblasts had the same MMP levels in all cases, thus, their contact with the CM of long-term exposed cells did not induce any tumour-related activity.

Conclusively, a reliable assessment of the carcinogenic potential of DBPs should consider the evaluation of a broad set of cancer hallmarks under conditions similar to the ones of the real-case scenario. In this Thesis, we analysed the most significant cell transformation markers, taking into account direct and indirect mechanisms of tumorigenesis. The results obtained in these studies support the non-carcinogenicity of the tested DBPs under a long-term, low-dosage regime. The lack of comparable studies for both DBP classes and the high disparity of results in previous evaluations of HAAs carcinogenicity adds value to the studies carried out in this Thesis.

Herein, the long-term carcinogenicity of these chemical species was assessed for the first time adopting an experimental design that includes human cells and concentrations similar to the ones found in disinfected waters, increasing the relevance and reliability of the conclusion of both studies regarding the carcinogenic potential of both DBP classes. Nonetheless, the specific species behind the increased cancer incidence and underlying processes that might be taking place in prolonged exposures to non-cytotoxic concentrations of DBPs require further analysis.

Additionally, the possibility that longer exposure times may have a significant effect on the parameters analysed cannot be ruled out. Also, even though we analysed sufficient cell transformation markers to obtain reliable negative results, the analysis of other indicators of cellular transformation such as gene dysregulation, for instance focusing on gene changes associated to the EMT process, would provide further information on the transforming ability of both HNMs and HAAs.

Finally, given the complex nature of DBPs exposure, possible interactions between two or more DBPs could be a key factor accounting for the observed carcinogenic effects in epidemiological studies. To date, the few studies focused on the carcinogenicity of DBP mixtures using drinking water obtained negative results (Richardson, 2007). Nevertheless, all previous studies have analysed the DBPs mixtures' effects through oral exposure in rats, therefore more extensive research is necessary to unravel possible additive or synergic effects of DBPs.

6. CONCLUSIONS

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Considering the objectives formulated in this Thesis, we have drawn the following conclusions:

- Regarding the setup of better models to evaluate DBPs' carcinogenic potential:
    1. We have established suitable models to analyse long-term exposure to DBPs that mirror the effects of these chemicals on their main entry route -the airways-, and the main target of DBPs carcinogenic effects -the urinary tract.
    2. The use of a wide range of cell transformation markers provides comprehensive information to evaluate the carcinogenicity of DBPs, proving itself as a reliable model to assess the carcinogenic potential of different DBP classes.

- The application of these models to assess the effects of long-term exposure to HNMs and HAAs led to the following conclusions:
    1. Mono-HAAs are capable of inducing oxidative DNA damage, which could lead to mutations, after short exposures to relatively high concentrations. However, neither oxidative or genotoxic DNA damage are detectable after a prolonged exposure to environmentally-relevant concentrations, suggesting that this effect doesn't have stable consequences.
    2. Long-term carcinogenic analysis of HNMs and HAAs showed that neither DBP class could induce a tumoural transformation. Thus, such evaluation of other classes is necessary to determine the species responsible for the increased cancer incidence.
    3. Although HAAs could not induce cell transformation, long-term exposure to HAAs triggered the acquisition of resistance to oxidising agents. The distinct repair capacity of DNA oxidative damage in HAA-exposed cells validates this hypothesis.

7. REFERENCES

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8. ANNEXES

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8.1. Annex 1: First paper

In vitro studies on the tumorigenic potential of the halonitromethanes trichloronitromethane and bromonitromethane

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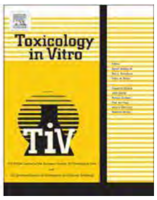

In vitro studies on the tumorigenic potential of the halonitromethanes trichloronitromethane and bromonitromethane

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ABSTRACT

Epidemiological data indicate that chronic exposure to water disinfection by-products (DBPs) may result in increased risk of cancer. However, the real carcinogenic potential of individual DBPs is not well known. In this study, we assessed the in vitro carcinogenic potential of trichloronitromethane (TCNM) and bromonitromethane (BNM), two halonitromethanes (HNMs) commonly found in DBPs' mixtures at comparably high concentrations. Human lung BEAS-2B cells were exposed for 8 weeks to TCNM and BNM, and the acquisition of different in vitro cancer-like features was evaluated. The results indicate that long-term exposure to non-cytotoxic doses of TCNM and BNM did not cause carcinogenic transformation as indicated by the absence of morphological changes, no effects on cell growth, no changes in the level of matrix metalloproteinases (MMPs) secretion, and no increased anchorage-independent cell growth capacity. Furthermore, TCNM- and BNM-exposed BEAS-2B cells were unable to enhance tumour growth directly or by indirect influence of the surrounding stroma. Our results indicate that the carcinogenic effects of DBP mixtures cannot be attributed to the evaluated HNMs. This is the first study evaluating the cell transformation effects of TCNM and BNM under a long-term exposure scenario using suitable hallmarks of the cancer process.

1. Introduction

Disinfection by-products (DBPs) are produced by reactions between the organic matter present in source water and the chemicals used to disinfect it (Ngwenya et al., 2013; Banach et al., 2015). DBPs in drinking water are a complex mixture, containing many different che-mical groups with a number of compounds having mutagenic and carcinogenic potential, as extensively reviewed (Richardson et al., 2007). Therefore, DBP exposure can suppose important long-term human health implications (Grellier et al., 2015), with bladder cancer as one of the main documented effects (Villanueva et al., 2007; Costet et al., 2011; Hrudey et al., 2015).

Due to their potential risk to public health, some DBPs are regulated in many countries, although some chemical species that belong to non-regulated classes could present health risks (Jeong et al., 2015; Kim et al., 2015; Li et al., 2015). Halonitromethanes (HNMs) constitute an emerging class of non-regulated DBPs found at low-μg/L concentrations (Weinberg et al., 2002; Krasner et al., 2006; Serrano et al., 2015). These soluble, low molecular weight compounds are produced when chlorine and/or ozone are used for water treatment. They are structurally similar to the halomethanes but have a nitro-group in place of hydrogen bonded to the central carbon atom. The presence of nitrogenous DBPs in drinking water is of great concern due to their higher genotoxicity and cytotoxicity as compared to those already regulated DBPs (Bond et al., 2011). Trichloronitromethane (TCNM) and bromonitromethane (BNM) are two well-known HNMs. Although TCNM is the most common HNM, special attention must be focused on BNM because brominated DBPs are more reactive than their chlorinated forms (Woo et al., 2002; Kim et al., 2015).

Genotoxicity assessment is usually accepted as a surrogate bio-marker of potential cancer risk. Due to the complexity and ethical issues posed by long-term carcinogenesis studies using mammalian models, in vitro genotoxicity assays are used to evaluate the potential for carci-nogenic risk. This approach has been exploited to determine the gen-otoxic potential of various DBPs (Richardson et al., 2007; Liviac et al., 2011; Manasfi et al., 2015; Teixidó et al., 2015). Nevertheless, a more direct way to measure the potential carcinogenic risk of individual DBPs remains to be developed. In vitro cell transformation assays (CTA) have been proposed as suitable alternatives to long-term animal studies to measure carcinogenic effects (Vasseur and Lasne, 2012). In fact, in

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2012 EURL ECVAM published its recommendation concerning CTAs using Syrian hamster embryo cells (SHE) and the mouse fibroblast cell line BALB/C 3 T3 for in vitro carcinogenicity testing (EURL ECVAM, n.d.). These recommendations are based on the increasing evidence that the cellular and molecular processes involved in in vitro cell transfor-mation are similar to those that occur during in vivo carcinogenesis (Creton et al., 2012). Nevertheless, these CTA are mainly based on the formation of foci as a marker of cell transformation, and subsequently confirming the acquisition of anchorage-independent growth ability. It is generally accepted that an exposed cell line becomes tumorigenic when a battery of different cancer hallmarks is evident (Hanahan and Weinberg, 2011). In consonance with this, different cell lines have been used to detect carcinogenesis onset. Specifically, the BEAS-2B cells have already been used to detect the tumorigenic effect of titanium dioxide nanoparticles (Vales et al., 2015), cadmium (Cartularo et al., 2016) and silver nanoparticles (Choo et al., 2016). In all these cases, changes in different cancer hallmarks were explored.

It is also interesting to note that the detection of the harmful effects induced by chemical exposures in vivo or in vitro, including those in-duced by DBPs, are usually measured in unrealistic scenarios of acute treatments at cytotoxic concentrations. Nevertheless, when the me-chanisms of induced carcinogenicity are studied, it is particularly im-portant to design studies involving more relevant types of exposure in terms of human risk; thus, in vitro long-term or chronic exposures with non-cytotoxic doses causing a biological response similar to that in humans would seem to be a good alternative system to assess realistic exposure conditions (Bach et al., 2014).

Using an experimental approach with long-term (8 weeks) exposure and non-cytotoxic concentrations, we have evaluated the potential transforming capacity of two HNMs (TCNM and BNM) in a human bronchial epithelial cell line (BEAS-2B). The model was chosen given its nature as one of the main DBP-entry routes is via the respiratory system (Richardson et al., 2007), and also because it has been successfully used for long-term carcinogenesis-assessment studies by other authors (Vales et al., 2015; Cartularo et al., 2016; Choo et al., 2016). It must be in-dicated that exposure to DBPs targets the respiratory tract, mainly during showering (Xu and Weisel, 2005), and was able to induce bronchiolar-alveolar carcinomas in mice long-term exposed via con-taminated drinking water (National Toxicology Program, 2007).

2. Materials and methods

2.1. General cell culture conditions and in vitro DBP exposure

Bromonitromethane (BNM, $BrCH_2NO_2$; CAS 563-70-2, 90% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and tri-chloronitromethane (TCNM, CCl_3NO2; CAS 76-06-2, 97.5% purity) from Riedel-de-Haën (Seelze, Germany). Human bronchial epithelial cells (BEAS-2B), was provided by Dr. H. Norppa (Finnish Institute of Occupational Health); human colorectal carcinoma cell line (HCT116) was provided by Dr. M.A. Peinado (IRO, Spain); and human lung fi-broblast cell line (MRC5) was bought from Coriell Institute Biorepository. All cell lines were maintained in DMEM high glucose medium (Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS; PAA®, Pasching, Austria), 1% of non-essential amino acids (NEAA; PAA®) and 2.5 μg/mL plasmocin (InvivoGen, CA, USA) at 37 °C in a humidified 5% CO_2 incubator. BEAS-2B cells were long-term exposed to non-cytotoxic concentrations of the HNMs (1 and 5 μM BNM; 5 and 25 μM TCNM) for 8 weeks. The DBP-containing medium was changed every 48 h, and sub-confluent cells were passaged weekly. Three separate closed vented 75-cm^2 flasks were maintained during the 8-weeks long treatment for each treatment, and DBP-ex-posed cells were in all cases compared with unexposed passage-mat-ched controls.

BNM and TCNM solutions were always freshly prepared before every treatment or change of DBP-containing medium. First, BNM and TCNM were weighed and diluted in distilled water to an initial con-centration of 1 M and 100 mM, respectively. Then, working con-centrations were prepared in culture medium. The manipulation of chemicals was conducted in a certified biological/chemical safety hood following the manufacturer's instructions.

2.2. Cytotoxicity measurement

BEAS-2B cells were plated in 6-well plates at a density of 50,000 cells per well and incubated overnight in complete medium. Cells were then placed in fresh medium with concentrations ranging from 2 μM to 120 μM for BNM and from 10 μM to 1200 μM for TCNM. After 24 h of treatment, the cells were washed with PBS, and their number was as-sessed after trypsinization by the Beckman counter method with a ZTM Series coulter counter (Beckman Coulter, CA, USA). Cytotoxicity curves were derived from averaging three independent experiments, and the IC_{50} values were calculated using GraphPad prism version 5.03.

2.3. Determination of cell proliferation

BEAS-2B long-term exposed cells and unexposed passage-matched controls were plated in 6-well plates at a density of 50,000 cells per well in regular conditions. At 24 h intervals, the cells were counted by the above-mentioned method. Proliferation was defined as the time ne-cessary for doubling the cell population, and it was calculated ac-cording to the equations referred by Roth (2006).

2.4. Anchorage-independent cell growth measurement

Colony formation in soft agar was assessed in long-term exposed BEAS-2B cells and passage-matched controls to determine the capacity for anchorage-independent growth (Bach et al., 2014). For this study, BEAS-2B cells were collected and individualised using a 30 μm filter. A total of 10,000 cells were suspended in a 1:1:1 mixture containing 1× DMEM (supplemented with 10% FBS, 1% NEAA, 1% L-Glu 200 mM and 2.5 μg/mL Plasmocin), 2× DMEM (with 2× supplements) and 1.2% Bacto Agar (DIFCO, MD, USA). The mixture was then plated in 6-well plates over a 0.6% base agar (in supplemented 2× DMEM) and in-cubated at 37 °C for 21 days. After this time, cell colonies were stained overnight with 1 mg/mL of (2-p-iodophenyl)-3-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (Sigma, MO). Plates were scanned and colony counting was performed using OpenCFU open-source Software.

A modified version of the assay was performed using 48-h condi-tioned medium (CM) from long-term exposed BEAS-2B cells and pas-sage-matched controls to assess their capacity to promote the malignant growth of tumour cells via secretome. A total of 10,000 individualised HCT116 cells were resuspended in CM and mixed in a 1:1:1 ratio with supplemented 2× DMEM and 1.2% bacto agar. The subsequent steps of the protocol were performed as described above. Plates were incubated for 15 days.

It has been described that transformed cells stimulate the secretion of promoting factors by cells from the stroma (Bach et al., 2016). Thus, to further analyse the cancer-like phenotype in long-term exposed BEAS-2B, their 48 h CM was used to grow the lung fibroblast MRC5 cells. Then, the 48 h CM of stimulated MRC5 stroma cells was collected and used for the soft agar assay with HCT116 cells as indicated above.

For all anchorage-independent cell growth experiments, HCT116 cells seeded at different cell densities or with different FBS concentra-tions were included as positive controls.

2.5. Secretion of MMP-2 and MMP-9 measurement

The activity of secreted matrix metalloproteinases 2 and 9 was ex-amined in long-term exposed BEAS-2B and passage-matched controls. Cells were cultured in basal DMEM medium (without serum or sup-plements) for 72 h to obtain a CM that was analysed by standard zymography, following the manufacturer's instructions (Bio-Rad, Hercules, CA). The area of protease activity was measured densitome-trically using the ImageJ analysis program. Medium containing 10% FBS was used as positive control in all cases.

The secretion of MMPs has also been described in tumour-induced stroma cells (Del Pozo Martin et al., 2015). To analyse whether long-term exposed BEAS-2B could prompt the secretion of these enzymes, MRC5 fibroblasts were grown in 48-h CM of long-term exposed BEAS-2B and passage-matched controls. Subsequently, the 48-h CM of sti-mulated MRC5 stroma cells was collected and used for zymography as indicated above.

2.6. Statistical analysis

Three separate 75-cm^2 flasks were maintained for each long-term treatment, and three independent experiments were performed for each assay including triplicates of the three independently-treated flasks of cells. Mean and SEM were calculated. One-way analysis of variance followed by Dunnett's multiple comparison test was performed to compare treated with untreated time-matched controls. In all cases, a two-sided $P < 0.05$ was considered statistically significant. All statis-tical analyses were performed using GraphPad Prism 5 version 5.03.

3. Results

3.1. BNM and TCNM are toxic for BEAS-2B cells at low micromolar level

Since the aim of the study was to determine the effects of long-term exposures to non-toxic doses of TCNM and BNM, an initial cytotoxicity measurement experiment was carried out using a wide range of com-pound concentrations to determine the appropriate concentrations to study the effects of long-term exposures to non-lethal concentrations of TCNM and BNM. The obtained results showed differences in the sus-ceptibility of the cells to the two compounds (Fig. 1). Thus, BNM was more cytotoxic than TCNM given their IC$_{50}$ values of 32 ± 10 μM and 111 ± 17 μM, respectively. Both compounds were clearly cytotoxic at 50 μM, with a mean viability of 32.4 ± 7.2% and 64.2 ± 7.4% for BNM and TCNM, respectively, when compared to control cells. Given these results, two different concentrations of each HNM inducing less than a 20% decrease in viability were chosen to carry out the sub-sequent experiments. The selected doses were 1 and 5 μM for BNM and 5 and 25 μM for TCNM.

3.2. Long-term exposure to HNMs does not induce changes in BEAS-2B cellular morphology or proliferation

Cells undergoing tumoural transformation are known to progres-sively decrease their doubling times and to start a process in which cells lose their intrinsic morphological characteristics known as anaplasia (Kumar et al., 2007). To determine whether the long-term exposure to TCNM or BNM induces tumoural transformation, cellular proliferation and shape were monitored as markers of cancer-like phenotypic changes throughout 8 weeks of long-term treatment. No noticeable changes in cell morphology were observed throughout the whole ex-posure time (Fig. 2A), and results analysed at the end of the exposure period indicated that neither BNM nor TCNM treatment increased the proliferation rates significantly when compared to time-matched con-trols (Fig. 2B).

3.3. Long-term exposure to HNMs did not alter BEAS-2B cell capacity to grow in soft-agar

One of the main characteristics of cancer cells is their anchorage-independent growth capacity (Hanahan and Weinberg, 2011). To assess this feature in the HNM long-term exposed cells, BEAS-2B cells were subjected to a soft-agar colony formation assay.

BEAS-2B cells formed a considerable number of small-sized colonies spontaneously, with an average size of 143.49 ± 0.20 μm. Nevertheless, no variations that suggest cell transformation were ob-served after the HNM treatments (Fig. 3). Representative pictures of the colony plates are indicated in Fig. 3A, where no significant changes are apparent. As shown, control samples formed a mean of 273 ± 21 co-lonies, while cells treated with BNM long-term-exposed cells displayed 332 ± 16 and 246 ± 40 colonies for its lowest and highest con-centrations, respectively. On the other hand, TCNM long-term-exposed cells formed an average of 288 ± 16 colonies at 5 μM concentration and 157 ± 25 colonies at 25 μM. When colony sizes were taken into account (Fig. 3C), the mean size was similar in all cases (142.65 ± 1.51 μm for controls vs. 145.68 ± 1.58 and 145.06 ± 0.77 μm for BNM 1 and 5 μM treatments, respectively, and 141.84 ± 0.74 and 142.24 ± 1.81 μm for TCNM 5 and 25 μM treat-ments, respectively). The lack of increase in the number of colonies and in the mean colony size, as an indicator of the cells capacity to grow independently of their substrate, implies that they have not acquired this tumoral marker.

3.4. Long-term exposure to HNMs did not alter the BEAS-2B secretion pattern

It is known that the interplay between the different cell populations and their environment determine the formation and malignancy of a tumour. Cells undergoing malignant transformation secrete paracrine signals that enhance the tumorigenic potential of nearby cells (Del Pozo Martin et al., 2015). Thus, the capability of long-term exposed BEAS-2B cells in promoting the growth of the tumoural cell line HCT116 in soft-agar was evaluated. As observed in Fig. 4, no differences in HCT116

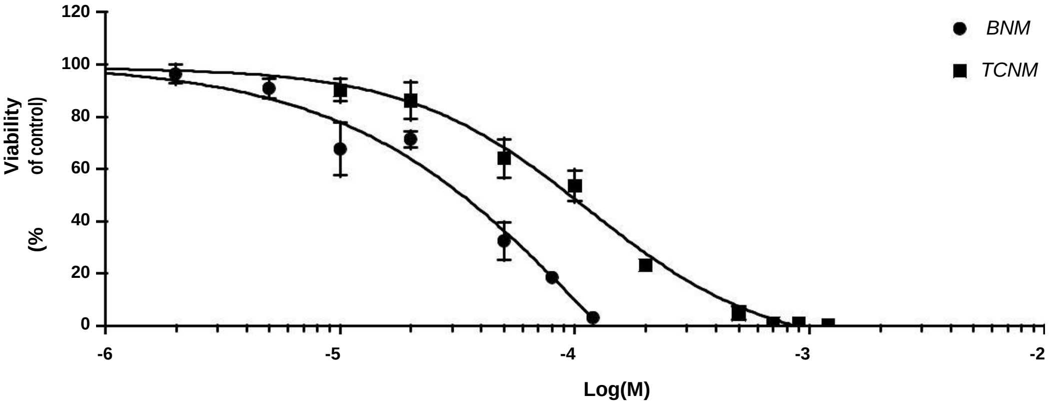
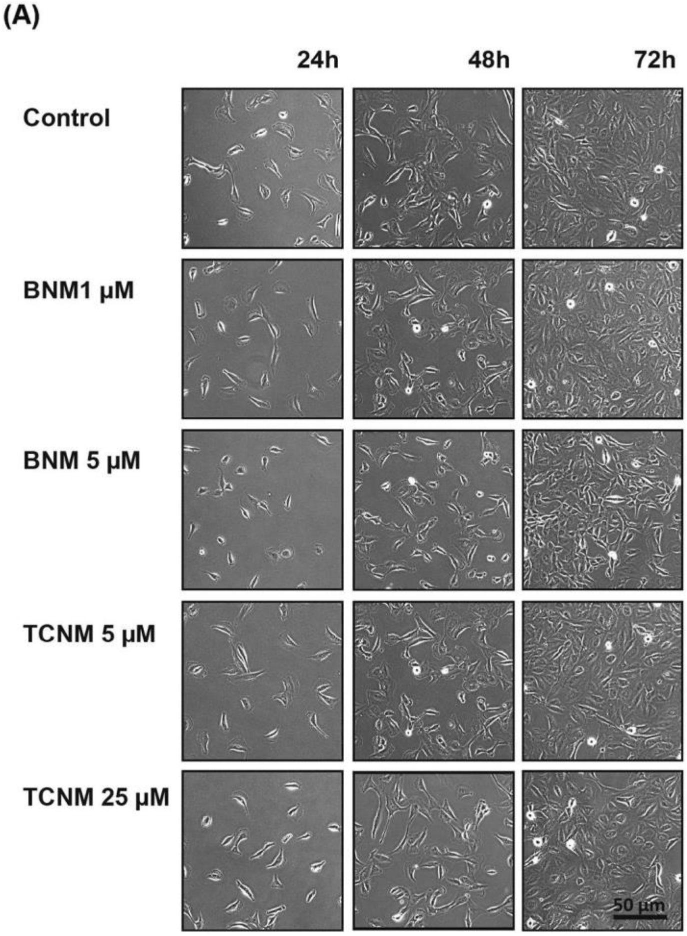


Fig. 1. BEAS-2B cell viability curves after 24 h of exposure to various concentrations of BNM and TCNM. Data are presented as mean values of three independent experiments ± SEM.



(B)

	Doubling time ± SEM (h)
Control	19.88 ± 0.64
BNM 1 µM	18.96 ± 1.17
BNM 5 µM	21.09 ± 1.63
TCNM 5 µM	22.74 ± 1.49
TCNM 25 µM	19.61 ± 1.50

Fig. 2. Cell proliferation and morphology of BEAS-2B cells after 8 weeks of BNM and TCNM long-term exposure. A) Representative pictures of cell morphology taken 24, 48 and 72 h after seeding. B) BEAS-2B doubling time values (mean values ± SEM).

colony number or size were observed after the exposure. Cells grown with untreated BEAS-2B CM displayed an average of 1338 ± 44 co-lonies. Similar mean values were displayed by HCT116 cells grown in CM of long-term exposed BEASe2B, showing an average of 1382 ± 91 and 1329 ± 62 for the CM of 1 and 5 µM BNM, and 1309 ± 68 and 1309 ± 61 colonies for the CM of 5 and 25 µM TCNM.

As CM contains cell-secreted factors known to enhance and not in-itiate tumour effects, the comparisons of big colonies or colony sizes are of significance when assessing the promotion of anchorage-independent cell growth. The average size of HCT116 cells exposed to control CM was 216.00 ± 1.17 µm, whereas HCT116 colonies grown in HNM-ex-posed BEAS-2B CM showed mean values of 214.70 ± 2.84 and 213.90 ± 4.34 µm for 1 and 5 µM BNM, and 210 ± 2.74 and 206.53 ± 5.70 µm for 5 and 25 µM TCNM, respectively. There were no significant differences in the percentage of big colonies among the treatments (Fig. 4C).

3.5. Long-term exposure to HNMs did not influence the lung stroma secretion pattern

Another method by which transformed cells induce malignant transformation is to prompt stroma cells to secrete growth factors that potentiate tumour effects (Cammarota and Laukkanen, 2016). To assess whether BNM or TCNM could enhance tumour growth through this indirect mechanism, MRC5 lung stroma fibroblasts grown in HNM long-term-exposed BEAS-2B CM were evaluated by the soft agar assay with HCT116 cells (Fig. 5). As shown, HCT116 cells grown in CM of control MRC5 presented an average number of colonies of 1822 ± 119 non-significantly different than the number of colonies formed by HCT116 cells grown in CM of exposed MRC5 cells, which was 1848 ± 127, 2306 ± 149, 1889 ± 178 and 2171 ± 204 for 1 µM BNM, 5 µM BNM, 5 µM TCNM and 25 µM TCNM, respectively. When mean colony sizes were analysed, the results consistently showed no HNM-associated effect. Thus, HCT116 cells grown in CM of control MRC5 cells showed a mean colony size of 198.61 ± 2.15 µm, whereas colony sizes of HC-T116 cells grown in CM of exposed MRC5 cells were 199.25 ± 1.66, 201.06 ± 4.74, 202.19 ± 2.83 and 200.56 ± 2.24 µm for 1 µM BNM, 5 µM BNM, 5 µM TCNM and 25 µM TCNM, respectively. The CM of MRC5-exposed cells was also unable to induce changes in the percentage of big colonies (Fig. 5C).

3.6. Long-term exposure to HNMs does not change the secretion of MMP-2 and MMP-9

Extracellular proteinases, such as matrix metalloproteinases (MMPs), are known to influence cancer cell invasion at a local and distant level (Kessenbrock et al., 2010). For this reason, increased MMP activity levels are generally used as indicators of cell malignant trans-formation, especially the tumoral-invasion-related MMP2 and MMP9. No significant changes were observed when the secreted MMP 2 + 9 were analysed in HNM long-term-exposed BEAS-2B cells compared to control cells (Fig. 6A). As MMPs are mainly secreted by stromal cells after stimulation by tumoural cells, we found it interesting and neces-sary to confirm these negative results by analysing the secretion of MMP 2 + 9 in MRC5 cells grown in CM of long-term-exposed BEAS-2B cells. Consistent with previous results, no changes in MMP secretion were observed (Fig. 6B).

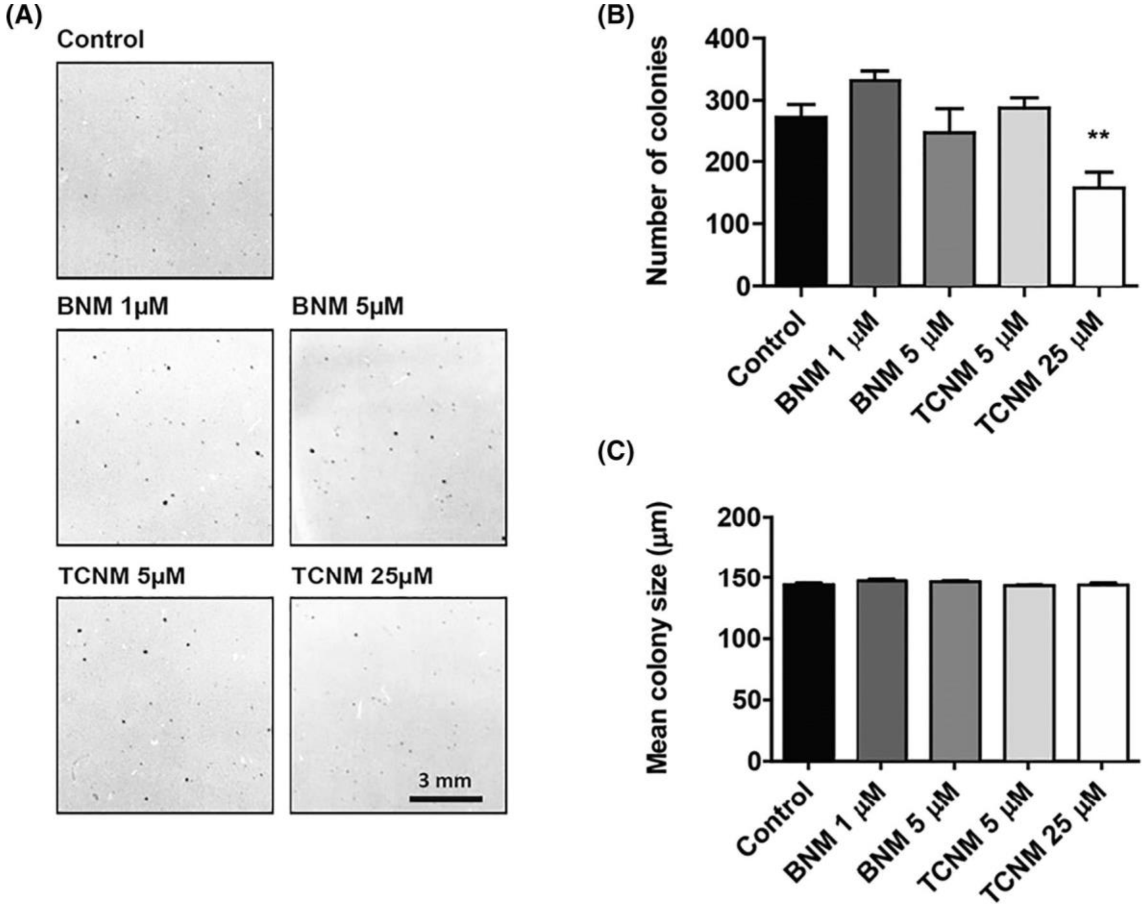


Fig. 3. Anchorage-independent growth capacity of BEAS-2B cells after 8 weeks of BNM and TCNM long-term exposure. A) Representative images of BEAS-2B colonies in soft agar. B) Mean number of colonies per well ± SEM. C) Mean colony size per treatment ± SEM. **P < 0.01 compared with time-matched controls.

4. Discussion

DBPs constitute a very complex mixture of chemicals. The amount and variety of their chemical components depend on both the disin-fection procedure used and the characteristics of the raw water source. So far, > 600 DBPs have been discovered, with many of them showing different levels of cytotoxicity, genotoxicity and carcinogenic properties (Richardson et al., 2007).

Although there is ample epidemiological evidence suggesting a link between cancer risk and DBP exposure (Villanueva et al., 2007, 2015; Hrudey et al., 2015), none of the individual species tested to date in the limited assays employed has shown sufficient carcinogenic potency to account for the cancer risks projected from the epidemiological studies (Bull et al., 2011). This implies that a more systematic approach to determine the carcinogenic potential of DBPs is required. In this sense, our study analysing the carcinogenic potential of two HNMs is an at-tempt to provide new information to fill in this gap.

There are very few in vitro studies together with several in vivo studies dealing with the carcinogenic risk of DBPs. Among the in vivo studies, McDorman et al. (2003a, 2003b) evaluated four DBPs by using rats phenotypically prone to the effects of renal carcinogens. Potassium bromate, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), chloroform, and bromodichloromethane were tested. No significant increases in renal adenomas and carcinomas were detected. Never-theless, elevated hyperplasia and tumours were observed when rats were exposed to the highest dose of chloroform, as well as in the mixture containing the highest doses of the four DBPs tested. In another study from the same group using the same DBP compounds, transitional epithelial hyperplasia and cell proliferation in the urinary bladder were observed in rats exposed to the highest dose of MX. Furthermore, treatment with the four individual DBPs and the mixture induced the development of aberrant crypt foci, considered as putative pre-neoplastic colon cancer lesions (McDorman et al., 2003b). A third study evaluating the effects of dichloroacetic acid (DCA) in early life observed that DCA is carcinogenic in mice, inducing increases in the incidence of hepatocellular tumours comparable to those of life-long exposures (Wood et al., 2015). In the particular case of BNM and TCNM, our data suggest that long-term exposures to non-cytotoxic doses of both com-pounds are not able to trigger any of the in vitro cancer phenotypic hallmarks analysed in lung epithelial cells. This would support the view that exposure to these particular DBPs does not increase the carcino-genic risk in humans. Accordingly, some studies support the view that carcinogenic risk is not conclusively associated to DBPs' exposure, i.e. a study analysing the incidence of tumours in TCNM-treated rats and mice indicated that this compound didn't show conclusive evidence of carcinogenicity (National Toxicology Program, 1978).

Although the in vivo studies are an accurate approximation of the actual tumour-inducing scenario, the high costs, and complexity of this type of studies make them non-applicable to the vast number of po-tential genotoxic/carcinogenic DBP compounds. This is the basis of our proposal to use in vitro cell transformation assays. Indeed, the perfor-mance of such assays in predicting carcinogenic potential has been established on several hundreds of chemicals. In fact, the Organization for Economic Co-operation and Development (OECD) has specific guidelines on "Cell transformation assays for the detection of chemical carcinogens" (Vasseur and Lasne, 2012), with accumulated evidence that the cellular and molecular processes involved in in vitro cell transformation are similar to those occurring during in vivo carcinogenesis (Creton et al., 2012). The assumption that in vitro cell trans-formation takes place when a battery of different cancer hallmarks is

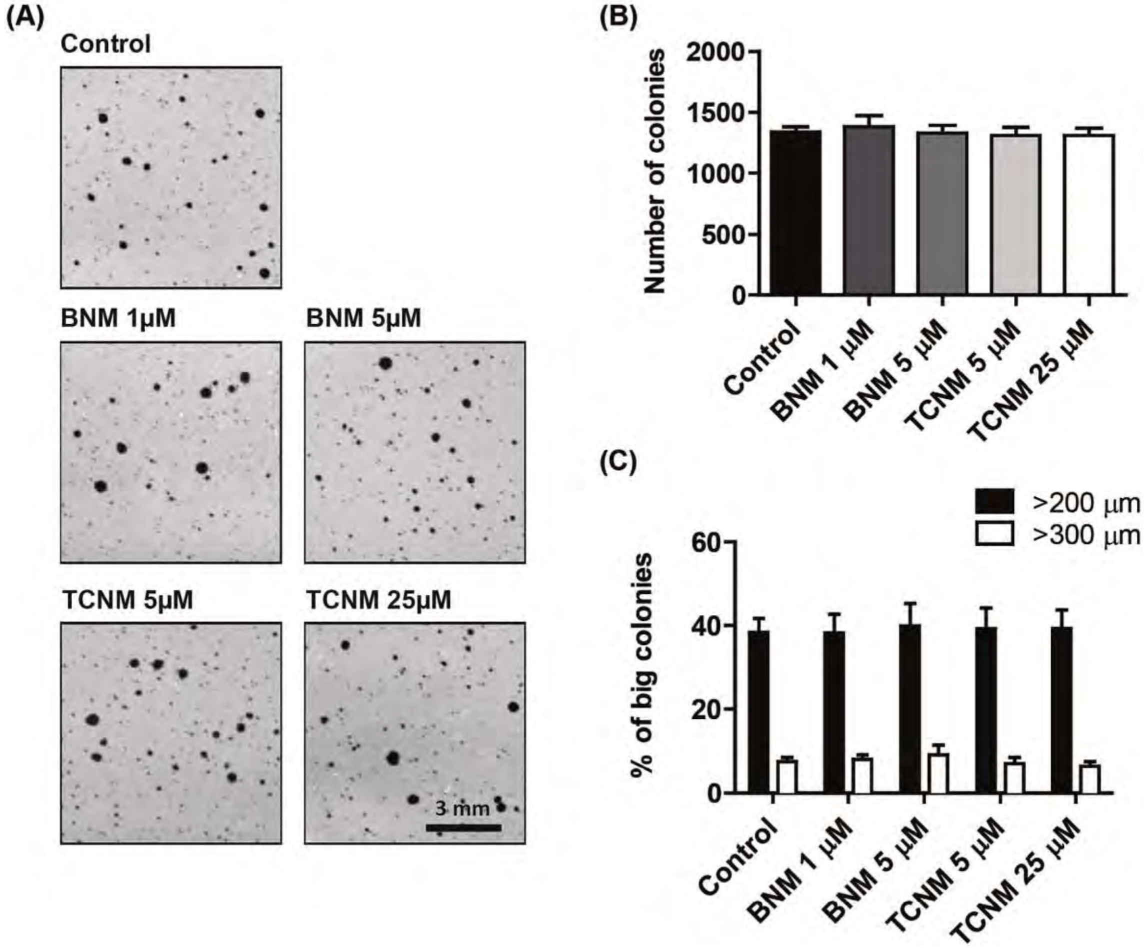


Fig. 4. Promotion of HCT116 anchorage-independent cell growth by the secretome of BEAS-2B after 8 weeks of BNM and TCNM long-term exposure. A) Representative images of HCT116 colonies in soft agar. B) Mean number of HCT116 colonies per plate ± SEM. C) Percentage of HCT116 big colonies ± SEM.

evident (Hanahan and Weinberg, 2011) has moved researchers to ex-plore different models. At this point, it should be remembered that the standard CTAs use animal cell models, no human cells. The use of human cell lines allows the selection of target cells according to the type of exposure to be mimicked. In line with this, the human bronchial epithelial BEAS-2B cells have shown its usefulness in different studies detecting the tumorigenic effect of different agents such as titanium dioxide nanoparticles (Vales et al., 2015), cadmium (Cartularo et al., 2016) and silver nanoparticles (Choo et al., 2016). In spite of the ad-vantages of these in vitro approaches, the limited number of studies carried out with DBPs is surprising, mainly taking into account the environmental risks posed by these chemicals. In one of the few available studies, the ability of MX to promote cell transformation was determined in mouse embryonic fibroblasts. MX was used in both the initiation and the promotion phase, after a previous exposure to 3-methylcholanthrene (MC). When MX was added during the promotion phase in the MC-initiated cells, it promoted the development of the transformation foci in a dose-dependent manner. Exposure to MX as the initiator slightly enhanced the development of foci, suggesting that MX may rather act via promoting tumour development (Laaksonen et al., 2001). In a posterior study, different chlorohydroxyfuranones such as MX, MCA [3,4-dichloro-5-hydroxy-2(5H)-furanone], CMCF [3-chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone], and MCF [3-chloro-4-me-thyl-5-hydroxy-2(5H)-furanone] were tested to demonstrate their ability to promote foci formation in the two-stage cell transformation assay in BALB/c3T3 cells. The results indicate that MX acted as a potent inhibitor of gap-junctional intercellular communication. This action was associated with its ability to promote malignant foci formation (Hakulinen et al., 2004). In addition, four DBPs namely tri-bromomethane, bromochloroacetic acid, dibromonitromethane and tribromonitromethane were tested for their potential to transform normal human colonocytes into malignant cells. The results indicated that all DBP-exposed colon cells acquired the ability to grow in soft agar to some extent; however, only cells exposed to tribromomethane were able to grow in media lacking serum and enriching growth factors (DeAngelo et al., 2007). Finally, the cell-transforming potential of the two unregulated DBPs iodoacetic acid (IAA) and iodoform (IF) was tested in mouse NIH3T3 cells. Exposure to IAA increased the fre-quencies of cells with anchorage-independent growth abilities. In ad-dition, IAA-transformed cells were found to form aggressive fi-brosarcomas after inoculation into Balb/c nude mice (Wei et al., 2013).

The above-mentioned studies show the usefulness of cell trans-forming assays to demonstrate the tumorigenic potential of DBPs. In the case of in vitro HNMs carcinogenic effects, only genotoxic damage has been used as a surrogate marker for transformation. The first of these studies analysed a series of HNMs, comparing their DNA damage po-tential in Chinese hamster ovary cells. After short-term exposure, all of the HNMs analysed displayed an increase in SCGE tail moment, in-dicating the induction of genotoxic damage, especially by brominated compounds (Plewa et al., 2004). Other authors have obtained similar results. The exposure of TK6 cells to TCNM and BNM for short periods of time resulted in oxidative and genotoxic damage. These lesions, however, did not translate into clastogenic or aneugenic effects, and could also be repaired after a recovery time, implying that genotoxicity doesn't translate into permanent effects (Liviac et al., 2009). Geno-toxicity, however, not always correlates with carcinogenicity

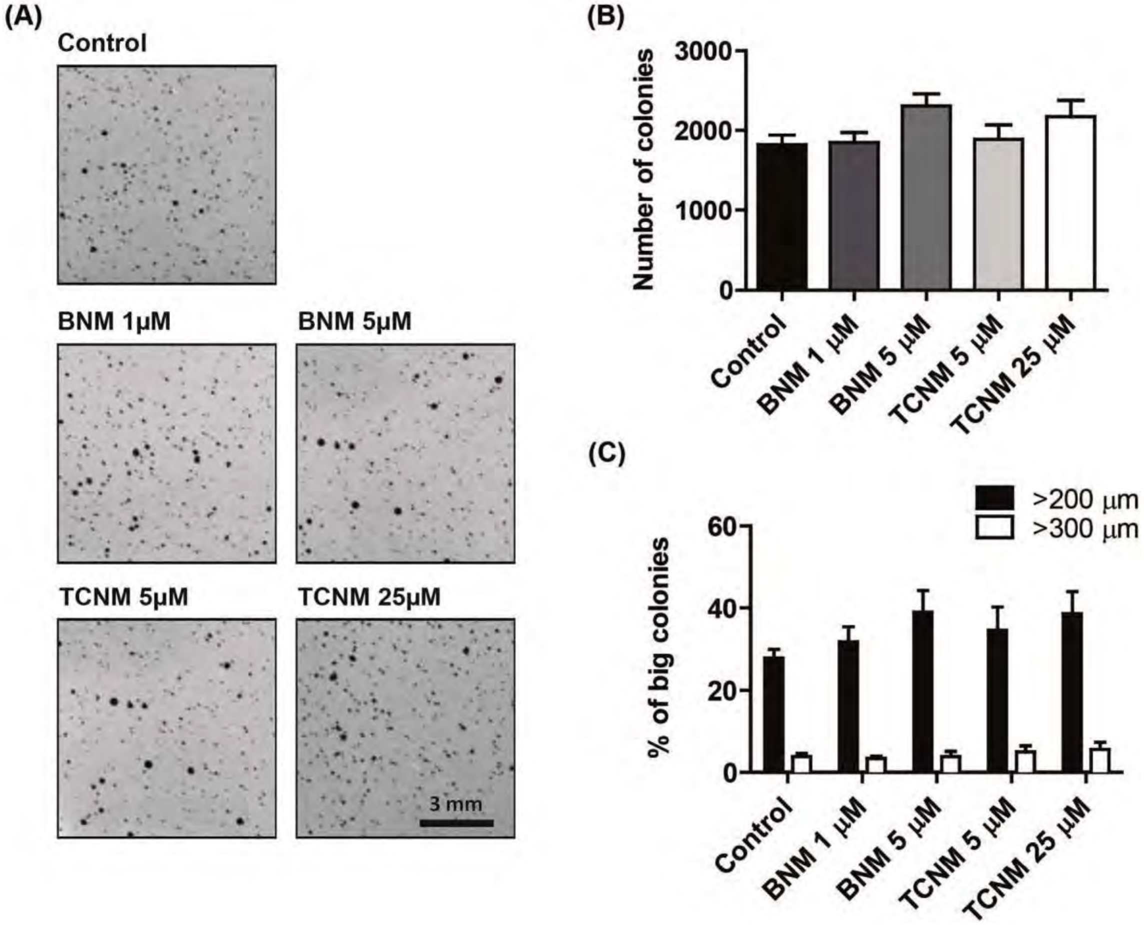


Fig. 5. Promotion of HCT116 anchorage-independent cell growth by the secretome of stromal MRC-5 cells grown in HNMs-long-term CM. A) Representative images of HCT116 colonies in soft agar. B) Mean number of HCT116 colonies per well ± SEM. C) Percentage of HCT116 big colonies ± SEM.

(Richardson et al., 2007).

Due to the complexity of the cell transformation process, it is pre-ferable to investigate the acquisition of a wide range of cancer-like phenotypic features to decipher carcinogenic risk potential. This is the basis of the strategy used in our study. Morphological changes in the exposed cells are considered to be typical cancer-like phenotypic in-dicators (Clancy et al., 2012). In addition, secretion of matrix me-talloproteinases (mainly MMP2 and MMP9) is also considered an appropriate biomarker of cell transformation since MMPs play crucial roles in tumour invasion, morphogenesis, angiogenesis, metastasis, and wound healing by remodeling the extracellular matrix (Oum'hamed et al., 2004; Page-McCaw et al., 2007). Moreover, assessment of the anchorage-independent growth of cells in semi-solid matrices is con-sidered an intrinsic property of transformed cells (Borowicz et al., 2014). The paracrine signals secreted by cells undergoing malignant transformation enhance the tumorigenic potential of nearby cells (Del

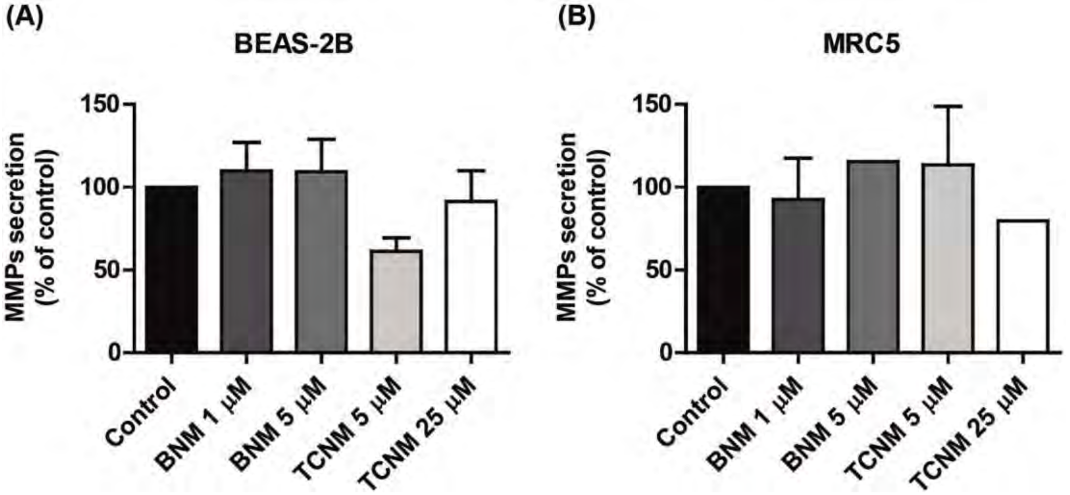


Fig. 6. Secreted MMP 2 + 9 activities after 8 weeks of BNM and TCNM long-term exposure. A) MMP 2 + 9 in long-term exposed BEAS-2B cells. B) MMP 2 + 9 in stromal MRC5 cells exposed to CM of long-term exposed BEAS-2B cells.

Pozo Martin et al., 2015); therefore, conditioned media obtained from these cell cultures can be used to determine their ability to induce anchorage-independent growth of tumoural or stromal cells. In this way, the usefulness of an indirect soft agar approach has recently been demonstrated (Bach et al., 2016).

In our work, the absence of these tumoral markers suggests that HNMs should be considered as non-carcinogenic DBPs. However, taking into account the environmental risks posed by these chemicals other aspects not explored in this work should be considered in future studies assessing HNMs transforming potential. We cannot discard the possi-bility that longer exposure times may have a significant effect on the parameters analysed. Also, even though the set of cancer phenotypic hallmarks analysed gave us reliable negative results, the analysis of other indicators of cellular transformation such as gene dysregulation and migration capacity would provide further information on the transforming ability of BNM and TCNM. Another interesting aspect to consider is that the interaction between two or more DBPs could be a factor accounting for the carcinogenic effects of these compounds. As DBPs are a complex mixture of chemicals, an additive or synergic effect between them should also be evaluated. The few studies focusing on the carcinogenicity of DBP mixtures using drinking water obtained negative results (Richardson et al., 2007). Nevertheless, all these studies have analysed the effect of mixtures as oral exposure in rats, so this phe-nomenon can't be ruled out without a more extensive evaluation.

5. Conclusions

As a summary, a battery of in vitro cancer-like phenotypic hallmarks must be performed to clearly predict the carcinogenic potential of a given environmental chemical. On this basis, the lack of observed (i) morphological and proliferation changes, (ii) variations in the secretion of matrix metalloproteinases, and (iii) anchorage-independent cell growth ability, both directly and indirectly, demonstrate that the two selected HNMs do not induce cell transformation under our experi-mental conditions, suggesting that they should be considered as non-carcinogenic DBPs. Our results support previous data implying that these chemicals do not pose a carcinogenic risk to humans. However, their potential negative effect on public health requires further evaluation to completely rule this hazard out.

Conflict of interest statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Transparency document

The http://dx.doi.org/10.1016/j.tiv.2017.08.013 associated with article can be found, in online version.

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8.2. Annex 2: Second paper

Hazard assessment of haloacetic acids in human urothelial (UROtsa) cells

Submitted paper

Hazard assessment of haloacetic acids in human urothelial (UROtsa) cells

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Running Title: Carcinogenic potential of water disinfection by-products

ABSTRACT

Disinfection by-products (DBPs) are compounds produced in the raw water disinfection processes. Although increased cancer incidence has been associated with exposure to this complex mixture, the carcinogenic potential of individual DBPs remains not well known; thus, further studies are required. Haloacetic acids (HAAs) constitute an important group among DBPs. In this study we have assessed the *in vitro* carcinogenic potential of three HAAs namely chloro-, bromo-, and iodoacetic acids. Using a long-term (8 weeks) and sub-toxic doses exposure scenario, the acquisition of different *in vitro* transformation markers was evaluated using human urothelial (UROtsa) cells, as a suitable target model. Our results indicate that long-term exposure to low doses of HAAs did not reproduce the genotoxic effects observed in acute treatments, where oxidative DNA damage was induced. No carcinogenic transformation was observed, as indicated by the absence of morphological changes, no effects on cell growth rate, and no increased anchorage-independent cell growth capacity. Interestingly, HAA-long-term exposed UROtsa cells developed resistance to oxidative stress damage, what would explain the observed differences between acute and long-term exposure conditions. According to that, data obtained under long-term exposure to sub-toxic can be more useful in terms of risk assessment then those obtained under acute exposure scenarios.

Keywords: haloacetic acids; chloroacetic acid; bromoacetic acid; iodoacetic acid; cell transformation; UROtsa cells; cancer biomarkers

1. INTRODUCTION

Chemical disinfection of water is a common procedure aiming to control waterborne infectious diseases. Nevertheless, different reactions occur between the organic matter present in raw water and the chemicals used to disinfect it.[1] As result of these reactions, disinfection by-products (DBPs) are produced. DBPs constitute a complex mixture, containing many different chemical groups with a number of compounds having mutagenic and carcinogenic risk.[2] Among them we can point out haloacetic acids (HAAs),[3] which have become a public health concern due to their potential toxic and carcinogenic risk.[4] Among the HAAs, chloroacetic acids (CAAs), bromoacetic acids (BAAs), and iodoacetic acids (IAAs) are found with an important prevalence in disinfected water.[5]

Due to their potential impact in public health, some DBPs are regulated in many countries. HAAs count for approximately the 25% of the halogenated DBPs.[5] Among this DBPs class, only five (bromoacetic acid, dibromoacetic acid, chloroacetic acid, dichloroacetic acid, and trichloroacetic acid) are currently regulated by the U.S. EPA, with a maximum contaminant level (MCL) for the sum of the five regulated HAAs of 60 µg/L.[6] One of the long-term human health implications produced by DBPs exposure is cancer,[7] with bladder cancer being one of the main documented effects in epidemiological studies.[8,9] Nevertheless, the potential carcinogenic risk of each individual DBP remains to be determined.

Long-term studies of carcinogenesis using mammalian models are lengthy and expensive, and present important ethical implications. For this reason the *in vitro/in vivo* genotoxic assessment of DBPs is usually accepted as a surrogate biomarker of their potential cancer risk.[2,10-12] Nevertheless, this approach underestimates the risk of non-genotoxic carcinogens, implying that a more direct way to measure the potential carcinogenic risk of individual DBPs *in vitro* remains to be developed.

To overcome this problem, *in vitro* cell transformation assays (CTA) have been proposed as suitable alternatives to long-term animal studies to measure carcinogenic effects. Cellular and molecular processes involved in the *in vitro* cell transformation are close to those taking place in *in vivo* carcinogenesis.[13] It is accepted that an exposed cell line becomes tumorigenic when different cancer hallmarks become evident.[14] Among the different hallmarks of cell transformation, morphological cell changes, anchorage-independent cell growth, secretion of matrix metalloproteinases (MMPs), and invasiveness have been used in different studies.[15-18] The Organization for Economic Co-operation and Development (OECD) analyzed the performance of three CTAs to screen the carcinogenic potential of chemicals. The Syrian hamster embryo (SHE) cells, and the mouse cell lines BALB/c 3T3 and C3H10T1/2 were evaluated and, as conclusion, the development of an OECD guideline with the SHE and BALB/c 3T3 models was proposed.[19] However, the acute treatment methodology employed by these assays may not reproduce the actual case scenario, where target cells are exposed to low doses of carcinogenic agents for a long period of time. This could be translated as an overestimation of the actual carcinogenic potential of chemical species which do not pose health risks at realistic concentrations.

Due to the aforementioned reasons, the aim of this study has been to test a new methodology to determine the potential carcinogenic risk of three HAAs, namely chloroacetic acid (CAA), bromoacetic acid (BAA) and iodeacetic acid (IAA), in a human urothelial cell line (UROtsa), which has shown to be a powerful tool to evaluate environmental insults to human urothelium.[20] This cell line has also been chosen considering that bladder cancer is one of the main effects associated to DBPs exposure. Our study has been carried out under long-term exposures (8 weeks) and low, non-cytotoxic doses. It is important to indicate that this experimental approach tries to mimic a more realistic exposure scenario to DBPs.

2. MATERIALS AND METHODS

2.1. Cell Culture Conditions and *In Vitro* DBP Exposure.

The human urothelial cell line (UROtsa), and the human cervix epitheloid carcinoma cells (HeLa) were maintained in DMEM high glucose medium (LifeTechnologies, NY, USA) supplemented with 10% foetal bovine serum (FBS; PAA®, Pasching, Austria), 1% of non-essential amino acids (NEAA; PAA®) and 2.5 μg/mL plasmocin (InvivoGen, CA, USA) at 37 ºC in a humidified 5% CO_2 incubator. Chloroacetic acid (CAA, $C_2H_3ClO_2$, CAS 79-11-8) and bromoacetic acid (BAA, $C_2H_3BrO_2$, CAS 79-08-3) were both purchased from Acros Organics (Geel, Belgium). Iodoacetic acid (IAA, $C_2H_3IO_2$, CAS 64-69-7) was purchased from Sigma-Aldrich (MO, USA). Three separate 75-cm^2 flasks of UROtsa cells were exposed to non-cytotoxic concentrations of the three HAAs (10 and 100 µM CAA; 0.005 and 0.05 µM BAA; 0.01 and 0.1 µM IAA) for 8 weeks, the DBP-containing medium was changed every 72 h, and sub-confluent cells were passaged weekly. CAA was diluted in distilled water to an initial concentration of 100 mM. BAA initial concentration was 50 mM, and for IAA was 10 mM. In all cases, working concentrations were freshly prepared in media before every treatment. The manipulation of chemicals was conducted in a certified biological/chemical safety hood following the manufacturer's instructions.

2.2. Analysis of Cell Viability.

UROtsa cells were plated in opaque 96-well plates in triplicates at a density of 20,000 cells per well and incubated overnight in complete medium. Next, it was replaced with fresh medium with concentrations up to 10 mM of BAA, CAA or IAA. Twenty-four h after the exposure, cells were washed with PBS and incubated in 0.44 µM of resazurin dye (Alfa Aesar, Karlsruhe, Germany) for 2 h. Fluorescence emitted by the reduction product resorufin was then measured using a microplate fluorimeter equipped with a 560 nm excitation / 590 nm emission filter set, being the resorufin produced proportional to the number of viable cells. Cytotoxicity curves derived from averaging three independent experiments, and the IC_{50} values were calculated using GraphPad prism version 7.00.The same protocol was followed to determine whether the 8 weeks treatment induced variations in cells resistance to an agent inducing oxidative damage. UROtsa cells previously exposed to the higher concentrations of the three HAAs for 8 weeks were exposed to increasing concentrations of $KBrO_3$ (3 mM to 15 mM) for 24 h. Resazurin incubation and following data analysis was performed as described previously.

2.3. Determination of Genotoxic and Oxidative DNA Damage.

Genotoxic and oxidative DNA damage were assessed by the alkaline comet assay with and without the use of formamidopyrimidine DNA glycosylase (FPG) enzyme in UROtsa cells exposed to the HAAs for 4 h and 8 weeks. The comet assay detects single- and double-stranded DNA breaks in naked supercoiled DNA. These DNA strand breaks allow loops of DNA to migrate during the electrophoresis, forming a comet tail. The use of enzymes as FPG allows the detection of oxidative damage. Untreated and long-term exposed UROtsa cells were seeded in 6-well plates in triplicates at a density of 200,000 cells per well. Untreated cells were then exposed to concentrations ranging from 750 to 1500 µM for CAA, 5 to 25 µM for BAA, and 2.5 to 8 µM for IAA; while long-term exposed cells remained exposed to the long-term treatment. To assess a possible resistance to oxidative damage after the long treatments, long-term exposed cells were as well exposed to 2.5 mM $KBrO_3$ for 30 minutes. After the corresponding exposure times, cells corresponding to positive controls were incubated 30 minutes in 200 µM MMS and 5 mM $KBrO_3$. At the end of the treatment, cells were washed twice with PBS and collected by tripsinization. A mixture 1:10 containing the cells and 0.75% agarose at 37 ºC is then prepared, and 7 µL drops are placed onto the Gelbond® film (GBF, 10.5 x 7.5 cm), with 3 replicate drops corresponding to each treatment. Two identical GBF were processed simultaneously for each experiment. Both films were then lysed overnight by immersion in ice-cold lysis buffer (2.5 M NaCl, 0.1 M Na2EDTA, 0.1 M Tris base, 1% Triton X-100, 1% lauroyl sarcosinate, 10% DMSO, pH 10) at 4 °C. One film was incubated for 30 min at 37 °C in enzyme buffer containing FPG and the other one in enzyme buffer alone. Both films were washed with electrophoresis buffer (0.3 M NaOH and 1 mM Na_2EDTA, pH 13.2), and placed into a horizontal gel electrophoresis tank. Films were incubated 35 min in the same buffer to allow DNA unwinding prior to electrophoresis, performed at 1 V/cm and 300 mA for 20 min at 4 °C. After the electrophoresis, both films were rinsed with cold PBS for 15 min, fixed in absolute ethanol for 2 h and air-dried overnight at room temperature. Prior observation, GBF were stained for 20 min with 1/10,000 diluted SYBR Gold (Molecular Probes). Once mounted on an acrylic slide and covered with an appropriate coverslip, comet tails were measured using the Komet 5.5 Image analysis system (Kinetic Imaging Ltd., Liverpool, UK). 100 cells were scored for each treatment, and the percentage of DNA in tail was the parameter used to measure the DNA damage.

2.4. Cell Proliferation.

Long-term exposed cells and unexposed passage-matched controls were plated in 6-well plates at a density of 100,000 cells per well in regular conditions. Cells were collected by trypsinization and counted at 24 h intervals by the Beckman counter method. The time necessary for doubling cell population was calculated according to the equations referred in http://www.doubling-time.com/compute.php.

2.5. Anchorage-Independent Cell Growth Capacity.

The colony formation capacity in soft-agar was assessed in long-term exposed UROtsa cells and passage-matched untreated controls as described elsewhere.[21] UROtsa cells were trypsinised and individualized using a 30-µm filter. 10,000 cells were suspended in a 1:1:1 mixture containing 1x DMEM (supplemented with 10% FBS, 1% NEAA, 1% L-Glu 200 mM and 2.5 µg/mL Plasmocin), 2x DMEM (with 2x supplements) and 1.2% Bacto Agar (DIFCO, MD, USA). Cells were then plated in triplicates in 6-well plates over a 0.6% base agar (in supplemented 2x DMEM) and incubated at 37 ºC for 21 days. After this time, cell colonies were stained overnight with 1 mg/mL of (2-p-iodophenyl)-3-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (Sigma, MO). HeLa cells were seeded following the same procedure at different concentrations, and in 1xDMEM supplemented with 10, 20 and 30% FBS as a positive control for cell growth and dose response. Plates were scanned and colony counting was performed using OpenCFU open-source Software. A modified version of the assay was performed using 72 h conditioned media (CM) from long-term exposed UROtsa cells and passage-matched controls to assess their capacity to promote the malignant growth of tumour cells. Triplicates of a total of 10,000 individualized HeLa cells were resuspended in CM and mixed in a 1:1:1 ratio with supplemented 2x DMEM and 1.2% bacto agar. The remainder of the protocol was performed as described above. Plates were incubated for 15 days.

2.6. Statistical analysis.

For each assay, three independent experiments were performed in triplicates and the mean and SEM was calculated. One-way analysis of variance followed by Dunnett's multiple comparison test was performed to compare treated with untreated time-matched controls. In all cases, a two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 7.00.

3. RESULTS

3.1. BAA and IAA are Considerably more Cytotoxic than CAA on UROtsa Cells.

To determine the effect of HAAs on UROtsa cells viability, a preliminary cytotoxicity experiment was carried out using a wide range of concentrations of CAA, BAA and IAA. Even though all three compounds were cytotoxic after 24 hours of exposure, the obtained results showed differences in the UROtsa cells' sensibility to each compound (Fig. 1). IAA showed the strongest cytotoxic effect, with an IC_{50} value of 2.4 ± 0.2 µM. BAA presented an IC_{50} of 6.7 ± 0.4 µM. Interestingly, CAA cytotoxicity was more than 100 times lower than the brominated and iodinated acids, with an IC_{50} of 794 ± 61 µM. Thus, these results seem to agree with previous data, suggesting that there is a correlation between cytotoxicity and the relative atomic mass of the halogen atom present in the molecule, and that the cytotoxicity of chlorinated compounds is sensibly lower compared to brominated and iodinated HAAs.

3.2. Short-term Exposure to HAAs Induces Oxidative Lesions, but no Genotoxic DNA Damage.

DNA damage has long been considered a surrogate marker for cancer risk. To assess the genotoxic potential of HAAs on UROtsa cells, the cell line was exposed to different non-cytotoxic concentrations of all three HAAs for four hours, and genotoxic and oxidative DNA damage were evaluated using the alkaline comet assay (Figure 2). No differences in DNA tail % were observed at the tested conditions in the alkaline comet assay, suggesting that no DNA breaks are formed under the tested conditions (Figure 2A). Conversely, significant oxidative DNA damage was observed for CAA at concentrations higher than 1 mM. In line with the results obtained in our cytotoxicity assays, exposure to BAA and IAA induced analogous effects at consistently lower concentrations (5 µM and 2.5 µM, respectively) (Figure 2B). For all three compounds, the maximum non-cytotoxic oxidative damage observed was comparable, with DNA tail percentages of 34.78%, 35.97% and 39.51% for CAA, BAA and IAA, respectively. These results suggest that, even though direct DNA breakage was not observed at the analysed concentrations, HAAs induce oxidative DNA damage in UROtsa cells, which could have mutagenic effects if not repaired promptly.

3.3. Long-term Exposure to Low Concentrations of HAAs did not Cause Genetic Damage.

As acute treatments might not simulate the real-case scenario of HAAs long-term exposure, we wanted to analyse the effects of a long-term exposure to the aforementioned compounds. To this aim, UROtsa cells were exposed for 8-weeks to two different non-cytotoxic concentrations of CAA (10 and 100 µM), BAA (0.005 and 0.05 µM) and IAA (0.01 and 0.1 µM). Results show that, as previously seen for acute treatments, long-term exposure to HAAs does not change the level of direct DNA damage compared to the untreated controls (Figure 3A). In this case, however, the exposure to HAAs did not induce oxidative DNA damage (Figure 3B). The DNA tail percentages measured were 3.64%, 2.04% and 3.14% for the highest concentrations of CAA, BAA and IAA, respectively, which are not significantly different to those observed in the controls. Taken together, our results show that, even though HAAs induce oxidative damage in UROtsa cells when subjected to an acute exposure, long-term exposures do not, suggesting that assays based on acute exposures might overestimate the real mutagenic potential of HAAs.

3.4. Long-term Exposure to HAAs Induced no Changes in UROtsa Cellular Morphology or Proliferation.

Since DBPs lacking genotoxic activity could nonetheless induce tumoral transformation, we tested the long-term carcinogenic potential of each of the selected HAA. As carcinogenesis is a complex, multistep process, different transformation hallmarks were evaluated. One of the initial effects observed in transformed cells is the progressive decrease of their doubling times and the loss of their characteristic morphology. Thus, cellular proliferation and shape were monitored as phenotypic markers of cancer onset throughout the 8 weeks of long-term treatment. No noticeable changes were observed in cell morphology between the untreated controls and the HAA-exposed cells (Figure 4A), and results analysed at the end of the exposure period indicated that none of the

HAAs tested increased the proliferation rate significantly when compared to time-matched controls (Figures 4B and 4C).

3.5. Long-term Exposure to HAAs did not Alter UROtsa Cells Capacity to Grow in Soft-Agar.

Another main characteristic of cancer cells is their anchorage-independent growth capacity.[14] UROtsa cells were subjected to a soft-agar colony formation assay to assess this feature after the HAA-long-term exposure. As shown in figure 5, all experimental conditions spontaneously formed colonies. Nevertheless, no significant changes were apparent after HAA treatments. Control samples formed a mean of 1827 ± 352 colonies, while CAA long-term-exposed cells displayed 1974 ± 245 and 1848 ± 202 colonies for its lowest and highest concentrations, respectively. On the other hand, cells exposed to BAA formed an average of 1904 ± 223 colonies at 0.05 µM concentration and 1873 ± 151 colonies at 0.005 µM. Finally, IAA exposed cells were able to grow 1932 ± 158 and 2090 ± 201 colonies for 0.1 µM and 0.01 µM, respectively (Figure 5A). When colony sizes were taken into account (Figure B), all the experimental conditions presented similar mean sizes (158.67 ± 3.84 µm for controls *vs.* 147.93 ± 1.86 and 159.34 ± 2.08 µm for CAA 10 and 100 µM, 154.74 ± 4.19 and 155.06 ± 1.51 µm for BAA 0.05 and 0.005 µM, and 147.90 ± 1.62 and 146.59 ± 0.76 µm for IAA 0.1 and 0,.1 µM, respectively).

3.6. Long-Term Exposure to HAAs did not Alter UROtsa Tumor-Inducing Capacity.

The interplay between the different cell populations and their environment play a critical role in the formation and malignancy of a tumour. Transformed cells secrete paracrine signals that potentiate the tumorigenicity of their surrounding neighbours.[22] The capability of long-term exposed UROtsa cells to promote anchorage-independent growth of the tumoural cell line HeLa in soft-agar was therefore evaluated. As observed in Figure 6A, no differences in HeLa colony number or size were observed after the exposure. Cells grown with untreated UROtsa CM displayed an average of 3755 ± 322 colonies. Similar mean values were displayed by HeLa cells grown in CM of long-term exposed UROtsa cells, showing an average of 3873 ± 272 and 3897 ± 271 for the CM of 10 and 100 µM of CAA, and 3762 ± 230 and 4112 ± 493 colonies for the CM of 0.005 and 0.05 µM BAA, and 3269 ± 451 and 2477 ± 413 colonies for the CM of 0.01 and 0.1 µM of IAA. Other than tumorigenesis-inducing factors, transformed cells also secrete factors known to enhance tumour effects such as cell proliferation. Thus, comparison of colony sizes could give us useful information when assessing the promotion of anchorage-independent cell growth. The average size of HeLa cells exposed to control CM was 196.50 ± 2.63 µm, whereas HeLa colonies grown in CAA-exposed UROtsa CM showed mean values of 208.18 ± 5.89 and 201.97 ± 5.91 µm for 10 and 100 µM, 205.67 ± 6.37 and 192.15 ± 5.31 µm for 0.005 and 0.05 µM BAA, and 191.05 ± 3.14 and 202.34 ± 10.57 µm for 0.01 and 0.1 µM IAA respectively (Figure 6B). Taken together, our experiments showed that long-term exposure of UROtsa cells to low concentrations of monoHAAs did not trigger any of the analysed transformation markers.

3.7. Long-Term Exposure of UROtsa Cells to HAAs Determines the Appearance of Resistance to Oxidative-Damage Inducing Agents.

It is known that chronic exposure to low concentrations of cytotoxic agents may result in the increased production of factors that are responsible for a decrease in the cytotoxic sensibility of the cells, a process called drug resistance. An evaluation of HAA long-term treated UROtsa cells sensibility showed that exposed cells developed resistance to the oxidative damage-inducing agent $KBrO_3$ (Figure 7A). The IC_{50} of this compound for the untreated control was 4.76 ± 0.17mM, whereas the IC_{50} of UROtsa cells that were exposed for 8 weeks to low concentrations of CAA, BAA and IAA was 6.66 ± 0.38 mM, 6.68 ± 0.39 mM, and 6.99 ± 0.47mM, respectively. Furthermore, the FPG-modified version of the alkaline comet assay showed that, despite that there were no significant differences in the levels of genotoxic DNA damage (Figure 8A) nor oxidative damage between HAA long-term exposed UROtsa cells and their time-matched controls, there was a decrease in the DNA tail % for all of the HAA-treated cells when exposed to $KBrO_3$ (Figure 8B). All in all, our results seem indicate that long-term exposure of UROtsa cells to monoHAAs does not induce transformation, but rather decreases the sensibility to oxidative damage-inducing agents.

4. DISCUSSION

There are epidemiological evidence showing a strong positive association between chronic DBP exposure and an increased risk of bladder cancer.[8.9] Despite this strong epidemiological base, very few of the chemical species individually tested to date have provided enough evidence to support their direct role on cancer development. This is a gap requiring further efforts.

Most of the little current knowledge on DBPs carcinogenic potential comes from animal model studies. The initial carcinogenicity studies showed a causal role of chloroform in the development of hepatocellular tumors in male and female mice.[23,24] This was the starting point for several *in vivo* studies that have served to regulate DBP concentration in drinking water, showing that DBPs could generate tumors in animals. Among the *in vivo* studies carried out testing HAAs, it was demonstrated that the oral administration of dibromoacetic acid induced the development of hepatocellular tumors in male and female mice and mesotheliomas and leukemia in rats,[25] while dichloroacetic acid also prompted the development of liver tumors in mice and rats.[26,27]

Even though animal models give us a good approximation to study the tumor-inducing effects of DBPs in a whole organism, there are several drawbacks to the selection of this model. Their high cost makes this model unsuitable for testing the vast amount of DBP species to be analyzed. In addition, the results obtained to date have not been conclusive enough, even giving contradictory results.[28,29] Furthermore, differences in the tumorigenic development pattern between rodents and humans make the rodent model at least questionable. Differences could be attributed to different factors: first of all, the known metabolic differences between species, which could explain the variation in the tissues where the tumor develops. The exposure route is also another important factor; most studies have used ingestion as the main entry route for evaluate DBPs, whereas airways and dermal absorption seem to play an important role on human intake of DBPs.[30,31]

Due to the problems posed by the *in vivo* carcinogenesis approach, the *in vitro* transformation assays appear as a sound alternative. Different studies have pointed out parallelisms between cellular and molecular processes involved in *in vitro* cell transformation and *in vivo* carcinogenesis.[13] According to that, the evaluation of transformation biomarkers after the exposure to water pollutants in *in vitro* cultured cells could give us relevant information on their carcinogenic potential. [14] In line with this, CTAs have been used to evaluate the carcinogenic potential of different chemicals, including DBPs. In fact, a recent study demonstrated that NIH3T3 cells treated with iodoacetic acid at micromolar concentrations for 24 h present DNA damage, and that 72 h of exposure can induce the formation of foci in the soft-agar assay, and tumors in nude mice.[32] Furthermore, the adequacy of CTAs for carcinogenic-potential assessment has been validated by international entities such as the Organization for Economic Co-operation and Development (OECD), proposing a series of guidelines for cell transformation assays for the detection of chemical carcinogens.[19] Although the established CTAs can give valuable information on the carcinogenic potential of DBPs; however, most of them use experimental conditions that do not faithfully reflect the exposure conditions to DBPs. On one hand, standard CTAs evaluate the carcinogenic potential over animal cell models, or human cells that are not the target of the tested chemicals; furthermore, the acute treatment regime usually used does not reflect the chronic exposure conditions of environmental chemicals such as DBPs. This could increase the chance of overestimating the actual risk that DBPs pose to human health. To overcome these issues, the standard regulated CTAs have been modified to get a more accurate perspective on the capacity of one compound to induce cellular transformation.[33,34] It is important to point out that in our study UROtsa cell line was selected due to its tissue origin, potential target, and also because its previous successful use in long-term studies of other environmental pollutants.[35.36] Also, the administration of subcytotoxic concentrations of HAAs for longer time periods should better reflect the cellular response to environmental carcinogens.

By using the aforementioned improvements, we analyzed the transformation potential of subtoxic concentrations of three monoacetic acids by evaluating different biomarkers considered to be involved cellular transformation. Due to the complexity of the carcinogenesis process to be sure that the compounds under study behave as a carcinogen it is recommended to have positive effects in more than one cell-transformation biomarker. [37] In our case, the set of transformation biomarkers analysed gave reliable negative results, suggesting that exposure to the selected HAAs should be considered as non-carcinogenic, under our conditions of study. Unfortunately, there is an important lack of studies determining *in vitro* cell transformation for HAA, As indicated only one study has been carried out,[32] showing the ability of iodoacetic acid to induce cell transformation in the mouse embryonic cell line by 72 h exposure. The discrepancy of our results with those reported by Wei et al. may be due to the cell line used, end-point analyzed or exposure procedure. This emphasize the need of extend CTAs evaluation of DBPs, including HAAs. Genotoxicity has been considered a surrogate biomarker of carcinogenic process, nevertheless in our conditions neither of the selected HAAs was able to induce direct DNA breaks contrarily what indicated by other authors where at least iodoacetic and bromoacetic acids were detected as genotoxics in the comet assay.[38,39] Again these discrepancies can be attributed to any of the variables before indicated. Nevertheless, we were able to show their ability to induce oxidatively damaged DNA what was a possible mechanism proposed by Dad et al.[39]

Although acute treatments with CAA, BAA, and IAA induced oxidative damage; the chronic exposure to low doses did not cause the same effect. This reflects the importance of the type of exposure when evaluating the potential risk of chemicals, including DBPs. It seems sound to thing that as much similar are the experimental conditions to those expected to occur in humans. Furthermore, our results show that the long-term treatment with sub-cytotoxic doses of HAAs generates resistance to DNA oxidative agents in UROtsa cells, implying that these cells develop protection mechanisms against these water pollutants. Adaptive response in front of situations generating oxidative stress is a known mechanism.[40,41] Unfortunately the relevance of this mechanism is not detected in acute treatments but seems to be important in long-term exposures. This point out how interesting is the use of long-term approaches to mimicry real exposure scenarios and to get sound results to be used in risk assessment evaluations. In spite of the reported negative findings, DBPs exposure can poses a risk to human health, prompting the exploration of other aspects in future studies. The analysis of other cellular transformation markers such as gene dysregulation could provide further information on HAAs transformation potential. Likewise, the possibility that longer exposure times may have an effect on the parameters analysed in our work should also be explored. Finally, given that disinfected water presents a complex mixture of chemicals, an *in vitro* assessment of possible interacting effects between them should also be carried out.

Summarizing, our results show that exposure to environmentally relevant doses of HAAs does not induce tumoral transformation *in vitro*. These results point out the necessity to explore new methodologies of carcinogenic potential assessment, in order to improve the CTAs currently used, so overestimation of the health risks posed by environmental contaminants can be minimized.

Conflict of interest statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Figure legends

Figure 32. UROtsa cell viability curves after 24 h of exposure to various concentrations of CAA, BAA and IAA.

Figure 2. Genotoxic **(A),** and oxidative DNA damage **(B)** in UROtsa cells after 4 h of exposure to increasing concentrations of CAA, BAA and IAA. Data represented as mean ± SEM. $*P<0.05$; $**P<0.01$; $***P<0.001$.

Figure 3. Genotoxic **(A),** and oxidative DNA damage **(B)** in UROtsa cells after 8 weeks of long-term exposure to CAA, BAA and IAA. Data represented as mean ± SEM. $*P<0.05$; $***P<0.001$.

Figure 4. Cell proliferation and morphology of UROtsa cells after long-term (8 weeks) exposure to CAA, BAA, and IAA. **A)** Long-term (8 weeks) exposed cells morphology 24, 48 and 72 h after seeding. **B-C)** UROtsa doubling time values (mean values ± SEM).

Figure 5. Anchorage-independent growth capacity of UROtsa cells after 8 weeks of HAAs long-term exposure. **A)** Mean number of colonies per well. **B)** Mean colony size per treatment.

Figure 6. Promotion of HeLa anchorage-independent cell growth by the conditioned media of UROtsa cells after 8 weeks of HAAs long-term exposure. **A)** Mean number of HeLa colonies per plate. **B)** HeLa mean colony diameter.

Figure 7. Cell viability curves of long-term exposed (8 weeks) cells after 24 hr of exposure to increasing concentrations of $KBrO_3$.

Figure 8. Genotoxic **(A)** and oxidative DNA damage **(B)** in long-term exposed UROtsa cells after 30 min of exposure to 2.5 mM $KBrO_3$. Data represented as mean ± SEM. $*P<0.05$; $***P<0.001$.

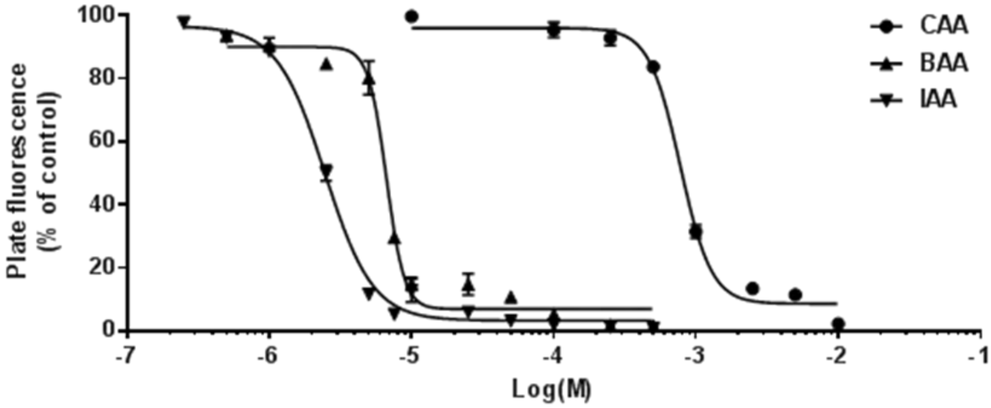


Figure 1

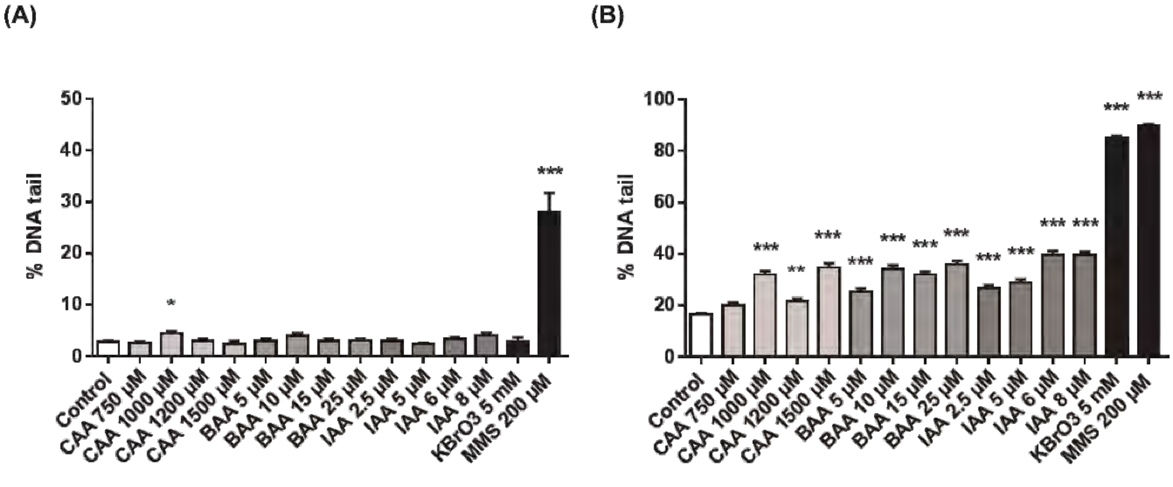


Figure 2

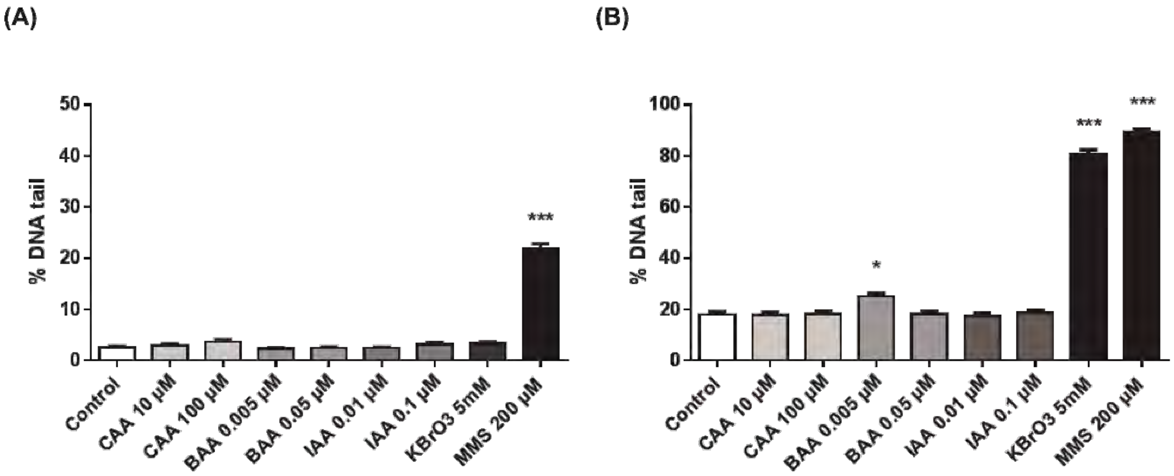
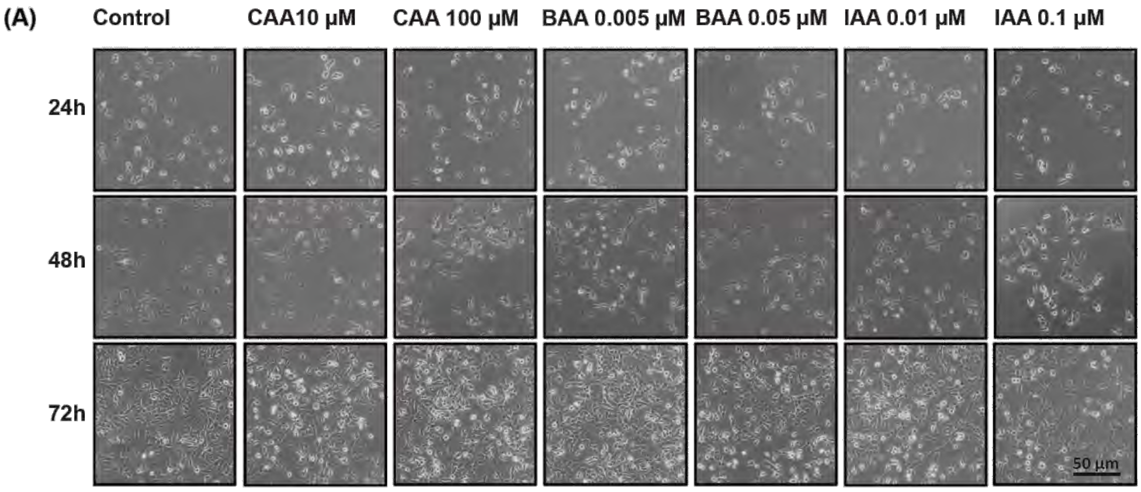
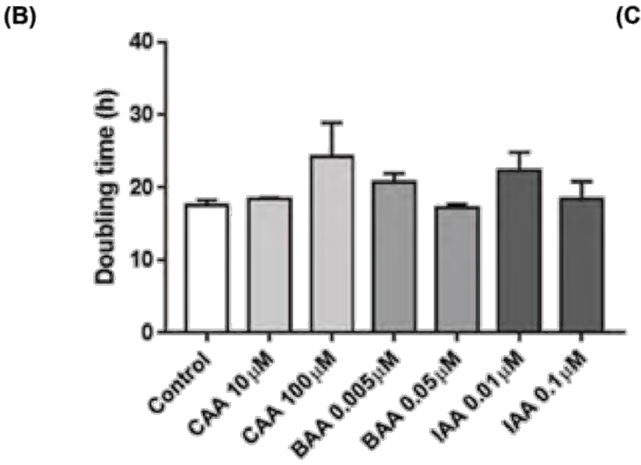


Figure 3


(A)
Control
CAA10 µM
CAA 100 µM
BAA 0.005 µM
BAA 0.05 µM
IAA 0.01 µM
IAA 0.1 µM
24h
48h
72h
50 µm



(C)

	Doubling Time ± SEM
Control	17.54 ± 0.71
CAA 10 µM	18.44 ± 0.14
CAA 100 µM	24.27 ± 2.14
BAA 0.005 µM	20.67 ± 1.25
BAA 0.05 µM	17.26 ± 0.40
IAA 0.01 µM	22.27 ± 2.56
IAA 0.1 µM	18.43 ± 2.40

Figure 4

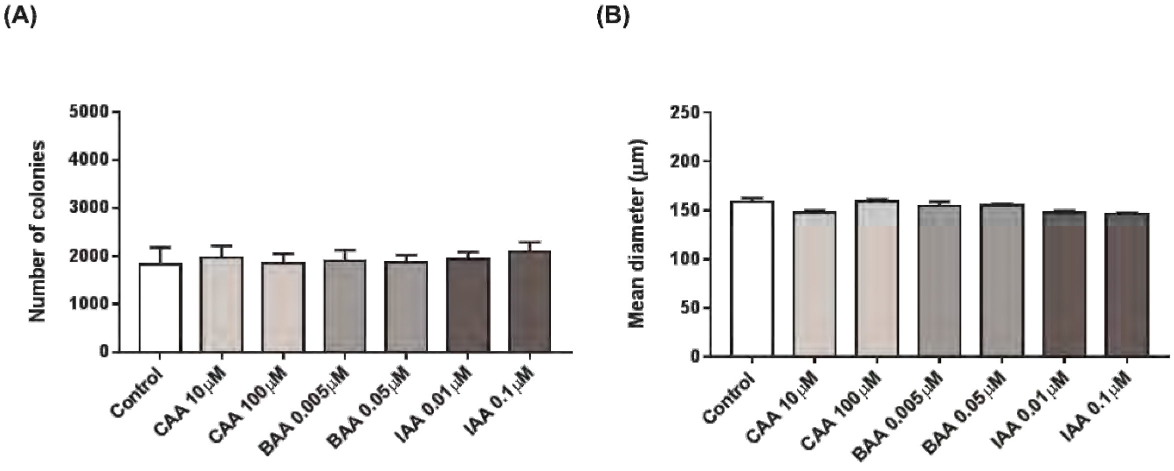


Figure 5

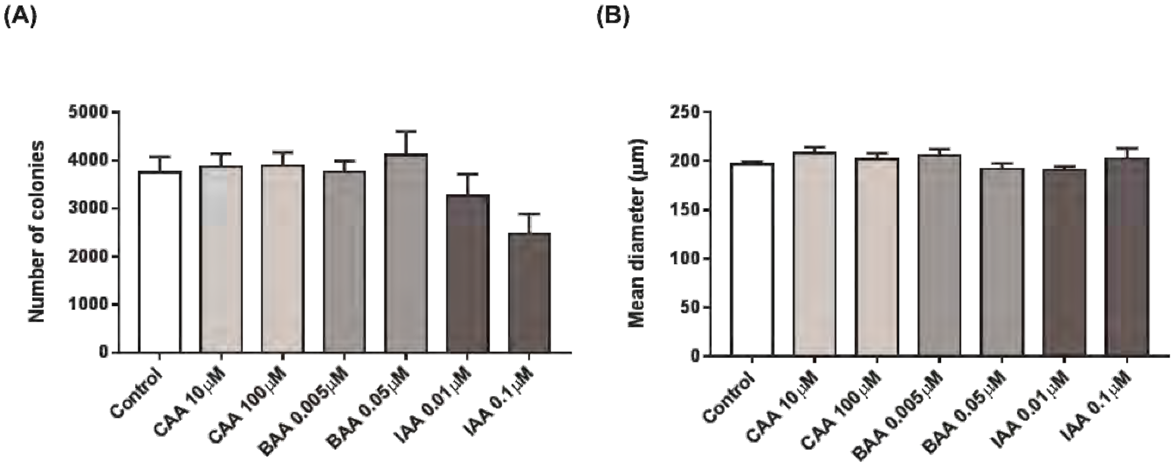


Figure 6

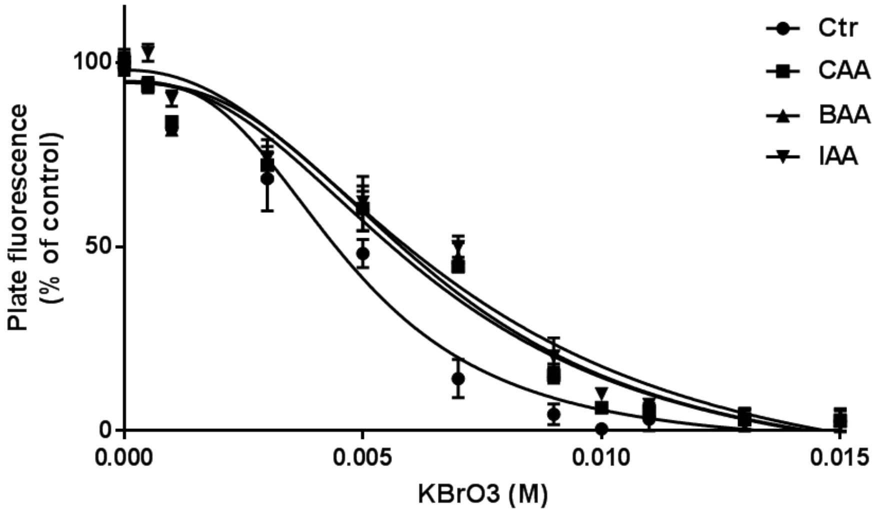


Figure 7

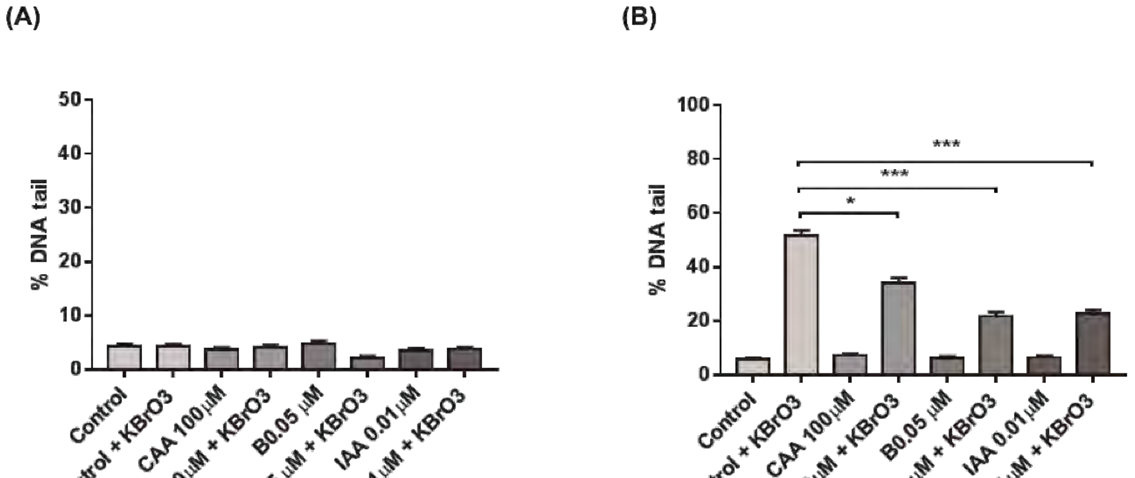


Figure 8