In vitro toxicity studies of plastics used in novel solar water disinfection reactors.

A thesis submitted to Dublin City University in fulfilment of the requirements for the award of the degree of Doctor of Philosophy

by

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- Ozores, P. Disinfection and Enhancement Parameters Task 4.5 Toxicological Evaluation. Project progress presentation at the Annual Congress Assembly of the *Waterspoutt* project held in Mekelle, Ethiopia May 2019.
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- Ozores, P. Screening of estrogenic substances leached into water. Poster presented at the 10th annual research day organized by the Biological Research Society of DCU, Ireland January 2018.

List of abbreviations

ABS: Acrylonitrile-Butadiene-Styrene

AET: Analytical Evaluation Threshold

ANOVA: Analysis of variance

AOP: Advanced Oxidative Processes

AP: Acid Phosphatase assay

BBP: Benzyl butyl phthalate

BHA: Butyl hydroxyanisole

BHT: Butylated hydroxytoluene

BPA: Bisphenol A

CALUX: Chemical Activated Luciferase gene eXpression bioassay

CHO: Chinese Hamster ovarian cells

CIEMAT: Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (translated as: Energy, Environmental and Technology Research Centre)

CSFBS: Charcoal stripped Foetal Bovine Serum

DBP: Dibutyl phthalate

DCU: Dublin City University

DEHA: Diethylhexyl adipate

DEHP: Diethylhexyl phthalate

DMEM: Dulbeco's Modified Eagle Medium

DMSO: Dimethyl sulphoxide

EAWAG: Department of Water and Sanitation in Developing Countries at the Swiss Federal Institute of Aquatic Science and Technology

E2: 17β estradiol

EA: Estrogenic activity

EDC: Endocrine disrupting compounds

EFSA: European Food Safety Authority

EPA: Environmental Protection Agency

EEQ: Estradiol Equivalent

ERs: Estrogen receptors

EtOH: Ethanol

FBS: Foetal Bovine Serum

FCM: Food Contact Material

FDA: Food and Drug Administration

GC-MS: Gas Chromatography and Mass Spectrophotometry

HDPE: high-density polyethylene

HLB: Hydrophilic-Lipophilic-Balanced

HPLC: High Performance Liquid Chromatography

HRE: Hormone Response Elements

ICI: fulvestrant

IMS: Industrial Methylated Spirits

IR: Infrared Radiation

LDPE: Low-Density Polyethylene

LLDPE: Linear Low-Density Polyethylene

MCF-7: Michigan Cancer Foundation- 7 cell line

NIAS: Non-Intentionally Added Substances

NP: 4- nonylphenol

OML: Overall Migration Limit

OP: 4-tert-octylphenol

PBS: Phosphate Buffer Saline

PC: Polycarbonates

PE: Proliferative effect

PE: Polyethylene

PEEK: Polyetheretherketone

PET: Polyethylene terephthalate

pNPP: para-Nitrophenylphosphate

PMMA: Polymethyl methacrylate

PP: Polypropylene

PPS: Polyphenylene sulphide

PS: Polystyrene

PTFE: Polytetrafluoroethylene

PVC: Polyvinyl chloride

ROS: Reactive oxygen Species

RPE: Relative Proliferative Effect

SODIS: Solar Disinfection

SML: Specific Migration Limit

SPA: Plataforma Solar de Almería (translated as Solar Platform of Almeria)

SPE: Solid Phase Extraction

US EPA: United States Environmental Protection Agency

UV: Ultraviolet

Waterspoutt: **Water** - **S**ustainable **P**oint-**O**f-**U**se **T**reatment **T**echnologies

WHO: World Health Organization

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Abstract

In vitro toxicity studies of plastics used in novel solar water disinfection reactors.

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Solar disinfection (SODIS) is a cost-effective point-of-use method for disinfecting water usually in a 2 L PET plastic bottle. To increase the volume of water disinfected, the project Waterspoutt (Water - Sustainable Point-Of-Use Treatment Technologies) developed three novel reactors. Polymethyl methacrylate (PMMA) tubular solar reactors capable of delivering >20 L of water, transparent 25 L polyethylene terephthalate (PET) jerrycans and polypropylene (PP) 20 L buckets. In vitro assays were used to investigate leaching of toxic substances from the plastic reactors. The E-screen assay was used to test for estrogenicity using MCF-BUS cells at a cell density of 4000 cells/well and an incubation period of 6 days at 37 °C. Cell proliferation was determined using the Hoechst assay. The Ames test using the Ames II kit by Xenometrix AG was used to test for mutagenicity. The novel reactors were filled with water and exposed to sunshine in Africa and Spain. The water was extracted using solid phase extraction with glass oasis cartridges and tested for leachates. Samples of raw and aged plastics used in the reactors were tested for extractables. No mutagenicity was detected in any sample. Samples of extractables showed the presence of estrogenicity when extracted at 37 °C or above for extended incubation time periods. No estrogenicity was found in the SODIS disinfected water produced by the PMMA reactors or the PP buckets when exposed to sunlight for up to 9 months. However, while water disinfected using the PET reactors showed no estrogenicity after 3- and 6-months exposure to the sun, estrogenicity was detected following 9-months exposure to sunlight. Laboratory-based toxicity studies on the advanced oxidative processes photo-Fenton and persulphate activation using PET and polycarbonate showed no mutagenic activity, however estrogenicity was detected. All levels of estrogenicity detected in this study were within the acceptable daily intake for 17 β-estradiol of up to 50 ng/kg bw/day.

Chapter I

1. Introduction

1.1. Global drinking water supply

Access to safe drinking water is essential to health, a basic human right and a component of effective policy for health protection (WHO, 2017). In recent times, the COVID-19 pandemic has demonstrated the critical importance of sanitation, hygiene and the need for adequate access to clean water for preventing and containing diseases. The Sustainable Development Goals (SDGs) were launched at the United Nations Conference on Sustainable Development in Rio de Janeiro in 2012. The SDGs replaced the Millennium Development Goals (MDGs), which started a global effort in 2000 to tackle the indignity of poverty. Sustainable Development Goal 6 (SDG 6) is one of 17 Sustainable Development Goals established by the United Nations General assembly in 2015. It calls for access to clean water and sanitation for all people.

Sustainable Development Goal 6 calls for safe drinking-water along the entire water service delivery chain and represents a higher level of ambition than the previous Millennium Development Goal target related to drinking-water. SDG 6 focuses on the type of infrastructure available and emphasizes the quality of the service that is delivered, including safety of drinking water. This necessitates ensuring that water safety risks are minimized from catchment to consumer, including in households where unsafe collection, storage and handling can result in contamination (WHO, 2019).

To ensure the supply of safe drinking water, WHO has published guidelines to inform water and health regulators, policymakers and their advisors, to assist in the development of national policies and regulations. Now in its fourth edition (WHO, 2017), the guidelines deal with all aspects of drinking water provision. The interrelationships among the individual chapters of the Guidelines are illustrated in Fig.1.1 and demonstrate the scope and detail of the work. However, despite considerable progress, billions of people still lack access to safe water. According to the World Health Organization (WHO), 2.2 billion people worldwide still lacked access to safely managed drinking water services in 2017 including:

- 1.4 billion people with *basic* services, meaning an improved water source located within a round trip of 30 minutes
- 206 million people with *limited* services, or an improved water source requiring more than 30 minutes to collect water

- 435 million people taking water from unprotected wells and springs
- 144 million people collecting untreated surface water from lakes, ponds, rivers and streams.

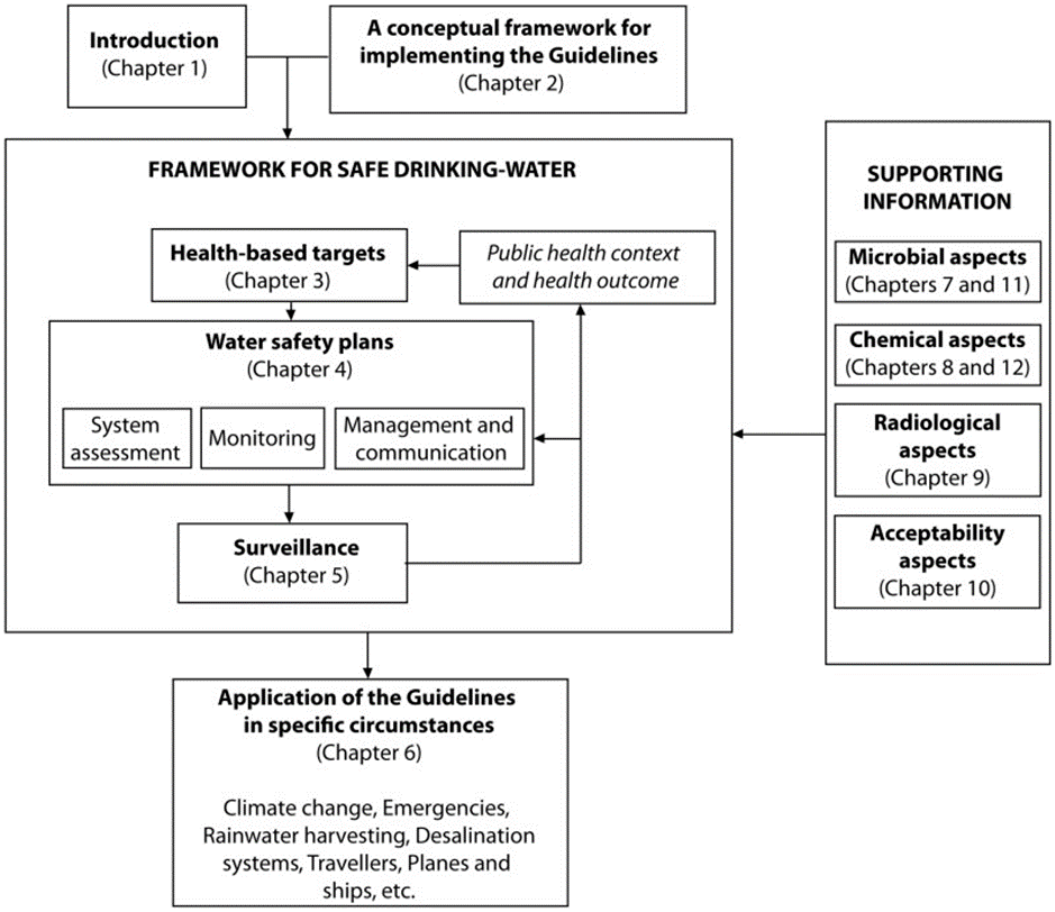


Figure 1.1 Inter-relationships among the individual chapters of the guidelines for drinking water quality in ensuring drinking-water safety *(WHO, 2017).*

In terms of water safety, microbial hazards continue to be the primary concern in both developing and developed countries. Where access to safely managed drinking water services is unavailable, a variety of household water treatment (HWT) technologies are available to ensure a safe drinking water supply. If used correctly, these HWT technologies disinfect the water leading to the killing or removal of all pathogens or disease-causing organisms. Disinfection can be achieved using heat, chemicals, filtration or irradiation.

In 2014, the WHO established the International Scheme to Evaluate Household Water Treatment Technologies. The Scheme aims to consistently and independently evaluate the performance of

household water treatment (HWT) technologies against WHO performance recommendations. The results of the Scheme evaluation are intended to guide HWT product selection by Member States and procuring UN agencies. In this regard, the Scheme fills an important global and national need for independent health-based evaluation of HWT, especially considering the large number of product manufacturers and product claims, and the limited capacity of low-income countries to conduct testing to verify these claims. They recommended the classifying of HWT technologies into three levels of performance: *** (3-star); ** (2-star) and * (1-star), based on their ability to remove pathogens from drinking-water as described in Table 1.1

Table 1.1 WHO performance criteria for HWT technologies (WHO, 2019)

Performance classification	**Bacteria** (log_{10} reduction required)	**Viruses** (log_{10} reduction required)	**Protozoa** (log_{10} reduction required)	**Interpretation** (with correct and persistent use)
★ ★ ★	≥ 4	≥ 5	≥ 4	Comprehensive protection
★ ★	≥ 2	≥ 3	≥ 2	
★	Meets at least 2-start (★ ★) criteria for two classes of pathogens			Targeted protection
-	Fails to meet WHO performance criteria			Little to no protection

A range of products were submitted to WHO for testing as part of the WHO International Scheme to evaluate HWT. The products were evaluated in Rounds I and II of the Scheme and met one of the three WHO-recommended performance levels: 3-star (***); 2-star (**); or 1-star (*) (Table 1.2). Treatment technologies in the areas of filtration, chemical treatment and irradiation are represented. Methods of irradiation include UV disinfection and solar disinfection (SODIS).

Table 1.2 Products submitted to WHO for testing as part of the WHO International Scheme to Evaluate HWT.

The products were evaluated in Rounds I and II of the Scheme and met one of the three WHO-recommended performance levels: 3-star (***); 2-star (**); or 1-star (*).

Treatment technology	Product[a]	Manufacturer	Evaluation Round	Performance classification
Membrane filtration	LifeStraw Family 1.0	LifeStraw (part of the Vestergaard Group)	I	**Comprehensive protection** ★★★
	LifeStraw Family 2.0		I	**Comprehensive protection** ★★
	LifeStraw Community		I	**Comprehensive protection** ★★★
	Uzima Filter UZ-1	Uzima Water Filters	II	**Targeted protection** (bacteria and protozoa only) ★
Ceramic filtration	Nazava Water Filters	PT Holland for Water / Nazava	II	**Targeted protection** (bacteria and protozoa only) ★
	SPOUTS Water Purifaaya Filter	SPOUTS of Water Ltd	II	**Targeted protection** (bacteria and protozoa only) ★
	Tulip Table Top Water Filter	Basic Water Needs B.V.	II	**Targeted protection** (bacteria and protozoa only) ★
Flocculation–biofiltration	BlueQ™ Two-Stage	Amway Corporation	II	**Targeted protection** (bacteria and protozoa only) ★
Flocculation–disinfection	AquaSure Tab10	AquaSure	II	**Comprehensive protection** ★★
	P&G™ Purifier of Water	The Procter & Gamble Company	I	**Comprehensive protection** ★★
Flocculation–disinfection–filtration	DayOne Waterbag™	DayOne Response, Inc.	II	**Comprehensive protection** ★★

Treatment technology	Product[a]	Manufacturer	Evaluation Round	Performance classification
UV disinfection	Mesita Azul	Fundación Cántaro Azul	II	**Targeted protection** (bacteria and protozoa only) ★
	Water Elephant	Years of Water	II	**Targeted protection** (bacteria and protozoa only) ★
	Waterlogic	Qingdao Waterlogic Manufacturing Company	I	**Comprehensive protection** ★★
Solar disinfection	AquaPak	Solar Solutions	II	**Comprehensive protection** ★★★
	JAMEBI Solar Water Pasteurizer	Relevant Projects Ltd	II	**Comprehensive protection** ★★
	SolarBag®	Puralytics	II	**Comprehensive protection** ★★★
	WADI	Helioz GmbH	I	**Targeted protection** (bacteria and protozoa; some protection against viruses) ★
Chemical disinfection	Aquatabs®	Medentech Ltd	I	**Targeted protection** (bacteria and viruses only) ★
	Aquatabs Flo		II	**Targeted protection** (bacteria and viruses only) ★
	Oasis Water Purification Tablets	Hydrachem Ltd	II	**Targeted protection** (bacteria and viruses only) ★
	WATA-Standard™	Antenna Technologies	II	**Targeted protection** (bacteria and viruses only) ★

1.2. Ensuring safe drinking water

One of the best-known household water treatment methods is to boil water. However, exposure to smoke from fires can increase the risk of respiratory disease and the cost of fuel or wood for boiling water could significantly drain the household budget, making it unaffordable for some communities and families (Clasen et al., 2008). The addition of chemicals such as chlorine to water is widely practiced and affordable; however, it can result in an unpleasant taste to the water. Filtration results in the removal of microorganisms from the water but its success depends on the level of sophistication of the filter used. Irradiation can lead to the killing of microbes in-situ depending on the source of the irradiation, however, this treatment may be too costly or too difficult to apply in developing countries or emergency situations (Borde *et al.*, 2016). As a consequence, inexpensive effective methods for purification of water at point-of-use are needed in developing countries. The most readily available source of irradiation especially in many developing countries is sunlight.

1.2.1. SODIS

2000 years ago communities on the Indian sub-continent placed open trays of drinking water outside to be "blessed" by the sun (Baker, 1981); this may be the first documented account of solar disinfection. Nowadays, and in the past 10 years, the technique of water solar disinfection has gained popularity as a household water treatment in rural areas of developing countries, like sub-Saharan Africa, where due to the lack of budget and resources people have no access to other treatment techniques such as boiling, chlorination and filtration (Clasen et al. 2007).

The implementation and application of this technique started with the research of Professor Aftim Acra at the American University of Beirut. With a primary degree in pharmacy, he published the first scientific paper in 1980 reporting the efficacy of sunlight to disinfect oral rehydration solutions. In 1991 this work subsequently motivated a research team of sanitary engineers, photo-chemists, bacteriologists and virologists based at the Department of Water and Sanitation for Developing Countries at the Swiss Federal Institute of Aquatic Science and Technology (EAWAG/ SANDEC) to embark on extensive laboratory and field tests to assess the potential of SODIS (Pesaro 1994).

SODIS is a low-cost, simple, effective and environmentally friendly household water treatment technique that in its simplest form, consists of filling transparent containers with contaminated water and exposing them to sunlight (***Fig 1.2)***. The containers are exposed to sunlight, for at least

5-6 hours to achieve disinfection through the synergic effects of heat and UV radiation (Pesaro 1994; Meierhofer and Wegelin 2002).

The EAWAG project also stated that suitable containers or reactors for solar disinfection were 2 L plastic PET bottles (made of polyethylene-terephthalate), due to their correct transmission of UV light radiation. Plastic containers have emerged as a more convenient option for SODIS than glass containers due to their weight, transportability, durability and safety in relation to the risk that glass containers pose when broken (Meierhofer and Wegelin, 2002).

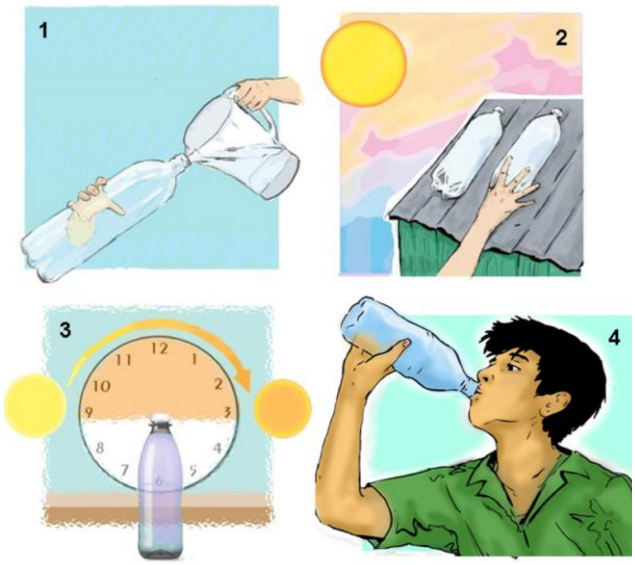


Figure 1.2 Schematic representation of SODIS process*. (McGuigan et al., 2012, p31, Fig 2)*

1.2.2. Technical aspects of SODIS

SODIS uses two components of sunlight, UV radiation and infrared radiation (IR). There are three different wavelength ranges of UV: 400–315 nm for UV-A; 315–280 nm for UV-B; and 280–100 nm for UV-C (***Fig 1.3)***. Most of the radiation of UV-C is absorbed by the ozone (O_3) layer in the atmosphere. It is estimated that due to the UV-blocking effects of plastic reactors less than 1% of the solar energy of UV-B can exert a biological effect during SODIS (Castro-Alférez *et al.*, 2017).

UV-A radiation has a germicidal effect and IR radiation produces heat and raises the temperature of the water. Both forces combine to produce a synergistic effect that enhances the efficiency of the SODIS process. Solar light plays a key role in the germicidal effects and/or inactivation of pathogens during SODIS. Both UV-A and the small portion of UV-B that is transmitted have direct and indirect effects on living cells.

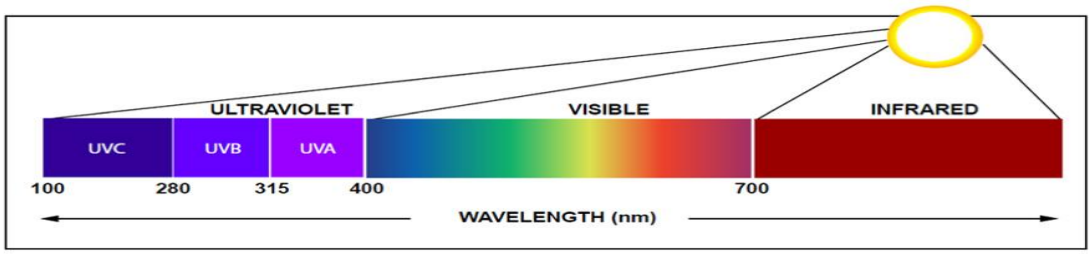


Figure 1.3 Representation of the wavelength spectrum

Cellular DNA across all living systems can absorb some of the energy received from UV radiation. The absorption of UV-B energy radiation by DNA leads to the formation of new bonds between adjacent pyrimidine bases, forming pyrimidine dimers (pairs connected by covalent bonds). The formation of these pyrimidine dimers (Regan *et al*., 1968) is problematic for the survival of cells because they change the structure of the DNA molecule in the area preventing base-pair formation with the complementary purines on the other strand of DNA. All of this poses difficulties for DNA polymerase during the process of DNA replication, yielding malfunction and production of mutations (by deletions of regions of DNA or introducing random bases)(Cundall, 1994). The wavelengths of UV-A that reach the surface of the earth are not energetic enough to modify DNA bases to the same extent as UV-B. The amount of direct DNA damage caused by UV-A is really minor. However, UV-A plays a major essential role in the inactivation of pathogens through indirect damaging pathways (Ravanat *et al*., 2001). UV-A is energetic enough to alter the electron transport chain and to play an important role in the formation of reactive oxygen species (ROS) inside the cell and in the water (***Fig 1.4***). These ROS can cause damage to DNA, which in the end could lead to inactivation of pathogens and/or disinfection of the water (Ubomba-Jaswa *et al.*, 2009; Giannakis *et al.*, 2016; Castro-Alférez, M. I. Polo-López, *et al.*, 2017). The main ROS formed inside the cells/pathogens during solar irradiation are hydrogen peroxide (H_2O_2) and superoxide radicals. Hydrogen peroxide is a common subproduct of the respiratory chain and its presence in the cells is regulated by ROS-scavenging enzymes (Imlay, 2008). The most common ROS-scavenging enzyme is superoxide dismutase (SOD) that detoxifies superoxide radicals; catalase (CAT) and peroxidases, detoxify hydroperoxides including H_2O_2 during the respiration process. However, during solar irradiation, there is an imbalance in the amount of ROS inside the cell which leads to oxidative stress. The reactive oxygen species and the oxidative stress generated by their imbalance have several damaging effects. Oxidative-driven DNA damage leads to the appearance of mutations and problems in replication; oxidative-driven protein damage like protein agglutination and structural modification leads to malfunction of the proteins and cellular metabolic failure; oxidative-driven lipid damage leads to an increase in the permeability of the membrane. Imbalance of ROS, especially H_2O_2, inside the cells/pathogens combined with UV radiation could lead to the release of iron into the cytoplasm from different iron clusters and iron-related enzymes (Imlay, 2003). The iron released into the cytoplasm and/or the iron attached to proteins or DNA that plays a role in their functionality and structure, could interact with H_2O_2 generating hydroxyl radical ($^{\bullet}OH$), in a reaction known as the Fenton reaction. The hydroxyl radical ($^{\bullet}OH$) is a highly oxidative ROS, that increases the oxidative stress damage to the cell. However, the damage that ROS can mediate in cells and/or pathogens is limited to their diffusion and half-life which in most cases of oxidative species is short. Therefore, most of the time they can only cause "local intracellular" damage (Giannakis *et al.*, 2016). On the other hand, depending on the presence and amount of oxygen in

the water, the amount of trace metals in the water such as iron, and the nature and amount of natural organic material (NOM) in the water, ROS could be formed extracellularly in the water matrix during solar irradiation. The oxygen dissolved in the water could absorb the energy of UV radiation and generate a singlet oxygen or superoxide radical. UV radiation of water containing trace levels of iron could lead to the catalysis of the Fenton reaction and sustain hydroxyl radical (˙OH) production in the water matrix. Finally, the absorption of UV radiation by NOM could lead to the generation of ROS and oxidative stress in the water (Giannakis *et al.*, 2016).

It has been suggested that the synergistic effects observed between IR radiation and UV radiation on water disinfection are attributed to an increase in the temperature of the water. This increase of the temperature contributes to the inactivation or denaturation of oxidative stress-scavenging enzymes and inhibition of the natural DNA repair that some pathogens have, ultimately leading to an accumulation of oxidative stress damage and mutations, which finally results in cellular death (Pesaro, 1994; Castro-Alférez, *et al.*, 2017).

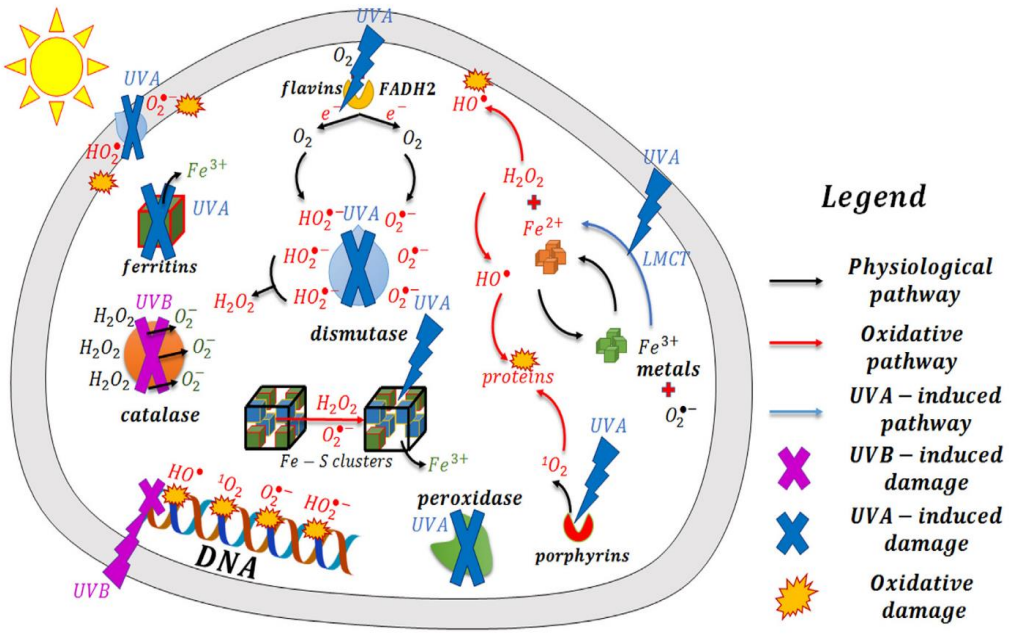


Figure 1.4 Schematic representation of light induced changes in cell homeostasis

UVB induces damage to DNA and CAT function. UVA affects the functions of enzymes and proteins related to ROS balance and production (flavins, FADH2, CAT, SOD, peroxidases, porphyrins) leading to accumulation of ROS, release of iron into the cytoplasm, Ligand-to-metal charge transfer (LMCT)- driven reduction of iron and intracellular Fenton reaction. (Giannakis et al.*, 2016, p 208, Fig 5)*

1.2.3. Efficiency of SODIS in the inactivation of pathogens

Escherichia coli is one of the most frequently used species of bacteria to study and assess the efficiency of disinfectant treatments of water due to its status as a faecal indicator organism (Gilcreas, 1966). Thus, this pathogenic bacterium among other waterborne pathogenic bacteria were the indicators on which early SODIS laboratory studies focused their research. To date, many of the waterborne pathogenic bacteria have been successfully inactivated following 6 hours of solar disinfection in the field (Wegelin *et al.*, 1994; Dejung *et al.*, 2007). However, other faecal coliforms have shown lower rates of inactivation (Sommer *et al.*, 1997; McGuigan *et al.*, 1998; Sinton *et al.*, 2002).

There are only a few studies on the efficacy of SODIS against viruses that cause waterborne diseases. The few studies available on the efficacy of SODIS on inactivating viruses show that these pathogens are the most resistant to solar disinfection. For example, while the inactivation of polio virus was achieved after 6 hours of simulated SODIS, the encephalomyocarditis virus required longer periods of simulated sunlight exposure (12.5 hours) for complete inactivation (Wegelin *et al.*, 1994; Heaselgrave *et al.*, 2006). Only 1 log of reduction of different classes of coliphages was achieved after 8–11 hours of sunlight exposure (Dejung *et al.*, 2007). The efficiency of SODIS in the reduction or inactivation of fungi has also been rarely studied. This lack of research is mainly due to the fact that for healthy adults there are few fungi that can be classified as waterborne human pathogens. However, some strains of *Candida albicans* and *Aspergillus fumigatus* could be considered a health risk for immunocompromised individuals such as those suffering from HIV/AIDS. *C. albicans* has been proven to be readily inactivated within 6 hours of solar exposure (Lonnen *et al.*, 2005; Sichel *et al.*, 2007).

Cryptosporidium parvum is a waterborne protozoan pathogen responsible for causing a debilitating form of diarrhoea that can be acute and fatal in immuno-compromised individuals such as those suffering from HIV/AIDS. *C. parvum* is able to survive in the environment for really long periods of time in an infective stage, known as an oocyst. In the ineffective stage, the pathogen is protected by a thick and robust wall structure, which makes conventional water treatments ineffective (Robertson *et al.*, 1992; Freire-Santos *et al.*, 2000). In 2010 Gómez-Couso *et al.*, (2010) highlighted the importance of heat and thermal stress in the inactivation of *C. parvum* during solar disinfection. In a later study it was shown that the oocyst infective form of *C. parvum* was reduced almost completely after 6 h of SODIS and completely after 12 h of SODIS (Méndez-Hermida *et al.*, 2005, 2007; Gómez-Couso *et al.*, 2012).

1.2.4. Possible enhancements of the SODIS treatment

Several approaches have been used to enhance the SODIS process in order to improve its efficiency, the speed of the process and the convenience to the user by up-scaling the volume of water disinfected.

One of the first approaches taken to enhance the efficiency of SODIS was heat treatment. In order to achieve synergistic effects between UV radiation and thermal stress for inactivation of pathogens a temperature of at least 40 °C is required. This high temperature is not commonly reached during regular sunlight exposure conditions (McGuigan *et al.,* 1998). Three different approaches could be taken to achieve thermal enhancements - painting sections of the bottles in black (Martín-Domínguez *et al.*, 2005), circulating the water in an enclosed black surface (Rijal and Fujioka, 2004) and use of a solar collector (Saitoh and El-Ghetany, 2002). Although painting sections of the bottles in black is one of the most economical approaches and although it is a successful enhancement, it is the least efficient of all of the three, followed closely by circulating the water in an enclosed black surface (Duffie *et al*., 1994). Then, there are highly efficient solar thermal collectors available, however, their cost is prohibitively high for poor communities in developing countries which are the common targets for SODIS system implementation (McGuigan *et al.,* 2012). Solar reflectors and mirrors can also be used for thermal enhancements however efficiency is not at the same level that is achieved with absorptive materials or blackening the reactors. The solar mirrors and reflective surfaces have been mainly used as an attempt to increase the radiation transmittance inside bottles or other forms of reactors such as flow reactors or batch collector reactors (Wegelin *et al*., 2000; Kehoe *et al.,* 2001; Martín-Domínguez *et al.*, 2005).

Different models of SODIS reactors with continuous flow of water and several static batch SODIS reactors have been designed in an effort to up-scale the volume of water disinfected thus increasing convenience for the user. Ubomba-Jaswa *et al.,* (2009) studied the efficiency of large batch reactors versus the efficiency of continuous flow recirculation reactors and observed that increasing the flow rate had negative effects on the inactivation of the bacteria. They concluded that to achieve complete inactivation of bacteria, constant illumination and uninterrupted UV dose is needed rather than repeated exposures to sub-lethal doses of UV radiation (***Fig 1.5***). Later, Polo-López *et al.,* (2011) studied the time required for SODIS treatment for complete inactivation of bacteria and found that the time of inactivation was successfully decreased by using a continuous multitube flow reactor (***Fig 1.6***). This reactor reduced the user-dependency by not only increasing the speed of SODIS treatment but also by increasing the total output of water treated per day.

1.3. Advanced Oxidative Processes

Figure 1.6. SODIS enhanced large batch reactor of 25 L, with a solar mirror. *Picture taken at Plataforma Solar de Almería, Spain (Ubomba-Jaswa* et al.*, 2009)*

Figure 1.5 SODIS enhanced large batch multitube flow-reactor of 100L with add-on CPC mirrors*. Picture taken at Plataforma Solar de Almería, Spain (Polo-López* et al.*, 2011)*

Other approaches taken to improve the efficiency of SODIS treatments includes the implementation of heterogenous photocatalysis by intentionally adding photocatalytic chemicals to the water, which will enhance the production of ROS. Most ROS will act indiscriminately destroying a wide variety of chemical contaminants and also causing lethal damage, especially the hydroxyl radical, in pathogenic microorganisms (Blake *et al.*, 1999; Fujishima *et al.*, 2000). These chemical oxidative reactions that generate ROS initiated by photocatalysis are known as advanced oxidative processes (AOPs). In the 1980s AOPs were initially proposed as a treatment for potable water and later were used as an application to remove organic and inorganic materials from wastewater (Munter, 2001). Initially, AOPs were conceived as oxidation processes to generate hydroxyl radicals ($^{\bullet}OH$), however later the AOP concept was extended to oxidative processes that generate persulfate radicals ($SO_4^{\bullet -}$) (Deng and Zhao, 2015).

1.3.1. Hydroxyl radical based AOPs

The mechanisms for hydroxyl radical production depend on the type of advanced oxidative technique used. The non-selective behaviour and high reactivity of the hydroxyl radical makes it one of the most used oxidizing agents in water treatment. Hydroxyl radicals have a very short lifetime, therefore their generation during the AOPs is achieved through different methods including a combination of oxidizing agents (such as H_2O_2 and O_3), irradiation (such as ultraviolet light or ultrasound), and catalysts (such as Fe^{2+}) (Huang *et al.*, 1993). Ozone (O_3) could be used as an oxidation catalyst and is a powerful oxidant by itself. However, oxidation through O_3 is a specific process that reacts primarily with the ionized and dissociated form of organic compounds, rather than the neutral form. Besides the generation of hydroxyl radicals could only be achieved under specific conditions, however the addition of other oxidants, such as H_2O_2 and/or irradiation significantly improves the amount of hydroxyl radicals generated (Gottschalk *et al.*, 2008).

UV and Fenton AOPs

Hydroxyl radicals can be generated by the excitation of a semiconductor catalyst through energetic photons during UV irradiation. A semiconductor metal frequently used in AOPs is iron. Fe^{2+} is able to activate H_2O_2 and generate reactive species such as hydroxyl radicals plus some Fe^{3+} ions in water in the so-called Fenton process (Ameta *et al.*, 2018). Of all the photocatalytic oxidative processes, the Fenton reaction is one of the best known. As previously described the generation of hydroxyl radicals through Fenton reaction is something that could happen inside the cells/pathogens during solar irradiation. However, by intentionally adding H_2O_2 and Fe^{2+} in small quantities to the water matrix during the solar irradiation process, the kinetics of the Fenton reactions and the amounts of hydroxyl radicals generated can be enhanced both outside and inside the cells. This combination of the Fenton reaction and UV irradiation is an AOP known as photo-

Fenton reaction. Photo-Fenton uses the increased oxidative capacities of iron ions combined with oxidizing agents such as H_2O_2 and the energy absorbed from UV radiation to generate hydroxyl radicals (˙OH) which lead to the inactivation of microorganisms (Cho et al., 2004; Rincón and Pulgarin, 2006) and the degradation through oxidation of organic and inorganic compounds in the water matrix (Neamţu and Frimmel, 2006; Klamerth *et al.*, 2012; Rocha *et al.*, 2013; Zhang and Li, 2014). However, the generation of hydroxyl radicals can be scavenged by new reactions catalysed by either of the Fenton reagents. In other words, the presence of the Fenton reagents in the water could promote the generation of catalytic reaction that eliminates the hydroxyl radicals from the water. Therefore, the proportion and volumes at which these reagents are added must be carefully controlled.

Hydrogen peroxide (H_2O_2) intentionally added to water can trespass the cells outer membrane and prompt ROS imbalance inside the cell. The generation of hydroxy radicals extracellularly damages cell walls and membranes increasing their permeability. Therefore the combination of solar irradiation and generation of the Fenton reaction intra- and extracellularly makes the photo-Fenton reaction an enhanced SODIS method for pathogen inactivation (Bauer, 1990; Kiwi *et al.*, 1993; Ruppert *et al.*, 1994). Direct and indirect actions of light, the significance of the photo-Fenton process on bacterial targets and approaches for enhancing photo-inactivation of bacteria by simple additions of H_2O_2, and iron (Fe^{2+}, Fe^{3+}) have been comprehensively described by Giannakis *et al.*, (2016).

Moncayo-Lasso et al. (2009) were one of the first to test the suitability of using the photo-Fenton reaction to treat water for drinking purposes. They concluded that photo-Fenton is a potential application for the inactivation of bacterial contaminants and the removal of natural organic matter (NOM) from superficial drinking water sources. Ndounla et al. (2013, 2014a 2014b, 2015) also investigated the application of photo-Fenton as a treatment for drinking water. Ndounla et al. (2013) demonstrated the efficacy of the photo-Fenton process to impair the re-growth of enteric bacteria in PET bottled water from a natural source in Burkina Faso. Subsequently, Ndounla et al. (2014a, 2014b) assessed the efficiency of the photo Fenton process, carried out in a compound parabolic collector (CPC) solar reactor, to disinfect a natural drinking water source from Burkina Faso. They concluded that although the weather conditions and sun exposure were not sufficient to ensure proper solar disinfection and ensure the impairment of reactivation of pathogens, the ongoing Fenton reaction during dark storage of the samples gave the opportunity to achieve an efficient disinfection of drinking water. Therefore photo-disinfection of natural well water was successfully carried out at real scale in a solar CPC reactor for multiple time intervals of 6 hours. However, in a later publication, Ndounla et al. (2015) concluded that more research was needed to improve photo-Fenton-disinfection of natural well water in a solar CPC reactor before its implementation at point-of-use level.

1.3.2. Sulphate radical based AOPs

Although the original AOPs involved the generation of hydroxyl radicals, other substances could be used to prompt a photocatalytic oxidative reaction. Research has shown that sulphate radicals generated by alternative oxidants such as peroxydisulfate can be used too as an AOP treatment. $S_2O_8^{2-}$ itself is a strong oxidant, that once activated either by heat, transition metals or UV radiation (***Eq 1.1)*** can generate more reactive sulphate radicals ($SO_4^{\bullet -}$). Sulphate radicals can also generate hydroxyl radicals but primarily tend to remove electrons from organic molecules that are subsequently transformed into organic radical cations (***Eq 1.2)***. The most commonly used transition metals in this sulphate radical based AOP are ferrous and ferric ions, however the activation by transition metals is not theoretically efficient (Anipsitakis and Dionysiou, 2004). The temperatures required for the generation of sulphate radicals through thermal persulfate activation broadly ranges within 35 and 130 °C (Tsitonaki *et al.*, 2010). The generation of hydroxyl and sulphate radicals through UV activation can be used to enhance SODIS and its

$$S_2O_8^{2-} \xrightarrow{\Delta \,/\, UV} 2SO_4^{\bullet -}$$

Equation 1.1 Sulphate radical base AOP achieved through UV persulfate activation

$$SO_4^{\bullet -} + H_2O \rightarrow OH\cdot + SO_4^{2-} + H^+$$

$$SO_4^{\bullet -} + OH^- \rightarrow OH\cdot + SO_4^{2-}$$

Equation 1.2 Generation of hydroxyl radicals from sulphates radicals

disinfection efficiency and be an economic effective replacement for H_2O_2 (Rodríguez-Chueca et al 2019).

However, concerns have been raised about the use of photocatalytic oxidative reactions in combination with common plastic SODIS reactors since hydroxyl radicals and other oxidative species generated during the reactions may be able to release plastic additives into the water.

1.4. Plastics

Plastics are primarily polymers made of carbon. They have been used by man for centuries. Medieval craftsmen used naturally-derived plastics such as keratin from animal horn or cellulose to make lanterns, windows and paper, respectively; plastics were used in their natural forms like chewing gum or shellac. Since the industrial revolution in the 1800s, advances in chemistry have led to natural plastics being either chemically modified or substituted by synthetic molecules yielding an array of now-familiar synthetic polymers such as polystyrene, polyester, polyvinylchloride, polythene and nylon. The foundation work on the polymer structures of plastics by Hermann Staudinger in 1922 marked the birth of a scientific and technological era where plastics became hugely transformative (Andrady, 2015). Production of plastic polymers has flourished over the past 80 years. Nowadays, plastics are everywhere and have yielded a diverse range of products of benefit for human health and the environment. Global plastics production reached 359 million tonnes in 2019 with around 40 % used for food packaging (*Plastics-the Facts 2019 An analysis of European plastics production, demand and waste data,* 2019).

1.4.1. Plastic production and manufacture

Plastics are resins made of polymers mixed with additives. These polymers are long chains of repeated structural segments called monomers which are covalently bonded together. The long chain-like structure of the polymers is responsible for the desirable properties of plastics (Andrady, 2015).

The production of plastic starts with the binding of monomers to form a polymer; if the same structural unit is repeated along the molecular chain it is considered a homopolymer, while if there are two or more structural units repeated then it is considered a heteropolymer (Andrady, 2015).

Plastic polymers are named according to the main monomer they are made of (***Table 1.3***). Polyethylene terephthalate (PET), polypropylene (PP), polymethylnmethacrylate (PMMA) and polycarbonates (PC) consist of polymerized units of the monomers ethylene terephthalate, propylene, methylmethylacrylare and carbonate respectively (Bhunia *et al.,* 2013). Thermoplastics and thermosets are the two main category types of plastics. Thermoplastic polymers (PET, PP, PMMA, PC) can be melted and solidified many times while thermoset polymers like some polyester appliances can only be melted and solidified once, making their final molten form irreversible (Yang et al. 2011).

Table 1.3 Summary and classification of different polymers (*table modified from Murphy, 2001)*

Polymer family and type	Abbreviation	Typical products and applications
Thermoplastics		
Homopolymers		
Acrylonitrile-butadiene-styrene	ABS	appliance housing, helmets, pipe fittings
High-density polyethylene	HDPE	milk bottles, wire and cable insulation, toys
Low-density polyethylene	LDPE	packing film, grocery bags
Polymethyl methacrylate	PMMA	impact resistant windows
Polypropylene	PP	bottles, food containers, toys, caps and biopharmaceutical devices
Polytetrafluoroethylene	PTFE	self-lubricated bearings
Polystyrene	PS	eating utensils, foamed food containers
Polyvinyl chloride	PVC	pipe, window frames
Heteropolymers		
Polyacetal		bearings, zippers, showerheads
Polycaprolactam	nylon 6	bearings, gears, pulleys
Polycarbonate	PC	compact discs, safety glasses, sporting goods, laboratory flask for cell culture
Polyetheretherketone	PEEK	machine, automotive and aerospace parts
Polyethylene terephthalate	PET	transparent bottles, recording tape, SUS (single use system)
polyphenylene sulphide	PPS	machine parts, electrical appliances
Thermoset		
Heterochain		
Epoxies		laminated circuits boards, aircraft parts, flooring
Phenol formaldehyde		electrical connectors
Polyester		boat hulls, automobile panels
Polyurethane		flexible and rigid foams for insulation
Urea and melamine formaldehyde		countertops, dinnerware

1.4.2. Additives commonly used in plastic production and manufacture

Before the shaping, moulding and finishing of a plastic product takes place, plastic resins are usually mixed with additives which are chemical compounds to improve performance, functionality and ageing properties of the polymer. Functional additives include plasticizers, antioxidants, light stabilizers, thermal stabilizers, flame retardants, acid scavengers, lubricants, pigments, antistatic agents, curing agents, blowing agents, biocides, slip agents and colorants including soluble azocolourants, organic pigments and inorganic pigments (Hahladakis et al 2018). All are added in specific amounts to alter properties like rigidness, colour, durability, thermal resistance or transparency in a process known as formulation and compounding. Almost all are not chemically bound to the plastic polymer and may migrate easily. Plasticizers, antioxidants, light stabilizers and thermal stabilizers are the most commonly used additives (Bhunia *et al.*, 2013; Andrady, 2015). Plastic packaging for food and beverages contains residues from substances used during manufacturing, such as solvents and unreacted monomers along with non-intentionally added substances (NIAS), such as impurities, oligomers, or degradation products. A recent publication by Groh *et al.*, (2019) features a database that listed over 12000 substances that are associated with plastic packaging.

Plasticizers

Plasticizers are a group of functional additives used to increase the flexibility, durability and pliability of the polymer. These additives could constitute between a 10- 70% of weight/weight ratio of the finalised polymer. Phthalic esters, such as bis (2-ethylhexyl) phthalate (DEHP) can constitute about 80% of the plasticizer volume for PVC production. Adipate (DEHA), di-octyladipate (DOA), diethyl phthalates (DEP) and dibutyl phthalate (DBP) are plasticizers that may be used in the polymerization of PET. Other plasticizers commonly added to other polymers are DBP, DEHP, diheptyl adipate (DHA), and butyl benzyl phthalate (BBP) (Hahladakis *et al.*, 2018).

Antioxidants

Antioxidants are a group of functional additives added to the polymer to slow oxidative degradation when exposed to ultraviolet light (Bhunia *et al.*, 2013). In particular, arylamines, phenolic and phosphite antioxidants scavenge highly reactive free radicals generated by heat, UV radiation and mechanical shear, a process that is often exacerbated by the presence of metallic impurities. Phenolic chemicals include compounds such as BHT, BHA, Irganox 1010 and BPA, and organophosphites include compounds such as TNPP and Irgafos 168. Phenolic antioxidants are normally used in low amounts while organophosphites are used in higher amounts however,

the amount of antioxidants used during the polymerization will depend on the chemical structure of the additive and of the plastic polymer (Hahladakis *et al.*, 2018).

Heat stabilisers

These additives prevent thermal degradation when the polymers are exposed to elevated temperatures, therefore they are mainly used in polymers that are going to be exposed to high temperatures such as PVC. There are three types of primary heat stabilisers based on the use of metals salts or metal compounds such as Pb, Sn, Ba, Cd and Zn (Hahladakis *et al.*, 2018).

Colorants

Colorants are not considered functional additives but rather aesthetic additives used to change the colour of the plastic polymer or to make it transparent. There are soluble colorants such as azocolorants and are mainly used to achieve a bright transparent colour in PS and PMMA polymers. These soluble colorants have a low resistance to light and heat. These conditions can easily provoke the release of the colorant from the plastic polymer. Other colorants, such as Cobalt (II) diacetate are organic colorants with low migration due to their insoluble nature.

Residual monomers and oligomers

Plastic monomers and oligomers per se do not pose significant toxicity to humans because the long-chain molecules that compose plastic polymers are too large in size to be absorbed via epithelial cells of the gastrointestinal tract. Furthermore, no human enzyme can hydrolyse these polymers into smaller molecules. So, although both monomers and oligomers can migrate from packaging materials into food and beverage, these substances do not pose a real risk to human health. However, most plastics contain numerous impurities, NIAS and additives that may be toxic (Groh *et al*., 2017).

Evidence that food contact chemicals can be taken up by gut epithelial cells and gut microflora has implications for gut health, particularly in subpopulations with increased intestinal permeability (Groh *et al*., 2017) Therefore food contact plastics are a relevant exposure pathway in humans for chemicals that are both intentionally and non-intentionally added to plastics (NIAS) (Andrady 2015; Zweifel et al. 2009).

1.5. Migrating substances from plastics

Regulations require safety assessments for all substances migrating from food contact plastics. Chemicals present in food contact materials (FCM) made of plastics can potentially migrate from the product into the medium in contact with the product. The process entails diffusion of chemical compounds through the polymers, desorption of the molecules from the polymer surface, sorption of the compounds at the plastic –food surface before absorption into the food (Hahladakis *et al.*, 2018). The migration rate is size-dependent. Small molecules such as monomers, residual solvents and the lower molecular weight additives migrate faster than higher molecular weight additives. The concentration of the additives presents in the plastic, the thickness and surface structure of the plastic are factors that influence the migration rate.

1.5.1. Regulations

In Europe, general legislation sets out the principles of safety and inertness for all plastic materials that come into contact with food and beverages. European Regulation No.1935/2004 (European Commission, 2004) states that materials that come in contact with food or drinking liquids, known as food contact materials, should not transfer any of their constituents in quantities large enough to endanger human health, bring an intolerable change in the composition of food/drinking liquid or deteriorate its organoleptic properties. Based on that regulation European Regulation No.10/2011 (European Commission, 2011) was created, which established a list of compounds authorized for the fabrication of plastic devices in contact with food/drinking liquids. This regulation states that the overall migration limit (OML) of a compound should not exceed 10 mg of the total constituents released per dm^2 of the plastic device surface. Some substances have specific migration limits (SML) established by their special toxicological data (European Commission, 2004, 2011). The regulation sets out detailed migration testing rules using 'simulants' appropriate for food types. Extractables from plastics are defined as substances that are forcefully released from the plastic materials with the use of solvents and exaggerated conditions of time and temperature, while leachates represent a subset of extractables released from the plastic-contact material under operating conditions (Dorival-García *et al.*, 2018). Therefore, a simulated-use extraction is an extraction process conducted using a method that mimics the expected operational conditions of the analysed device. An exaggerated extraction uses harsher extraction conditions to release a greater amount of substances. The most common practice is to perform both in order to reveal information of the released substances that can be practically expected and to estimate the highest amount of extractables that can be extracted under the worst-case conditions. ('Extractables and leachables: Regulatory requirements for vaccine and biologic products', no date).

Many studies have been performed to investigate if migrating substances found in food and water surpass the OML or the SML stipulated values, or if these migrating substances have biological effects. A few studies have performed toxicological analysis of different kinds of plastics used in the fabrication of food/water containers and biopharmaceutical products. Some studies have focused on how environmental factors such as light, temperature, radiation, time of storage, aging and the reuse of the container affect the rate of migration of substances from plastics. Migration of chemical substances is highly dependent upon processing and storage conditions, the nature of the packaging material and the compounds it may contain, as well as the chemical properties of the water. Examples of chemicals leaching from bottled water are: antimony (Westerhoff *et al.*, 2008; Keresztes *et al.*, 2009), bisphenol A (Casajuana and Lacorte, 2003; Amiridou and Voutsa, 2011; Guart *et al.*, 2011), phthalates (Biscardi et al. 2003; Casajuana and Lacorte 2003; Leivadara et al. 2008; Monarca et al. 1994; Schmid et al. 2008), adipates (Schmid *et al.*, 2008) and 4-nonylphenol (Amiridou and Voutsa, 2011).

1.5.2. Phthalates

Despite strict regulation phthalates are amongst the most heavily used plasticizers used in the industry; they are mostly but not exclusively used in manufacture of polyvinyl chloride (PVC) and other plastic polymers like PET, used in containers and packaging to improve their plasticity and flexibility (Cao, 2010). Dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), diethylhexyl phthalate (DEHP) and bis-ethylhexyl adipate (DEHA) are the most toxicologically evaluated phthalates and have been reported to contaminate water when packaged, stored and transported in plastic containers, but have never been reported to exceed their SML values (0.3 mg/kg, 30 mg/kg, 1.5 mg/kg and 18 mg/kg respectively) (Bach *et al.*, 2012).

Phthalates readily leach and migrate into water because they are not covalently bound to the plastic polymer matrix (Muncke, 2011; Khetan, 2014). The phthalate concentration of PET bottled mineral water may vary with pH (Montuori *et al.*, 2008); storage time (Biscardi *et al.*, 2003), storage temperature (30 °C–60 °C) (Casajuana and Lacorte, 2003; Schmid *et al.*, 2008; Bach *et al.*, 2013) and exposure to sunlight (Bach et al. 2014; Leivadara et al. 2008; Schmid et al. 2008).

Several studies have shown that phthalates inhibit the synthesis of testosterone and thus impair the male reproductive system, impair foetal development during pregnancy and cause male infertility in rodents (David, 2006; Foster, 2006; Christiansen *et al.*, 2009). Studies have also linked the actions of some phthalates with obesity (Desvergne et al. 2009); DEHP in particular has been shown to promote adipogenesis in mouse liver (Feige *et al.*, 2007). Although phthalates are generally less potent than endogenous estrogens, they are a cause for concern due to their

persistence in the environment, resistance to chemical or enzymatic degradation, and sequestration and storage in adipose tissue. A large number of preclinical *in vitro* studies, animal studies and epidemiology studies cite non-monotonic dose responses for phthalates found in plastics (Vandenberg *et al.*, 2012). Research proposes that the current reference dose defined by governments for common phthalates (BBP, DBP and DEHP) may promote steroid hormone-dependent breast cancer (Chen *et al.*, 2016). Very low concentrations of BBP, DBP, and DEHP were proliferative, stimulated the critical tumorigenic PI3K/AKT signalling pathway in breast cancer cells and were estrogenic. Being a target pathway for cancer treatment it is a concern that phthalates can stimulate PI3K/AKT pathway at levels similar to those to which the average population is currently exposed. Phthalate exposure in young girls at levels that correlated with the use of food and food containers was linked with early puberty (Chen *et al.*, 2015). Do *et al.*, (2012) provided evidence that doses of DEHP lower than the safe reference dose increased maternal serum testosterone levels and anogenital distance in pregnant mice. Together these studies suggest that exposure to phthalates at low levels can contribute to adverse health effects.

1.5.3. Antioxidants

These additives are used to avoid the oxidation of plastic materials. Although most food contact-materials and PET bottles are usually manufactured without antioxidants(Zweifel, Maier and Schiller, 2009), traces of some antioxidants, like butylated hydroxytoluene (BHT), have been found in PET containers or water bottles (Higuchi et al. 2004; Tombesi et al. 2004).

BHT is a lipophilic organic compound used as a food additive and plastic conservative because of its antioxidant properties (Yehye *et al.*, 2015). Although this compound has never exceeded the established SML of 3mg/kg there are *in vitro* studies that indicate that this compound at micromolar concentrations may inhibit testicular development (Botterweck *et al.*, 2000; Hughes *et al.*, 2000; Wada *et al.*, 2004).

BHT is normally used in combination with Butylated hydroxyanisole (BHA) a synthetic lipophilic compound also used as an antioxidant and preservative in food, food packaging and animal feed (Lam *et al.*, 1979). Through the use of the E-screen assay, based on the proliferative effect of estrogen in target cells, some research has shown that BHA has a weak estrogenic activity (Jobling *et al.*, 1995; Okubo and Kano, 2003) and that the effects of BHA and endogenous estrogen are additive (Veld et al. 2006). *In vivo* studies show that one outcome of the endocrine disruption of BHA was to decrease the uterine weight of female rats (Kang *et al.*, 2005). Another study tested this compound *ex vivo* and suggested that BHA promoted the appearance of carcinogenic effects in lung tissue, so it could be also considered a mutagen (Gressani *et al.*, 1999). Safety assessment of other food contact antioxidants is required.

1.5.4. Alkylphenols

Other bioproducts of plastic manufacturing or product breakdown are alkylphenols, in particular, 4-nonylphenol (NP) and 4-tert-octylphenol (OP); both show structural features similar to the endogenous hormone, 17 β-estradiol. They bind specifically to estrogen receptors and target estrogen signalling pathways (Sheikh *et al.*, 2016). NP is considered an endocrine disrupting chemical that acts as an agonist of estrogen (Khetan, 2014). Despite its weaker agonistic potency relative to 17 β-estradiol, its ability to persist and bioaccumulate enables it to produce a slow, weak but continuous estrogenic disruption (Cionna *et al.*, 2006). OP, on the other hand, has shown one of the strongest agonistic activities in an *in vitro* bioassay based on the induction of vitellogenin synthesis in Atlantic salmon hepatocytes (Tollefsen et al. 2003).

Biomonitoring studies of grey mullet fish showed that exposure to NP at an environmentally relevant concentration (250 mg/kg body weight) induced a measurable increase in plasma vitellogenin, an estrogenic marker in adult fish but not in juveniles (Sheikh *et al.*, 2016). Interestingly, the same study showed that NP at both 0.25 and 250 mg/kg body weight inactivated hepatic Cytochrome P4501A1 in both juvenile and adult fish. Cytochrome P4501A1 is a critical enzyme system for detoxifying apolar xenobiotics. This study highlighted the importance of not only of testing low doses but also selecting appropriate cellular biomarkers for providing an early indicator of environmental exposure.

OP has shown *in vivo* effects in the maturation of reproductive organs in rats (Laws *et al.*, 2000). It has also been shown in computational binding studies that it could act through the estrogen signalling pathway (Sheikh *et al.*, 2016).

1.5.5. Bisphenol A

Bisphenol A (BPA) is a synthetic estrogenic chemical substance within the group of alkylphenols used for the fabrication of certain plastics, such as PC bottles and epoxy resins. It is one of the chemicals most widely-produced each year. BPA is readily leached into food and the liquid external environment due to the hydrolysis of ester bonds in the polymer matrix (Khetan, 2014).

Since concern was first raised regarding leaching of substances from plastics (Colborn et al. 1996), reports from toxicological studies verifying the toxicity of BPA prompted the European Food Safety Authority (EFSA) to reduce the reference dose of BPA for the fabrication of plastic materials and to impose some restrictions about the use of BPA in infant bottles (European Commission, 2011).

BPA has been shown to have an impact on estrogen signalling pathways, which lead to reproductive and behavioural impairments in rodents at doses that were lower than that

recommended by the corresponding food agency authority (Chevalier and Fnichel 2015; Usman and Ahmad 2016; Vandenberg et al. 2007, 2009). BPA has also been linked with the appearance of certain cancers (Soto and Sonnenschein, 2010) and higher susceptibility to breast cancer has been found in rodents exposed to BPA (Rochester, 2013).

BPA is considered to have a weak estrogenic activity due to its lower affinity for the estrogen receptor. Nevertheless, it is likely, that BPA exerts its actions as an endocrine disruptor by targeting the estrogenic signalling pathway (Acconcia et al. 2015; Sheikh et al. 2016).

It has also been suggested that BPA acts as an antagonist of the androgenic receptor (Lee *et al.*, 2003; Xu *et al.*, 2005) causing androgenic disruption.

Several studies have shown that different cell lines are affected by the presence of BPA. In some cases it has been demonstrated that it acts as an endocrine disruptor (Schug *et al.*, 2011; Michalowicz, 2014), some others suggest that BPA has a genotoxic effect in several cell lines like Chinese hamster ovary (CHO) cells (Tayama et al. 2008), a cell line that is widely used in the biopharmaceutical industry, and also in human cell lines like human hepatoma cells (HepG2) and human adenocarcinoma cells (H295R)(Zhang *et al.*, 2011; Fic *et al.*, 2013). Finally, several animal studies have also associated BPA with metabolic diseases and obesity by causing dysfunction in the thyroid axis (Yang et al. 2016).

1.5.6. Metals

Some inorganic xenestrogen species are used as catalysts in the production of polymers like PET and could be found in the final plastic product. Some of these metals are Co, Cr, Fe and Mn, however, very few studies have examined the migration/leaching of these substances from plastic containers because of the low levels of these metals in the final polymer (27 mg/kg, 0.1 mg/kg, 1.3 mg/g and 0.3 mg/kg respectively).

Another metalloid, antimony trioxide (Sb_2O_3) used as a catalyst for PET manufacture is normally present as a residue in PET at a level between 170-300mg/kg (Westerhoff *et al.*, 2008; Keresztes *et al.*, 2009; Welle and Franz, 2011). The International Agency for Research on Cancer (IARC) has reported evidence of the carcinogenicity of Sb_2O_3 in experimental animals (IARC, 1989). Studies have shown adverse health effects such as nausea, vomiting and diarrhoea with short term exposure and higher blood cholesterol and lower blood sugar after long term exposure (Westerhoff *et al.*, 2008; Aghaee *et al.*, 2014).

In summary, toxicity and exposure information is only available for a small proportion of intentionally added substances. More studies to test the hazard properties of chemicals that are intentionally added as well as the unknown NIAS during plastics manufacture are needed.

Muncke *et al.*, (2020) identified methodological gaps that need to be addressed urgently in order to protect public health. Studies of chemicals at doses that are lower than the reference dose or no observed adverse effect level are needed to make regulatory decisions. Methodological approaches to addressing mixture toxicity and a framework to address aggregate and cumulative exposures are needed. Furthermore, the scope of toxicological testing of the finished food-contact plastic container should be expanded to include non-cancer related endpoints such as screening for endocrine disruptor activity and mutagenicity.

1.6. Toxicity of migrating substances

The current legislation for toxicological testing of FCM made of plastic is focused on the identification and quantification, through diverse instrumental chemical analysis, of single substances and their reported toxicity, mainly and almost exclusively focusing on genotoxicity. Analytical techniques normally employed to detect migrating substances from FCM are gas chromatography-mass spectrometry (GC/MS), liquid chromatography-mass spectrometry (LC/MS) and inductively coupled plasma-mass spectrometry (ICP/MS), after which a quantification of the analytical evaluation threshold of the detected compounds is carried out. However, plastic FCMs usually contain and/or release non-intentionally added substances (NIAS). NIAS are usually generated through the interaction of the FCM and its contents or through common use stressors. NIAS toxicity can only be tested if a substance's chemical identity is known and if it is available as a pure chemical. It has been estimated that as many as 12000 substances (intentionally added as well as non-intentionally added) may be present in FCMs (Muncke *et al.*, 2020). Quantification of single substances that migrate into food or water is therefore a huge challenge for analysts. If analytical standards of high purity are not available or not known, which is the common case with NIAS, it will be difficult to detect, identify and quantify each substance in the total migrate of new plastic prototypes. Another problem with instrumental techniques as an approach to toxicological testing of plastic FCM is that it does not take into account the mixed-effects exerted by mixtures of chemicals migrating from food contact materials.

Groh and Muncke (2017) carried out a comprehensive review of toxicity testing of FCM and described three main categories of toxicity that can be exerted by migrating substances from food contact materials cytotoxicity, genotoxicity, and endocrine disruption potential. During this review, Groh and Muncke (2017) suggested that *in vitro* bioassays may offer a robust and economic solution to screen the toxicity of food contact materials (FCM). Non-instrumental analytical techniques, such as bioassays give information about the biological effects or biological

interactions, although, unlike instrumental techniques, bioassays do not allow identification of the different compounds of a mixture and quantification is inexact and has to be achieved indirectly through extrapolation (Fang *et al.*, 2016).

1.6.1. Mutagens and genotoxic chemicals

In biology a mutation is defined as a permanent alteration of the genome or other genetic elements. Mutations play a role in processes like evolution, cancer and development being therefore part of both normal and abnormal biological processes. There is a huge and extensive classification of mutations that depend on their effect on structure, function, replication and inheritance of the genome as well as their effect on protein sequence, protein function and metabolism of the organism (Winter et al. 1998).

There are four main sources of mutation: i) spontaneous mutations, ii) mutations that occur during the replication of DNA, iii) mutations induced during DNA repair and, iv) induced mutations which are alterations caused by environmental conditions or mutagens.

A mutagenic compound is a physical, chemical or biological agent that changes the nucleotide sequence or alters the organisation of the DNA (genotype) of a living being; these agents increase therefore the ratio of natural and "induced" mutations (Molnar et al. 2009).

On the other hand, genotoxicity is defined as the ability of some physical, chemical or biological agent to cause damage to the genetic material, which not only includes DNA but also all the components related to its functionality and regulation in cell homeostasis. Therefore, damage to transcription and reparation proteins and factors, alterations in the assembling proteins for chromosome formation or deregulation of the histone modification machinery could be considered genotoxic effects (Brusick 1987; Molnar et al. 2009).

Thus, a mutagenic compound will be genotoxic but not all genotoxic compounds are mutagenic.

By the middle of the 20th century, there was almost no evidence to support the role of mutations in cancer; it was not until 1973 that the connection between mutagenesis and carcinogenesis was made thanks to the development of the Ames test (Ames et al. 1973). Throughout this decade many other mutagenicity assays were developed, and consequently, a new approach to the carcinogen/mutagenicity and genotoxicity prediction emerged in concordance with the new paradigm. Since then, decades of research have shown us the critical role and relationship between mutagenicity and carcinogenesis (Claxton *et al.*, 2010).

All of these assays led to the discovery that the environment in which we live is replete with chemicals and agents with mutagenic and/or genotoxic activity which pose a challenge for environmental researchers and public health regulatory authorities. Since then, everyday new

previously unrecognised and ubiquitous toxins are discovered making the regulators and public health authorities expand their knowledge on the topic, reconsider the approaches used for testing toxicity or redefine some of the definitions that led to the differentiation between genotoxic and mutagenic substances/agents.

1.6.2. Endocrine disrupting chemicals

Several definitions of an endocrine disruptive chemical (EDC) exist. Endocrine disrupting chemicals (EDCs) are described by the WHO and European Food Safety Authority as "exogenous substances that cause *adverse health effects in an intact organism or its progeny* as a consequence of changes to endocrine function" (Kavlock *et al.*, 1996). Nevertheless, there are some researchers and agencies in the US, like the U.S. Environmental Protection Agency (US EPA), that define EDCs more robustly as "external agents that alter the normal function of the endocrine system by deregulating the synthesis, secretion, transport, metabolism, release, uptake, signalling or degradation of endogenous hormones" (Zoeller *et al.*, 2012). The Endocrine Society in 2011 issued a clarifying statement that interference with hormone action was a clear predictor of adverse outcome and thus an EDC was an exogenous chemical or mixture of chemicals that *interferes with any aspect* of hormone action (Zoeller *et al.*, 2012).

In 1996 with the publication of *Our stolen future* (Colborn et al. 1996) the issue of anthropogenic chemicals that act as endocrine disruptors and the hazard that they pose to our health came into prominence. Since then characterizing and screening for exposures to endocrine disruptors has been recognised as priority research in a scientific statement of the Endocrine Society (Diamanti-Kandarakis *et al.*, 2009).

There are five attributes of EDCs that warrant specific attention. (i) Unlike the usual dose-response curve familiar to toxicologists, EDCs may, like endogenous hormones exhibit non-monotonic dose-response curves. They may follow a U-shaped dose-response curve, which implies that effects are shown at low doses but not at higher doses or they may follow an inverted U-Shaped curved which implies they exhibit a greater response at dose levels intermediate between low and high doses (Khetan, 2014). Thus some of these chemicals do not show a threshold dose below which exposure is safe and a dose-response relationship for individual leachate chemicals from plastic can be an important piece of evidence in the determination of the risk posed by plastics in some toxicological studies (Welshons et al. 2003; Welshons et al. 2006).

(ii) EDCs show multiple molecular mechanisms by which they could interfere with hormonal action. There are two main ways of hormone signalling a) the genomic signalling pathway and b) the nongenomic signalling pathway or rapid-response pathway. The activation of the genomic signals is mediated by the binding of a lipophilic hormone to a cytoplasmic or nuclear receptor

(NR), which stimulates dissociation of accessory heat shock proteins leading to receptor activation. Once the receptor has been activated it will form a homodimer complex that will translocate to the nucleus and will interact with specific DNA regions known as hormone response elements (HRE) and trigger or suppress the transcription of hormone-responsive genes and production of a target protein. The genomic pathway is known as the classical pathway and it takes longer (hours) to appreciate a net effect in cell behaviour. The nongenomic pathway, on the other hand, is mediated by binding of less lipophilic hormones or xenestrogens to receptors in the cell membrane. These receptors then activate second messenger molecules which trigger a signal transduction cascade leading to a rapid change in cell behaviour (seconds, minutes).

In both signalling pathways, the endocrine system releases very low concentration of hormones to exert effects; consequently, it may be assumed that EDCs, that mimic or antagonise endogenous hormonal signals will exert their effects at low concentrations (Welshons *et al.*, 2003). Also, it must be considered that small and precise concentrations of endogenous hormones are needed to maintain the homeostasis of the endocrine system. Small changes in hormone production, metabolism, uptake, release and degradation would hugely affect the homeostasis of the endocrine system, therefore even low-dose EDCs could lead to a deregulation of the homeostasis of hormones posing an adverse health effect (Welshons *et al.*, 2003; Khetan, 2014).

(iii) There are critical windows of susceptibility for EDC exposure. Prenatal, postnatal and young adulthood are critical windows of development that are highly susceptible to the detrimental effects of exposure to low dose EDC mixtures. Neonatal or prenatal exposure to EDCs causes irreversible disruptive effects on the maturation of the reproductive system and behavioural responses. Therefore, disruptive effects produced during development may become evident later in life during maturation and reproduction. The long time between exposure and effect makes diagnosis difficult and is therefore of concern (Khetan, 2014).

(iv) Moreover, some EDCs may exert epigenetic modifications and thereby affect cellular differentiation and development. The genomic pathway of some endogenous hormones induces epigenetic changes during development by activating or repressing expression of certain genes that translate to proteins with the potential to change cell phenotype. The epigenome allows the correct differentiation of tissues and the correct organisation of cells during embryonic stages, while in early postnatal stages it is implicated in the development of the brain and reproductive system (Janesick and Blumberg, 2011). Endocrine disruption during development could lead to deregulation of the epigenome causing permanent alterations, which could have a transgenerational effect. So EDCs can have effects not only on the individual but also transmit health problems through generations (Newbold, 2010).

(v) EDCs exist as mixtures in the environment. While individual components may not exert an adverse effect on their own, in combination they may exert either additive or synergistic effects. Most plastic products release xenestrogenic chemicals that pose potential adverse health effects in humans (Yang et al. 2011).

In essence, all the functional changes that EDCs could produce in living beings, together with the huge number of compounds that could act as EDCs and the technical limitations in their detection, make the understanding of human exposure to man-made chemicals complex. This has forced researchers to focus their investigations on a few chemicals. Some researchers have stated that this approach may not represent adequately the total exposure and therefore the total toxicity (Daughton, 2004). Fortunately, bioanalytical techniques can characterise the biological effect of a complex sample in which both unidentified chemicals and mixtures of compounds are included, helping therefore to overcome this issue (Wagner and Oehlmann, 2011).

1.7. *In-vitro* bioassays for toxicological assessments

In a commentary directed mainly at policymakers, decision makers and scientists of every discipline Muncke *et al.*, (2020) affirmed that known hazardous chemicals are authorised for use in food contact plastics and that humans are exposed daily to mixtures of chemicals migrating from food contact containers into food. They argue this is as a result of gaps in chemical risk assessment and propose risk assessment should encompass biological risk assessment to safeguard human health. This section will highlight challenges to risk assessment, identify approaches to modernise risk assessment and describe current bioassays to test mutagens, genotoxins and endocrine disruptive chemicals in food contact materials.

There are many challenges to evaluating the toxicity of migrating substances from plastic polymers. One minor challenge is the determination of the original source; the leachates could come from water pollution or water processing rather than from the plastic container itself. A major challenge is the lack of standardization in the preparation of the samples.

The lack of consistency in sample preparation makes it difficult to compare test results across different laboratories. It is apparent that very many protocols for preparing plastics for *in vitro* testing exist and that they should be standardised and internationally harmonised. A recent review of scientific studies on FCM by Groh and Muncke, (2017) advised that *in vitro* test-based workflows for assessing the safety of food contact plastics use the solvents and procedures outlined by the Regulation (EU) 10/2011 (EC 2011) (European Commission, 2011), or guidelines issued by the U.S. Food and Drug Administration (FDA, 2002). Furthermore, agreement on

certain “worse-case” extraction procedures would also be of benefit for the risk assessment process. Extractable studies are the most widely carried out because they allow the researchers to create a “worst-case scenario” and compare extraction under normal use conditions with extraction under exaggerated conditions which will give a margin of safety. However, to be able to. However, the conditions used to get the sample of extractables, such as UV radiation, temperature, other stress conditions and period of incubation vary enormously from study to study. Identifying conditions that would allow release of all chemicals that could possibly migrate from plastic would allow for positive samples to be further assessed using milder conditions that more closely reflect anticipated use conditions. The different solvents used for extraction as well as some concentration techniques used adds variability to the results that could be obtained. The use of more than one solvent increased the likelihood of detecting estrogenic activity due to polar and non-polar, hydrophilic and hydrophobic substances expected in plastic extracts. Due to cytotoxic effects most organic solvents could offer false negative/positive responses during *in-vitro* bioassays and thus some solvents are not suitable for bioassays. The most commonly used solvents are water, saline solutions, ethanol and DMSO. Nevertheless, the concentration of ethanol and DMSO solvents must never exceed 1% v/v when dissolved in cell culture media due to their cytotoxic effects. This limitation on the concentration of solvent vehicles that could be used in cell culture models determines the maximum neat concentration of leachates that could be tested (Körner *et al.*, 1999). Some labs tested plastic extracts directly; others further concentrated extracts using SPE before analysis. Solid-phase extraction (SPE) is a sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Analytical laboratories use optimized and standardized solid phase extraction protocols to concentrate and purify samples for analysis, however standardization and optimization of solid phase extraction have rarely been conducted for bioassay studies. There is a huge number of cartridges, sorbents and elution solvents that could be used and that offer variability on the results. Therefore, the choice of extraction method, the conditions used are critical. The solvent vehicle in which the sample is diluted is also critical because it has to minimise the loss of chemicals (known and unknown) that could migrate from the plastic while prioritizing the compatibility with the chosen biological model to minimise cytotoxicity (Chevolleau *et al.*, 2016). Ideally, in order to properly design the toxicological assessments of the plastic food contact materials an extensive knowledge of the plastic material, its additives, the intended content and the potential interactions that could lead to the release of harmful chemicals would be required, however some or most of this information is generally unknown when carrying out toxicological studies.

Another major challenge critical for obtaining high-quality reliable data is the sensitivity and specificity of the selected assay. A wide selection of assays for *in vitro* testing of plastics and plastic bottled water have been described in the literature. Each *in vitro* bioassay requires careful interpretation of test results and acknowledgement of limitations. For example, most models of genotoxicity are bacteria, which are prokaryotic cells, making a human extrapolation particularly difficult. Bacteria will not be able to show effects on chromosomes. Furthermore, when the biological model does not possess metabolic activity the use of S9, an exogenous mammalian metabolic activation system, is required to avoid false positive/negative results and to improve the reliability of the assay (Kirkland *et al.*, 2007). *In vitro* cell culture models for screening estrogenic activity of leachates or extractables from food contact materials are not subject to influences from human digestion, metabolism or toxicokinetics. Therefore, the extrapolation of *in vitro* findings to human health-relevant *in vivo* endpoints poses a challenge (McKim, 2010). One of these challenges is the difficulty of using *in vitro* data for predicting chronic exposure effects in humans. Besides the challenges of extrapolating from *in vitro* data to human health effects are further aggravated by the fact that neither accumulative effects or low doses-effects are taken into consideration by the thresholds used by regulatory authorities. Test results may also be influenced by the choice of cells and their metabolic activity, which in turn may affect the sensitivity and specificity of the assay. *In vitro* bioassays must be sufficiently sensitive to have a low rate of false negatives (i.e, detect biological effects at low concentrations) and sufficiently specific to have a low rate of false positives (i.e. detect specific biological effect).

All of these variables have to be taken into account and could explain the inconsistencies of results and conclusions between different studies. In conclusion standardisation of methods for sample preparation and agreement on cellular models would greatly advance the process of hazard assessment of all new plastic prototypes.

1.7.1. Assays for the detection of genotoxicity and mutagenicity

In vitro bioassays could be used to complement chemical assays to determine the biological activation of the mixture and interaction effects that cannot be inferred from concentration levels of the individual detected components. The identification of substances capable of inducing mutations has become an important procedure in safety assessment.

Some of the genotoxic and mutagenic assays most widely used are cytotoxic assays performed in Chinese Hamster Ovarian (CHO) like the CHO/HGPRT assay (Hsie *et al.*, 1975). This assay is used to evaluate the potential of a chemical or extract to induce mutations at the *hgprt* locus of CHO cells. In this assay, V79 CHO cells having one functional copy of the gene coding for HGPRT, an enzyme important in DNA synthesis, which is exposed to a toxic nucleoside analogue

6-thioguanine (6-TG) which halts growth. Cells with functional mutations in HGPRT are resistant to the toxic effects of 6-TG and are able to proliferate in the presence of 6-TG. Therefore, an increase in cell survival and colony formation in response to a test chemical correlates with the mutagenic potential of the chemical.

Another cytotoxic assay widely used is the $Tk^{+/-}$ mouse lymphoma mutation assay (Clive *et al.*, 1972). In this assay, thymidine kinase (TK) proficient cells are exposed to the pyrimidine analogue trifluorothymidine (TFT), which inhibits cellular metabolism and halts further cell division. Cells with functional mutations in TK, are resistant to the cytotoxic effects of TFT and are able to proliferate to form colonies in the presence of TFT. Therefore, an increase in proliferation as a response to a test chemical co-cultured with TFT correlates with the mutagenic potential of the test substance.

The Zimmermann test is able to detect mutagenic activity such as point mutations, gene reversions and conversions when yeast DNA is exposed to test samples (Zimmermann, 1971).

The Comet assay is one of the major tools in environmental pollution biomonitoring for assessing DNA damage *in vitro* and *in vivo*. The approach essentially consists of analysis of DNA fragments that during electrophoresis, migrate from the nuclear core, resulting in "a comet" formation.

The Ames test, developed in the 1970s by Bruce Ames, Professor of Biochemistry at UC-Berkeley, is widely used as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA (Mortelmans and Zeiger, 2000). National and international guidelines for performing the assay have been established by agencies including the Organisation for Economic Co-operation and Development. The bacteria used in the test include strains of the bacterium *Salmonella typhimurium* that are auxotrophic for histidine and *E. coli* WP2 strains that are auxotrophic for tryptophan. The tester strains are constructed to detect either frameshift or point mutations. The salmonella strain TA98 which detects frameshift mutations and TA100 which detects point mutations have been reported to be very sensitive, responding to a broad range of mutagenic compounds and are suggested as the basic strains (Guan *et al.*, 2017). Rat liver S9 fraction may be added to the test to mimic mammalian metabolic conditions (Mortelmans and Riccio, 2000). The standard Ames assay as described in the OECD Test Guideline uses the plate incorporation and pre-incubation methods in 100 mm plates. Miniaturised versions of the assay using 6-well and 24-well plates and the Ames II^{TM} assay, a second-generation bacterial reverse mutation assay developed as a screening assay, are useful for high-throughput, pre-screening purposes (Pant *et al.*, 2016).

The Ames test has been widely used to detect mutagenic activity in complex environmental mixtures such as surface waters, especially river waters. Some projects tested (Katsonouri et al. 2012; Ohe et al. 2004) recycled, surface, ground and drinking tap water samples and assessed

mutagenicity using two commercially available Ames test, with the aim to introduce a simple and effective way of testing water. Surface water showed no mutagenicity, 53% of recycled water samples tested positive for mutagenicity and one of three groundwater samples tested positive for mutagenicity but only in the absence of metabolic activation using S9 and not in its presence. Preliminary findings on tap water samples showing a positive mutagenicity result at two independent sampling times implied excessive chlorination during water treatment and mutagenic chlorine-by product formation as causative agents of mutagenicity. Rainer *et al.*, (2018) reviewed the suitability of the Ames assay to address genotoxicity of FCM migrates and discussed the limit at which genotoxins can be detected in complex mixtures and if such limits of detection (LOD) would be compatible with safety. They discussed that although it was desirable to identify and chemically characterise with instrumental chemical analysis all the migrants from FCM that exceed a certain threshold, it is not always possible. They also discussed that this kind of approach does not take into account the mixture effects. They suggested that the use of *in vitro* bioassays, such as the Ames test, offer a cheap and high throughput alternative to chemical analysis and *in vivo* assays.

1.7.2. Assays for the detection of estrogenic endocrine disruption

The first *in vitro* bioassay developed for screening estrogenic activity was the E-Screen bioassay, also known as the MCF-7 cell proliferation bioassay. It measures cellular estrogen-dependent proliferation of the MCF-7 human breast cancer cell line. It is a quantitative assay that determines the cell number achieved by seeding MCF-7 cells in the presence of a test sample at different concentrations. Estrogenic activity can be determined by comparison with cell numbers achieved in the absence of estrogens (negative control), in the presence of 17 β estradiol (positive control) and in the presence of fulvestrant (an estrogen receptor antagonist). Though the proliferative response is maximal after 6 days, the assay is very sensitive and suitable for high throughput analysis. As cell proliferation can be mediated by nuclear receptor cross-talk with membrane and cytoplasmic signaling molecules, the use of an estrogen receptor antagonist assures specificity and validates a positive response. It has been used to assess the estrogenic activity of environmental matrices (Etteieb *et al.*, 2015), bottled waters (Wagner and Oehlmann, 2011; Real *et al.*, 2015), red wine (Klinge *et al.*, 2003), polystyrene food containers (Fail *et al.*, 1998), (Hirano *et al.*, 2001), food packaging plastics and consumer products (Yang *et al.*, 2011; Bittner *et al.*, 2014a, 2014b) and raw material chemicals (Molina-Molina *et al.*, 2013). A systematic survey of 455 unstressed commercially available plastic resins (HDPE, PP, PET, PS, PLA and PC) and products (Flexible packaging, Food wrap, Rigid packaging, Baby bottle component, Deli containers and Plastic bags) carried out by Yang *et al.*, (2011) showed that many of these products released chemicals which had > 15% of the maximum response to E2 as determined in a robotized E-Screen bioassay.

Other *in vitro* bioassays that have been developed for screening hormonal activities are based on transcriptional activation of nuclear hormone receptors. Various mammary, human cell lines or yeast strains have been stably transfected with a specific nuclear receptor, either estrogen receptor, androgen receptor, glucocorticoid receptor coupled to a reporter gene allowing detection of agonist or antagonist activities. The first version of these assays was the yeast estrogen screen (YES) and yeast androgen screen (YAS). In the YES assay, the yeast strain is stably transfected with *hERα* the human estrogen receptor alpha gene and an expression plasmid containing the reporter gene *lac Z* under the control of estrogen responsive elements. When an estrogenic substance binds with the receptor, production of yeast β galactosidase (the *LacZ* product) is activated. Enzyme activity, a measure of the estrogenic activity of chemical compounds is measured spectrophotometrically at 540 nm by conversion of the chromogenic substrate chlorophenol red-β d- galactopyranoside (CPRG) into chlorophenol red. The YAS assay is similarly constructed but with the stably integrated human androgen receptor. Test samples are considered positive if, after 20-24 h exposure, the maximum response induced is equal to or exceeds 10% of the response of the positive control (17β estradiol or testosterone). Both assays can also measure antagonist activities of test samples. Due to the absence of endogenous receptors, nuclear cross talk is not an issue. However, chemicals must be able to cross cell walls of yeast in order to avoid false negative results (Leusch *et al*., 2010; Mertl *et al.*, 2014). YES has been used to assess the estrogenic activity of PET bottled waters (Pinto and Reali, 2009; Wagner and Oehlmann, 2009), plastic extracts (PET, PP, PE, PS) and composite films (Kirchnawy *et al*., 2014; Mertl *et al.*, 2014). YAES and YAAS have been used to assess the anti-estrogenic and anti-androgenic activities of glass and PET bottled waters, (Wagner *et al.*, 2013), plastic resins and polycarbonate bottles (Guart *et al.*, 2013).

Subsequently, fully validated test methods using recombinant human cell lines stably transfected with plasmids containing hERα and an estrogen-responsive luciferase reporter gene were established (OECD guideline 455, 2012) for detecting estrogen receptor agonists. Assays using the human cervical cancer HeLa-9903 cell line and human ovarian BG1Luc-4E2 cell line demonstrate functional activation of the hERα receptor by estrogen. When an estrogenic substance binds with the hERα receptor, dimerization of estrogen-bound receptors occurs before nuclear activation of estrogen-responsive elements that regulate transcription of luciferase gene. The luciferase product in the presence of luciferin substrate emits light which is quantified as relative light units using a luminometer. Test samples are considered positive if, after 20-24 h exposure, the maximum response induced is equal to or exceeds 10% of the response of the positive control (17β estradiol). A series of reporter gene assays based on the same principle was used for measuring androgenic, progestagenic, glucocorticoid and thyroidogenic activities in PET- and glass bottled waters (Plotan *et al.*, 2013; Chevolleau *et al.*, 2016) and in BPA-free plastics (Bittner *et al*., 2014a, 2014b). An extract of polypropylene which was subjected to

microwave radiation showed significant and concentration-dependent estrogenic activity when screened using the hERα-HELa 9903 stably transfected transcription activation assay (Riquet *et al.*, 2016). Anti-androgenic and estrogenic activity of PET- and glass bottled water exposed to sunlight for 2,6 and 10 days) or to high temperatures (40, 50, 60 ℃) was assessed using reporter gene assays with human breast epithelial cell line MDA-MB453-kb2 and hepatocellular carcinoma HepG2 cell lines (Bach *et al.*, 2013, 2014). As alluded to already, nuclear receptor cross talk can be an issue when developing bioassays using mammalian cell lines that contain multiple steroid hormone receptors. Interactions between ERα and the receptor for androgens, glucocorticoids or progesterone may trigger a unique signaling or gene expression profile. To circumvent this issue, Sonneveld *et al.*, (2005) developed a panel of reporter cell lines engineered from the human osteoblastic osteosarcoma U2-OS bone cell line and validated their use for activation of androgens and estrogens receptors. The U2-OS cell line does not express high levels of steroid receptors other than the stably introduced receptor of interest. Without nuclear receptor cross talk, this panel of engineered cells has allowed efficient and convenient measurement of androgen-, estrogen-and glucocorticoid-receptor interacting compounds (Sonneveld *et al.*, 2005; Wilson *et al.*, 2016; Sharma *et al.*, 2017; McComb *et al.*, 2019).

In addition to transcription activation-based assays, another strategy for testing the potential of chemicals to disrupt endocrine function is to examine their effect on an upstream event, in particular receptor translocation to the nucleus. In the inactive state, the steroid hormone receptor is located in the cytoplasm bound to various heat-shock proteins (Pratt and Toft, 1997). When activated by ligand binding, the bound receptor detaches and translocates to the nucleus where it interacts with hormone receptor regulatory elements to stimulate transcription of target genes. The recombinant U2-0S cell line stably transfected with human steroid receptors fused to an enhanced GFP is a model used to measure receptor translocation. The redistribution of the GFP-R from cytoplasm to nucleus may be visualised under the microscope. The high content analysis platform (CellInsightTM NXT High Content Screening (HCS) platform (Thermo Fisher Scientific, UK) is a powerful tool for identifying translocation changes of receptors to nuclei. It permits measurements of nuclear morphology, in particular cell number, nuclear intensity and nuclear area and has been used in a large number of studies of receptor translocation (Wilson *et al.*, 2016; Shannon *et al.*, 2017, 2019; McComb *et al.*, 2019). It allows attributing endocrine disruptive activity to compounds that do not disrupt receptor transactivation but do disrupt receptor translocation.

It is widely accepted that *in vitro* bioassays are important components of initial screening as they allow thresholds for follow up action to be defined. Mueller (2004) summarised in a table of his review the limitations and advantages of detection methods for assessing estrogenic activity of samples. In summary, most ligand-binding assays do not measure direct activation of the estrogenic receptor (ER) and do not give information about the physiological response; the yeast

bioassay does not give a physiological response but it gives information about the activation of ER; other reporter gene bioassays based on mammalian cells are also based on analysis of ER-mediated gene expression and are high throughput but need specific cell lines with active genes or markers. The E-screen bioassay gives information about the physiological response upon binding of xenestrogens to cells that endogenously express ERs, however it does not give information about the mechanistic data.
For the purpose of this study high throughput information about the physiological response of leachates and extractables was more important than the mechanistic data or the activation of ER, or the data provided by instrumental techniques therefore the E-screen bioassay was selected.

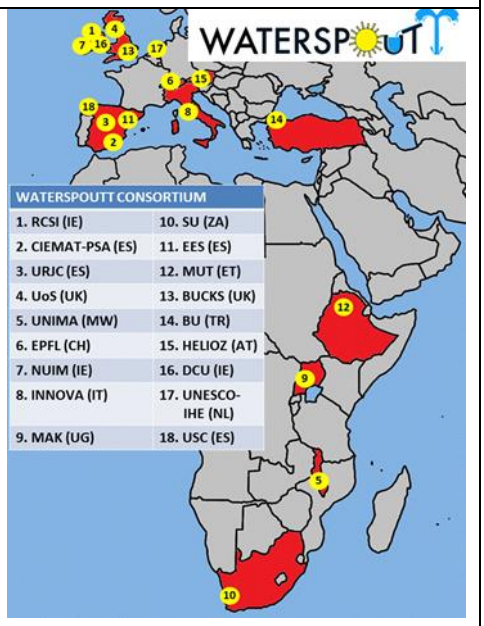
1.8. Waterspoutt project

Waterspoutt (**Water** - **S**ustainable **P**oint-**O**f-**U**se **T**reatment **T**echnologies) was a large collaborative research project funded by the Horizon 2020 programme (grant agreement no. 688928). It was a four-year project that started in June 2016. The objective was to develop a range of large volume (≥20 L) sustainable solar disinfection (SODIS) technologies that would provide affordable access to safe drinking water to remote and vulnerable communities throughout Sub-Saharan Africa and other resource-poor countries (http://www.waterspoutt.eu/). The multidisciplinary consortium comprised 18 partners, four of whom were from Africa and the others from Europe. Partners represented academic institutions, SMEs and international research organisations from 13 different countries (Table 1.4).

The project work was divided into a number of work packages (WPs) with the scientific research in WPs 1–4. (**Fig 1.6**). In addition to the critical aspects of Coordination and Management (WP7), and Dissemination and Commercialization (WP6) a work package (WP5) on Social Science was put in place to explore;

- the social, political and economic context of water use and needs of specific communities
- the effect of gender relations on uptake of solar water harvesting and SODIS reactor technologies
- governance practices and decision-making capacity at local, national and international level that impact the use of integrated solar technologies for point-of-use drinking water treatment
- challenges faced at household, community, regional and national levels for the adoption of integrated solar technologies for point-of-use drinking water treatment.

Table 1.4 Partners involved in the Waterspoutt project

Partner		**Country**	
Royal College Surgeons Ireland	1	Ireland	
Dublin City University	16	Ireland	
National University Ireland Maynooth	7	Ireland	
Plataforma Solar de Almería	2	Spain	
University Rey Juan Carlos	3	Spain	
University of Strathclyde	4	Scotland	
University of Malawi	5	Malawi	
EPFL (Ecole Polytechnique Fédérale de Lausanne)	6	Switzerland	
Innova S.p.A.	8	Italy	
Makarere University	9	Uganda	
Ecosystem Environmental Services	11	Spain	
Mekelle University	12	Ethiopia	
Buckinghamshire New University	13	England	
Bogazici University	14	Turkey	
Helioz GmbH	15	Austria	
UNESCO-IHE Institute for Water Education	17	Netherlands	
University of Santiago de Compostela	18	Spain	
University Stellenbosch	10	South Africa	

The focus of the project was the development of three large scale novel solar reactors. These included a solar rainwater reactor (WP1) for use in South Africa and Uganda. A transparent jerrycan (WP2) for use in Ethiopia and a solar ceramic filter which developed into a transparent 20 litre bucket for use in Malawi (WP3). In all three cases, the plan was to use plastic material in the reactors. Following investigations and research, the solar rainwater reactor was made of polymethyl methacrylate (PMMA), the transparent jerrycan was made of polyethylene terephthalate (PET) and the transparent bucket was made of polypropylene (PP). Due to concern about leachables from the plastics contaminating the water, toxicity studies were designed to test the plastics and the water disinfected by the reactors. This task, carried out by DCU, was part of WP4 and underpinned the work of WP1, WP2 and WP3. One of the tasks of WP4 was the development of processes to enhance solar disinfection. These processes or advanced oxidative processes were also tested for toxicity by DCU.

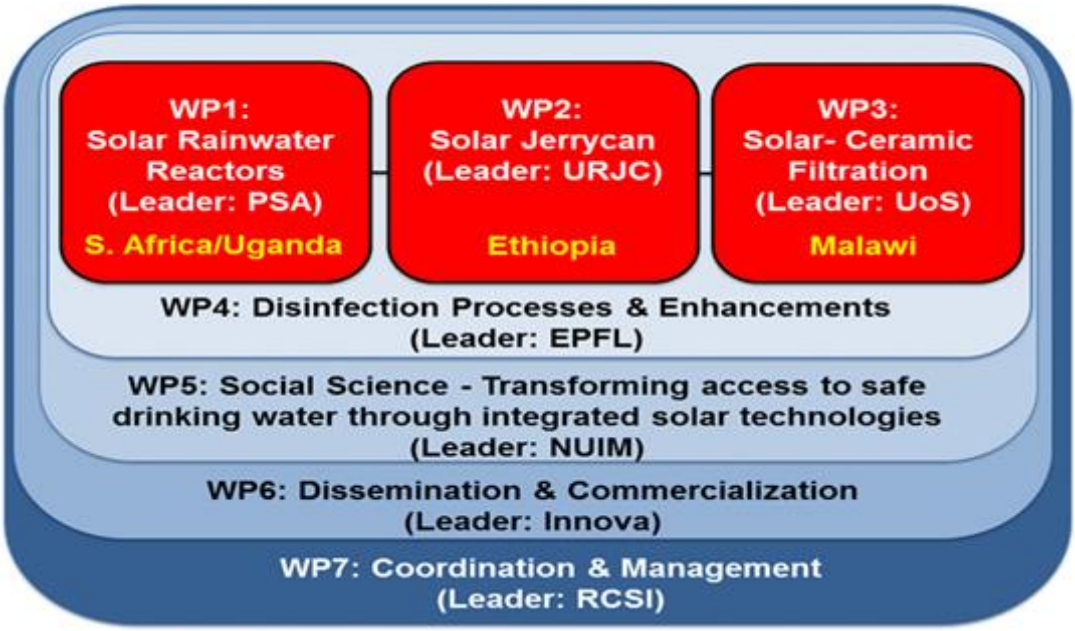


Figure 1.7 An overview of the work packages (WPs) in WATERSPOUTT.

1.9. Aims and objectives

In 2001, Wegelin *et al*., posed the question, 'Does sunlight change the material and content of polyethylene terephthalate (PET) bottles'? They pointed out that while SODIS was designed to destroy disease-causing microorganisms in water, the process also might transform the plastic material of the reactor used into photoproducts. Nevertheless, no SODIS study since that date, other than the study by Ubomba-Jaswa *et al*., (2010) who used the Ames test, addressed this critical question and no previous study used the E-screen bioassay to evaluate the estrogenic activity of possible endocrine disrupting chemicals migrating from plastic SODIS reactors. This study was designed to test solar disinfecting processes for toxicity using the Ames test to test for mutagenicity and the E-screen assay to test for estrogenic activity. Testing large scale SODIS reactors made with a range of plastics introduced further novelty to the work. The extractables of these large-scale SODIS reactors were used to assess the "worst-case scenario", while the simulated-use performance of the reactors was assessed by analysing solid phase extracted leachates that may be present in the water treated by the reactors. Thus, the main objective of this research, as part of the *Waterspoutt* project, was to carry out toxicity testing on solar processes developed in WPs 1-4 to determine if any toxicity was associated with the use of plastics in novel SODIS reactors developed by the project.

The aims of the project were

- To validate the use of the E-screen for use in the project
- To perform toxicological assessments of endocrine disrupting activity and mutagenicity of the possible leachates, present in water samples from the three novel SODIS reactors exposed to sunshine at both the pilot plant in Almeria, Spain and in field studies in Africa,
- To perform toxicological assessments of endocrine disrupting activity and mutagenicity of the possible extractables from the raw plastic samples and aged plastic samples from the PMMA, PET and PP plastics used in the constriction of the three novel SODIS reactors and
- To do preliminary toxicological assessments of the advanced oxidative processes photo-Fenton and persulfate activation to determine if they promote the release of mutagenic and/or estrogenic substances

The methodology used in the project is described in Chapter II. The results are described in Chapters III-VII, followed by the discussion in Chapter VIII. Conclusions and recommendations for future work are described in Chapters IX and X, respectively.

Chapter II

2. Materials and Methods

2.1. Materials

2.1.1. The Plastics

Three different plastic polymers were used in the construction of the novel large-volume batch solar reactor prototypes studied in Sections 4, 5 and 6: Polymethylmethacrylate (PMMA), Polyethylene Terephthalate (PET) and Polypropylene (PP).

- Polymethylmethacrylate (PMMA)

The PMMA plastic pieces used in this study were obtained from EES, Ecosystem Environmental Services, Barcelona, Spain www.ecosystemsa.com The plastic was also used by EES to manufacture the PMMA reactors. The PMMA was supplied by GEHR GmbH and was food grade quality.

- Polyethylene Terephthalate (PET)

The 25 L Jerrycans were supplied by Envases Soplados, Spain www.envasessoplados.com

The PET material was food grade quality.

- Polypropylene (PP)

The 20 L PP buckets with lids were produced by ArKay Plastics Ltd. (Malawi) www.arkayplastics.com from nucleated random PP copolymer for injection moulding applications.

Three plastics were used in the advanced oxidation processes studied in Section 7. They included;

- PET from the 25 L Jerrycan described above
- PET from a commercial Coca-cola bottle
- Polycarbonate (PC) from a commercial water cooler container

2.1.2. The novel SODIS Reactors

PMMA reactors

Two PMMA reactor prototypes were constructed. The Prototype I solar reactor (140 L treatment volume) consisted of three PMMA reactor tubes (200 mm diameter) that were positioned in the centre of a V-trough solar mirror (constructed from anodized aluminium). The reactor tubes were positioned at a 34° angle (equal to the local latitude to optimise the average annual solar UV irradiance input to the solar reactor) and were inter-connected by UV-A transparent PMMA tubing (Fig 2.1). The Prototype II solar reactor (88 L treatment volume) consisted of the same materials and design as prototype I, with the exception that eight PMMA tubes (100 mm diameter) were substituted for the three 200 mm diameter tubes used in the Prototype II system (Fig 2.2).

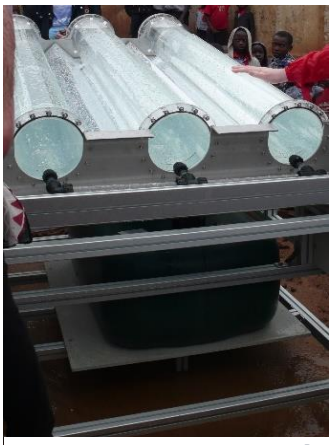


Figure 2.2 Prototype I tube reactor

Figure 2.1 Prototype II tube reactor

PET reactors

Two sizes of PET reactors were used in the study. 1.5 L-PET bottles and 25 L transparent PET jerrycans. The transparent jerrycan was 52 cm tall and with a base of 24 by 26 cm with an average wall thickness of 0.55 mm. The container was originally designed to hold olive oil and the material used was food grade quality (Fig 2.3)

Figure 2.3 PET transparent jerrycan SODIS reactor

PP buckets

20 L PP buckets with lids were produced by ArKay Plastics Ltd. (Malawi) from nucleated random PP copolymer for injection moulding applications. The main characteristics of the buckets were: wall thickness 1.60 mm, height 32.8 cm, diameter 30.8 cm, illuminated surface 0.101 m^2 and UV stabilizer 8.86 g (Fig 2.4)

Figure 2.4 Picture of 20 L PP bucket with lid.

2.2. Methods

2.2.1. Overview of sampling approach for SODIS studies (Sections 4, 5 and 6)

Testing for leachates and extractables

Polymethyl methacrylate (PMMA), polyethylene terephthalate (PET) and polypropylene (PP) used in the construction of the novel SODIS reactors were tested for extractables and leachates. Samples of raw and weathered plastic were tested for extractables and the plastics in contact with water were tested for leachates. An overview of the testing is described in Fig 2.5

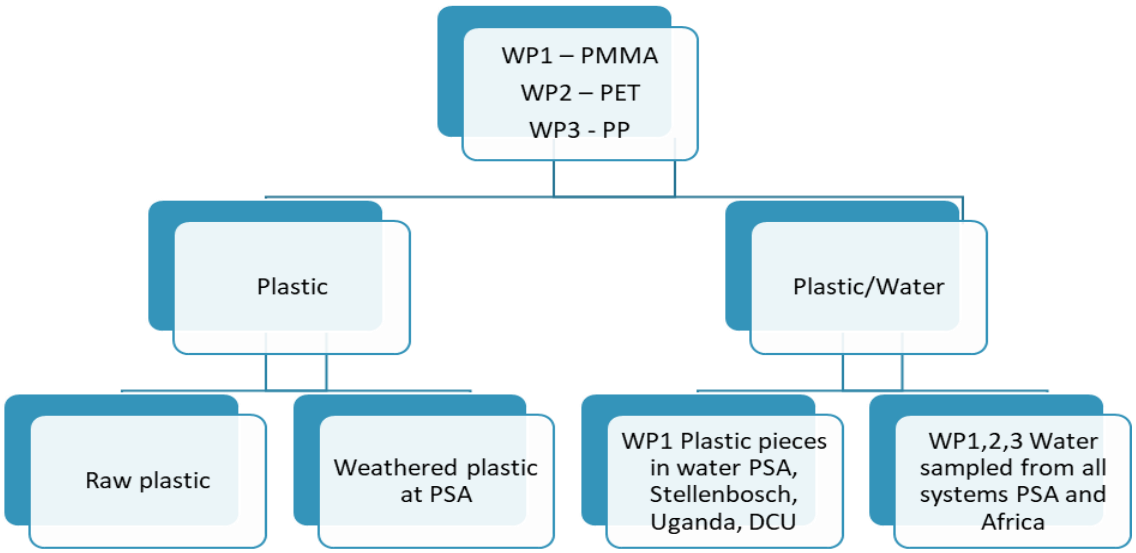


Figure 2.5 Schematic summary of the sampling plan for the plastics used in the novel reactors developed in work packages (WP) 1-3 in the Waterspoutt Project.

2.2.2. Testing raw and aged plastic for extractables

The raw plastics and weathered plastics were tested for extractables. Pieces of PMMA, PET and PP were weathered by exposure to sunshine at PSA for 3-, 6- and 9-month periods (Fig 2.7).

The plastic pieces to be extracted were cut into 4 mm square pieces, sprayed with IMS in a class II downflow re-circulating biological safety cabinet (Haier BioMedical model HR40-IIB2) and sterilised with UV light for 30 minutes.

The plastics were then transferred to clean Bijou Glass Vials with a polypropylene cap (Fisher scientific) and extracts were prepared according to the method of Yang *et al.* (Yang *et al.*, 2011). A schematic summary of the process is presented in **Fig 2.6**

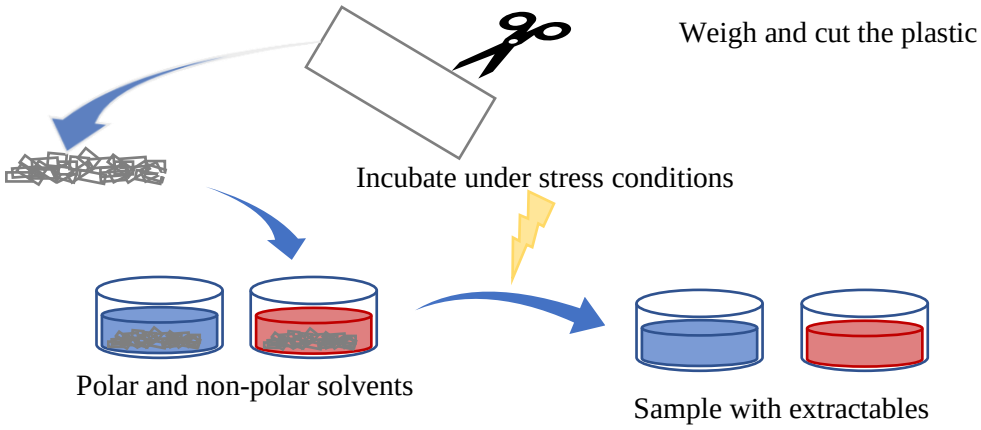


Figure 2.6 An overview of procedure used to obtain extractable from plastics

Three solvents were used;

Experimental culture medium DMEM/F12 and ethanol were used to extract the plastics for the E-screen assay

PBS and ethanol were used to extract the plastics for the Ames test.

All the solvents were used in a ratio of 4 mL of solvent per 1 gram of plastic.

The extractions were carried out using a range of times and temperatures of incubation: for 24 hours and 72 hours, 37 °C for 5 hours in one day, 5 hours each day for 3 consecutive days,

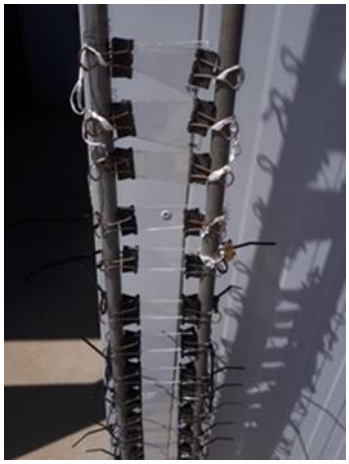


Figure 2.7 Pieces of plastic exposed to the sun at PSA

24 hours and 72 hours and 50 °C for 5 hours in one day and 5 hours each day for 3 consecutive days.

The stress conditions used are described in Table 2.1.

Following incubation, the entire volume was removed from each vial and transferred to a sterile glass vial and stored at -20 °C for analysis.

For the toxicological assessments of the estrogenic disrupting activity (Section 2.2.7), the extractables in culture medium DMEM/12 were diluted 100-fold and 10-fold in DMEM/F12 medium. For the toxicological assessments of the estrogenic disrupting activity (Section 2.2.7), the extractables in ethanol were diluted 100-fold in DMEM/F12 medium.

During the performance of the Ames test (Section 2.2.8) for toxicological assessments of mutagenicity, the extractables in ethanol and PBS were diluted 25-fold with the exposure medium.

Table 2.1 Stress conditions for extraction of the plastics

	Temperatures of extraction							
	Room temperature≈ 20°C		**37°C**				**50°C**	
Periods of extraction	24h	72h	5h/day for 1 day (5h out of a total of 24h)	5h/day for 3 days (15h out of a total of 72h)	24h	72h	5h/day for 1 day (5h out of a total of 24h)	5h/day for 3 days (15h out of a total of 72h)

2.2.3. Testing plastic pieces and reactors for leachates

Water samples from the PMMA, PET and PP reactors were tested for leachates. Pieces of PMMA submerged in water were also tested.

Figure 2.8 PMMA plastic pieces submerged in water

Pieces of PMMA in water

While waiting for the PMMA reactors to be manufactured, pieces of PMMA were immersed in water and exposed to the sun to test for leachates at

PSA, Stellenbosch, Uganda and at DCU. 5 cm cylinder pieces of PMMA (each approx. 57 g) were prepared by EES and sent to the relevant partners. In the case of PSA, the cylinders were further cut into pieces and put into a glass bottle (30 pieces of 2x5 cm) (Fig. 2.8). In all other cases, 2 cylinders were immersed in sterile water in a covered glass vessel. DCU used distilled water, Almeria used distilled water and local well water. Uganda and Stellenbosch used local rainwater. The vessels were set up in triplicate and exposed to local sunlight for up to 9 months. Similar treatments were set up in the dark as controls. The treated water was extracted using the solid phase extraction method (Section 2.2.6) and tested for toxicity (Sections 2.2.7 and 2.2.8).

Figure 2.9 Harvested rainwater tank used to supply water to the PMMA reactors in Uganda

PMMA reactors

One of each prototype was set up in South Africa and in Uganda. In South Africa, the reactors were installed at Stellenbosch at locations in Bonfoi next to a local church building in the Skoolplaas farming community (GPS coordinates: 33°56'38.5"S 18°46'26.3"E) and in Enkanini informal settlement (GPS coordinates: 33°55'28.1"S 18°50'35.8"E). In Uganda, the reactors were set up at two rural schools in the Makondo District, South of Kampala at the Arise and Shine School and at Kabuyoga Primary School. Water samples taken from the reactors were extracted locally using the solid phase extraction method (Section 2.2.6) and tested for toxicity at DCU (Sections 2.2.7 and 2.2.8). Water from the harvested rainwater tanks (Fig. 2.9) feeding each system was also sampled as a control.

PET and PP reactors

2 L PET bottles and 25 L PET Jerrycans and 20 L PP buckets were filled with local well water. Vessels in triplicate were exposed to sunshine at PSA for 3, 6- and 9-month intervals. Control vessels were set up in the dark. The water was extracted at PSA and analysed for toxicity at DCU.

Figure 2.10 PET and PP reactors exposed to the sunlight at PSA

2.2.4. Measuring physicochemical parameters for water

The conductivity and salinity (chloride, nitrite, nitrate, phosphate, sulphate, sodium, ammonium (NH_4), calcium, potassium and magnesium) of water sampled at PSA was tested.

The conductivity of the samples was measured using a conductivity meter GLP31 CRISON.

The salinity or the ion concentration of the water was measured using a Metrohm ion chromatograph Model 850, that consisted of two 872 extension modules: one module for determination of anions and polycarboxylic acids through a gradient analysis in a column METROSEP A Supp 7-250 (250mm X 4.0mm ID); the second module for determination of cations and amines through an isocratic analysis in a column METROSEP C4-250/4.0 (250mm X 4.0 mm ID). The mean and SD of salinity measurements were calculated using Microsoft Excel 2019 and the different samples were compared with a t-test using Microsoft Excel 2019.

2.2.5. Advanced Oxidation Processes

An *in vitro* experiment was set up to test the toxicity of water subjected to three solar treatments – SODIS, photo-Fenton and persulfate activation using three plastics - polyethylene terephthalate (PET) from a transparent jerrycan (PETjc), PET from a coca-cola bottle (PETcc) and blue polycarbonate (PC) from a container used in a water dispenser.

Procedure

Pieces of plastic were disinfected with 70 % IMS and cut into small pieces measuring 4x4 mm^2.

Plastic pieces, PETjc (8.3 g), PETcc (15 g) and PC (14.5 g), were submerged in 1 L of milliQ water in a glass borosilicate vessel. The quantities of plastic used corresponded to the internal surface-to-volume ratio for each original container.

Solar irradiation was simulated by a Hanau Suntest (AM1) lamp having a wavelength spectral distribution with about 0.5 % of emitted photons <300 nm (UV-C range) and about 7% between 300 and 400 nm (UV-B, A range). The emission spectrum between 400 and 800 nm follows the solar spectrum. Light intensity in all experiments was 1000 W m^{-2} and it was monitored with a Kipp and Zonen (CM3) power meter (Omni instruments Ltd, Dundee, UK).

The irradiation experiments were performed at room temperature (25 °C) and the temperature of the solution increased up to approximately 30 °C during irradiation. All experiments were carried out in equilibrium with air agitation at 700 rpm.

Three different treatments were used. Solar disinfection (SODIS) with no additives, photo-Fenton using 10 ppm (290 μM) H_2O_2 (Riedel-de Haën (Germany)) and 1 ppm (18 μM) Fe^{2+} and a persulfate activation process using 24.3 ppm (90 μM) peroxydisulfate (PDS) and 1 ppm (18 μM) Fe^{2+} (($FeSO_4 \cdot 7H_2O$) ≥ 99%, peroxy-monosulfate triple salt (PMS, Oxone TM)).

Two time periods of irradiation were investigated: 6 consecutive hours for 1 day and 6 consecutive hours a day for 7 consecutive days (1 week). Corresponding control vessels were incubated in the dark. Following incubation, water samples were extracted using solid phase extraction and analysed for estrogenic activity using the E-screen method and for mutagenicity using the Ames test (Sections 2.2.7 and 2.2.8 respectively).

2.2.6. Solid phase extraction of water

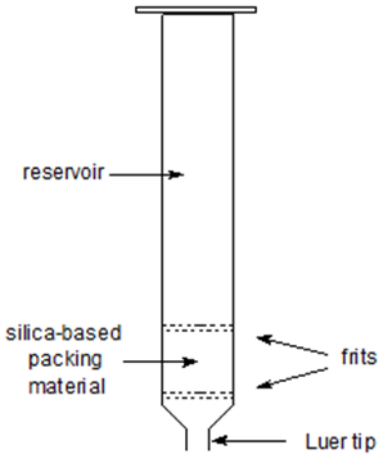


Figure 2.11 Cartridge scheme

Water samples were extracted using a modification of the solid phase extraction method described by Wagner and Oehlmann (2011).

Oasis HLB Glass Cartridges (5 cc / 200 mg LP by Waters Chromatography Ireland Ltd) were conditioned by pouring 4 ml acetone into the cartridge. The acetone was collected, discarded and the step repeated once.

The cartridge was then equilibrated by pouring 4 ml water into the cartridge. The water was collected, discarded and the procedure was repeated once.

The cartridges were set up on a Waters 20 position extraction manifold (Waters Corporation, Milford, MA, USA) connected to a Waters vacuum pump (220 V, 50 Hz).

The water sample (1 L) was loaded onto the cartridge and extracted under vacuum using a flow rate of 12 ml/min.

The effluent was collected and discarded. The dry cartridge was eluted with 4 ml HPLC grade methanol which was collected in a clean Bijou Glass Vial with Polypropylene Cap (Fisher scientific) 100 μl DMSO was added to the vial.

The methanol was removed under a gentle stream of nitrogen yielding final extracts in 100 μL DMSO.

The glass vial with PP cap was stored at -20 °C until analysed.

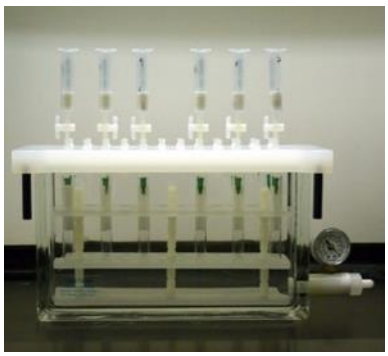


Figure 2.12 SPE manifold

2.2.7. The E-screen procedure

2.2.7.1. Preparation of estrogen receptor agonists and antagonist.

17β estradiol (E2)

17 β-estradiol (molecular weight 272.38) was supplied by Merck, Sigma-Aldrich as a 250 mg powdered aliquot. DMSO (1 mL) was pipetted into the borosilicate glass tube containing the β estradiol powder until it completely dissolved giving a concentration of 0.9 M. One mL was transferred to a 100 mL glass sterile bottle containing 89 mL of DMSO yielding a concentration of $1x10^{-2}$ M. Serial dilutions were subsequently carried out in experimental DMEM/F12 media (0.01 ρM – 1 μM).

Bisphenol A (BPA)

Bisphenol A (molecular weight 228) was supplied by Merck, Sigma-Aldrich. 228 mg of BPA were weighed out and transferred to a sterile 100 mL glass bottle containing 100 mL of DMSO yielding a concentration of 10^{-2} M. The bottle was agitated until the BPA was completely dissolved. Serial dilutions were subsequently carried out in experimental DMEM/F12 media (0.01 ρM – 1 μM).

Fulvestrant (ICI)

Fulvestrant (molecular weight 606) was supplied by Merck, Sigma-Aldrich as a 25 mg powdered aliquot. DMSO (1 mL) was pipetted into the borosilicate glass tube containing the ICI powder yielding a concentration of 0.0412 M. The powder was allowed to dissolve and was gently resuspended using a pipette. This stock was then transferred to a 15 ml glass sterile tube containing 3 mL of DMSO, generating a 0.01 M solution. 1 mL of this stock was then added to 9 mL of DMSO yielding a concentration of $1x10^{-3}$ M solution. Serial dilutions were subsequently carried out in experimental DMEM/F12 media (0.01 ρM – 1 μM).

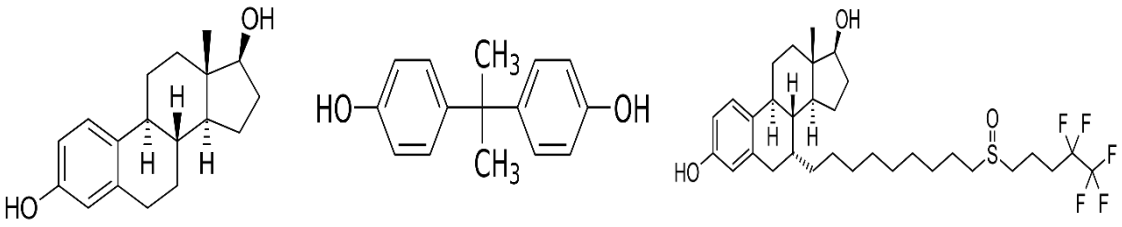


Figure 2.13 Structure of some naturally occurring and some synthetic compounds that have an effect on the metabolism of estrogen. From left to right. 17 Beta-estradiol, Bisphenol A (BPA) and Fulvestrant (ICI).

2.2.7.2. Preparation of MCF-7 cell culture media

MCF-7 Cell maintenance medium

Dulbecco's minimum essential medium (DMEM) was supplied by Merck, Sigma-Aldrich as 500 mL bottle. It was routinely aliquoted and stored in 100 mL glass sterile bottles at 4 ºC up to the expiry day as specified on the bottle. It was supplemented with 5% (v/v) Foetal Bovine Serum (FBS) by the addition of 5 mL serum to 95 mL DMEM.

MCF-7 Cell culture Medium "experimental medium"

DMEM/F12 phenol red-free medium was supplied by Merck, Sigma-Aldrich as 500 ml bottle. It was supplemented with 2 mM L-glutamine by addition of 5 mL of 200 mM L-Glutamine to 495 mL of DMEM/F12 phenol red-free medium. Then the medium was aliquoted and stored in 100 mL glass sterile bottles at 4 ºC.

Aliquots of DMEM/F12 phenol red-free medium were supplemented with 2.5 mL (2.5 % v/v) charcoal-stripped foetal calf serum (CSFCS) and 2.5 mL (2.5% v/v) new born calf serum for cell treatment experiments.

All media were stored for up to 4-6 weeks at 4 ºC after which time fresh culture media were prepared.

All cell work was carried out in a validated class II down-flow re-circulating biological safety cabinet (Haier) and strict aseptic technique was adhered to at all times. This involved swabbing the biological safety cabinet with 70 % industrial methylated spirits (IMS) and VIRKON (1 % w/v) before and after use as well as all items prior to their use in the cabinet.

2.2.7.3. Cell lines

The cell line used during the course of this project was MCF-7 cell line that was originally derived from a pleural effusion of a patient with metastatic mammary carcinoma. Two different stocks of this cell line were used, one of them was purchased from the European Collection of Authenticated Cell Cultures (ECACC #86012803), the other one MCF-7 BUS was generously gifted by the group C. Sonnenschein and A. Soto (Tufts University, Boston), who cloned the cells (MCF-7) from passage 173 of the original MCF-7 cells, received from C. McGrath of the Michigan Cancer Foundation; they were at post-cloning passages 115 at the time of our study.

MCF-7 Cell Maintenance

MCF-7 cells were maintained in a T75 flask in FBS-containing media prepared as previously outlined and the media was replaced every 2-3 days. When the cells reached 80 % confluency, they were sub-cultured and reseeded in a new T75 flask and in a T25 flask at a lower density.

Cell count

Cells were trypsinised and resuspended in DMEM. A 100 µl aliquot of cell suspension was mixed with an equal volume of 0.4 % (w/v) Trypan Blue. 20 µL of this solution was then transferred to a haemocytometer, enclosed by a coverslip and observed under a light microscope.

Viable cells appeared as small round and refractive, while non-viable cells absorbed the trypan blue and appeared swollen and dark blue. Cells were counted from four large corner squares and the centre square as observed under the 10X objective. The total number of cells counted was divided by 5 to give the average number of cells per square. This average was then multiplied by the dilution factor of 2 and by 10^4 (accounting for 10^{-4} ml volume underneath coverslip), yielding the total number of cells per mL.

Using the equation below the volume of suspension required to yield the required seeding density is determined:

$$Volume\ of\ suspension = \frac{required\ cells\ per\ mL}{\text{calculated cell concentration}}\ x\ final\ volume$$

Equation 2.1 Cell count from haemocytometer

Subculture of MCF-7 cells

The steps followed for subculture were as follows: media was removed and discarded; MCF-7 cells were washed with 6 mL of pre-warmed phosphate buffer saline (PBS). The PBS was decanted; the cells were incubated with 4 mL of pre-warmed Trypsin at 37 ºC in 5 % CO_2 for 3-5 minutes. Once the cells were fully detached from the base of the flask, they were transferred to a sterile universal centrifuge tube containing 6 mL of PBS; subsequently, the cells were centrifuged at 1000 rpm in Heraeus centrifuge for 5 minutes. The supernatant was discarded and the pellet was resuspended and reseeded at 1:4 (v/v) dilution. Only cells at passage number <30 were utilised.

Preparation of MCF-7 stocks for freezing

Stocks of the MCF-7 cell lines were prepared using confluent cells grown in fresh medium. The cells were harvested using trypsin as specified above, suspended in 5 ml of DMEM medium and centrifuged in a centrifuge (Heraeus) for 4 minutes at 1000 rcf. After centrifugation, the supernatant was discarded and the pellet was resuspended in 2 mL of cell freezing medium. The cells were transferred to cryovials in 1 mL aliquots and then frozen by placing the cryovials in -20 ºC freezer for 2 h followed by -80 ºC storage for up to three months and transfer to liquid nitrogen for long term storage.

Thawing of cells

Immediately prior to the removal of a cryovial from the liquid nitrogen for thawing, a sterile T75 flask containing pre-warmed growth medium was prepared for the rapid transfer and dilution of the thawed cells to reduce their exposure to DMSO freezing solution which is toxic at room temperature. The cells were allowed to attach overnight. After 24 h, the cells were fed with fresh medium to remove any residual traces of DMSO.

2.2.7.4. Description of the procedure of E-screen assay

The E-screening assay was carried out in accordance with the method described by Soto *et al.* (1995) with minor modifications.

Sub-confluent MCF-7 cells were grown in a T75 flask in phenol red DMEM medium (described in section 2.2.7.2 of these chapter) supplemented with 5 % (v/v) FBS. Once the cells reached 80% confluency, they were trypsinised, washed with PBS and resuspended in phenol red DMEM medium until a concentration of $4x10^4$ cells / mL. Aliquots of 100 µL of the cell stock were subsequently seeded into 96 well plates at 4000 cells / well.

The cells were allowed to attach for 24 hr after which they were washed with 100 µL of PBS and cultured in 100 µL of hormone-free DMEM/F12 medium (described in section 2.2.7.2 of this chapter) containing controls (medium alone (blank), 1 % v/v DMSO in medium (negative), 1 nM E2 (positive), sample extracts and sample extracts with ICI (0.1 µM). All treatments were in triplicate. Each plate was set up in triplicate.

Six days later the assay was terminated during the late exponential phase of proliferation and cell number per well was determined. Instead of counting the cells directly, cell number was assessed

by measuring acid phosphatase activity (AP assay), NAD(P)H oxidoreductase enzyme activity (MTS assay) or by quantification of DNA (Hoechst assay).

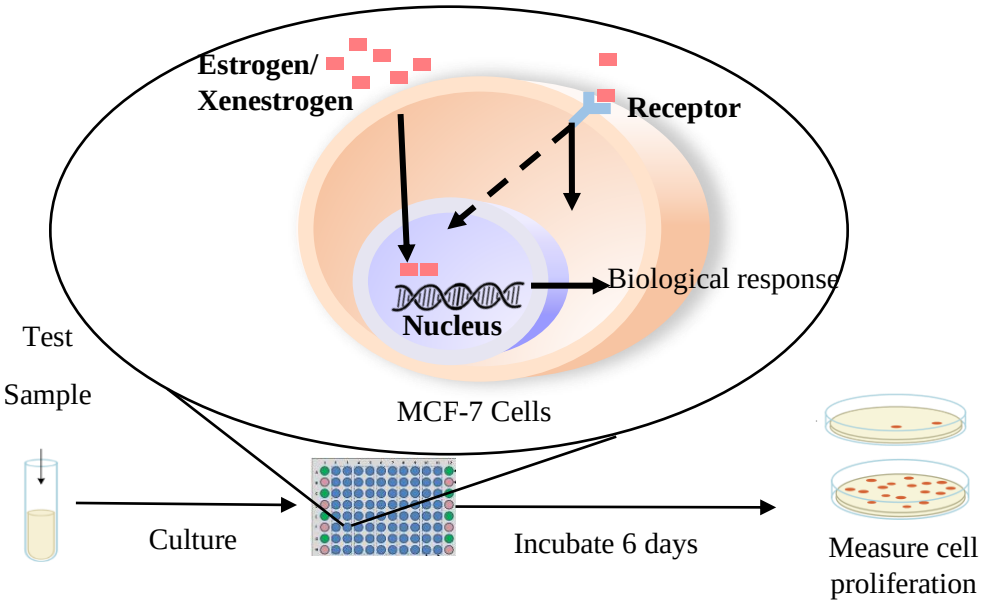


Figure 2.14 Schematic representation of the E-screen assay

Proliferative Assays

- Acid phosphate assay (AP assay)

The acid phosphatase (AP) assay is based on the ability of the AP enzyme, located in the lysosomes of cells, to hydrolyse the *p*-nitrophenyl phosphate (*p*NPP) yielding *p*-nitrophenyl chromophore. All the assay reagents were prepared in advance. PBS and 1 M NaOH solution were both stored at room temperature. Sodium acetate buffer 0.1% TritonX-100 pH 5.2 was provided by Merck, Sigma-Aldrich at a 3 M concentration stock, from which a dilution was made to yield a 0.1 M stock solution. This solution was subsequently stored at 4 ºC in the dark for up to 1 month. The *p*- nitrophenyl phosphate (pNPP) substrate was added to the sodium acetate buffer prior to performing the assay yielding a 10 mM solution. After six days of incubation, the medium with the extract was removed from the 96 well plate by flipping it over tissue paper previously impregnated with VIRKON. Each well was then rinsed with 50 µL of PBS. Subsequently, 100 µL of the freshly prepared pNPP substrate in sodium acetate buffer was added to each well; plates were incubated at 37 ºC for 2 h. The reaction was terminated by the addition of 50 µL of 1M NaOH to each well. This induced an electrophilic shift in the *p*-nitrophenyl chromophore yielding a yellow colour. After 10-15 minutes the plate was read at 405 nm on a Tecan infinite 200 PRO NanoQuant microplate reader.

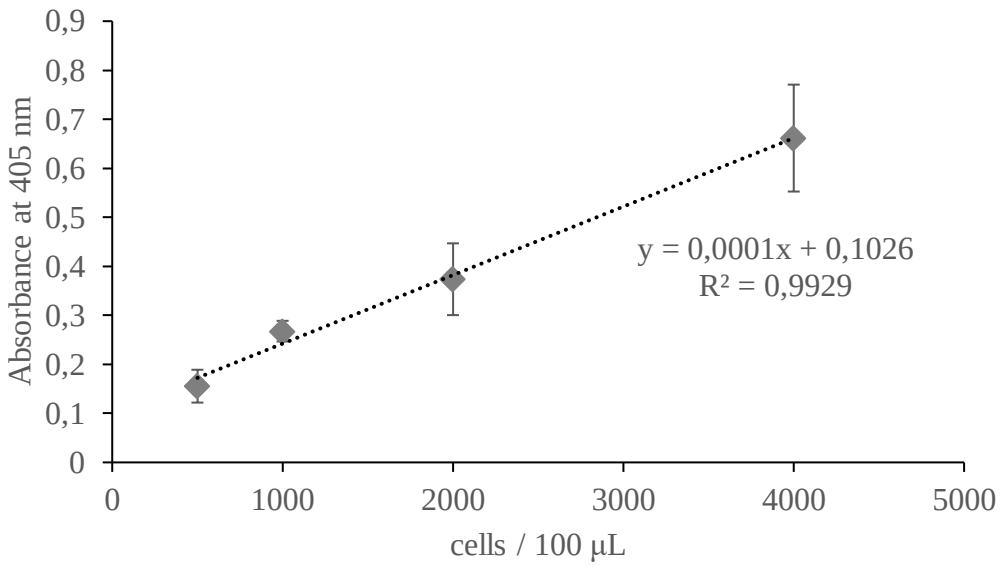


Figure 2.15 Standard curve for the validation of the AP assay used to measure proliferation of MCF-7 cells. Absorbance of p-nitrophenolate product of acid phosphatase activity of cells seeded between 0-4000 cells/well in a 96-well plate was measured at 405 nn by Infinite 200 PRO NanoQuant following 6 days of incubation at 37 °C, 5% CO_2. The graph represents the mean and standard deviation of octuplicates in one experiment

- MTS proliferation assay

The MTS proliferation assay is a sensitive colorimetric assay for quantification of viable cells in proliferation and cytotoxicity assays. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a coloured formazan product that is soluble in cell culture media. This conversion is carried out by NAD(P)H-dependent succinate dehydrogenase enzymes in metabolically active cells.

The CellTiter 96® Aqueous One Solution Cell Proliferation Kit Assay was purchased from Promega; The CellTiter 96® AQueous One Solution Reagent was stored at -20 ºC in dark and thawed at room temperature for at least 90 minutes prior to performing the assay. After six days of incubation 20 µL of the reagent was pipetted into each well of the 96-well plate containing the cells with samples in 100 µl of culture medium. After 1 h of incubation at 37 ºC in a humidified 5% CO_2 incubator absorbance was read at 490 nm on a Tecan infinite 200 PRO NanoQuant microplate reader.

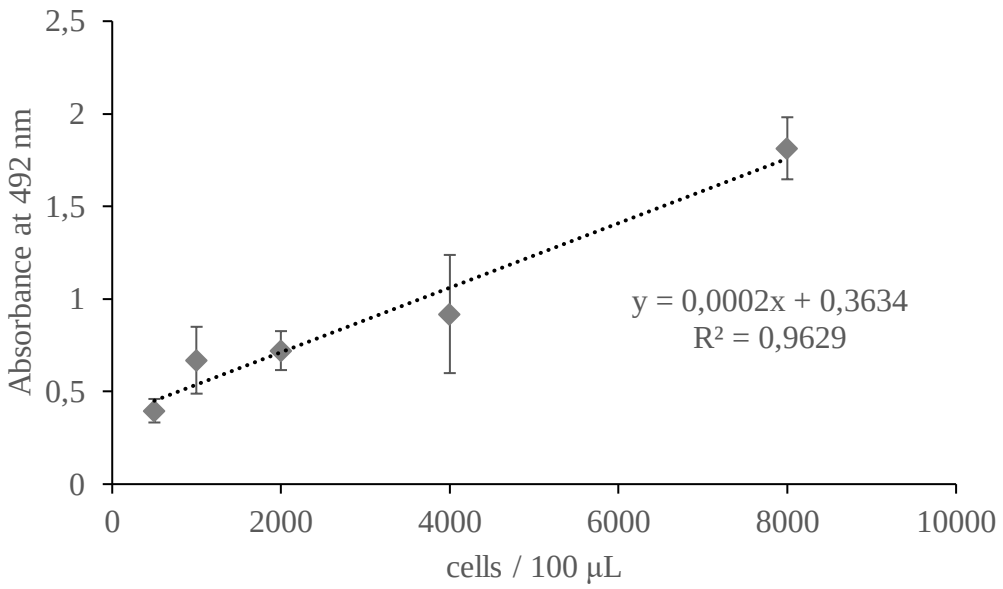


Figure 2.16 Standard curve for validation of the MTS assay to measure proliferation of MCF-7 cells. Absorbance of product of succinate dehydrogenase activity of cells seeded between 0-8000 cells / well in a 96-well plate was measured at 492 nm by Infinite 200 PRO NanoQuant following 6 days of incubation at 37 °C, 5% CO_2. The graph represents the mean and standard deviation of octuplicates in one experiment.

- DNA quantification

The bisbenzimidazole derivative Hoechst 33258 exhibits fluorescence enhancement upon binding to A-T rich regions of double-stranded DNA. DNA binding specificity is enhanced under high ionic strength conditions. Assay buffer (2 M NaCl, 50 mM NaH_2PO_4, pH 7.4) was prepared in advance and stored at 4ºC up to three months. Bisbenzimide H 33258 powder was provided by Merck, Sigma-Aldrich in 100 mg aliquot that was resuspended in 100 mL of distilled water to generate a Hoechst dye stock of 1 mg/ml which was stored at 4 °C in a light-tight container. Working assay solution was prepared fresh prior to each assay by mixing 2 µL of concentrated dye stock solution for every 1 ml of assay buffer required. After six days of incubation, the wells were emptied by overturning onto paper towels and subsequently stored at –80 °C until ready to scan. The plates were thawed at room temperature and 100 µL of distilled water was added and incubated for 1 h at 37 ºC in the incubator. Then the plates were frozen again at -80 ºC for 1 h and thawed at room temperature to promote the lysis of the cells and the exposure of the DNA content by thermic shock. 100 µL of aqueous Hoechst 33258 in buffer were added to each plate (final Hoechst 33258 concentration of 1 µg/mL per well) and the fluorescence was measured using excitation and emission filters at 360 nm and 460 nm, respectively.

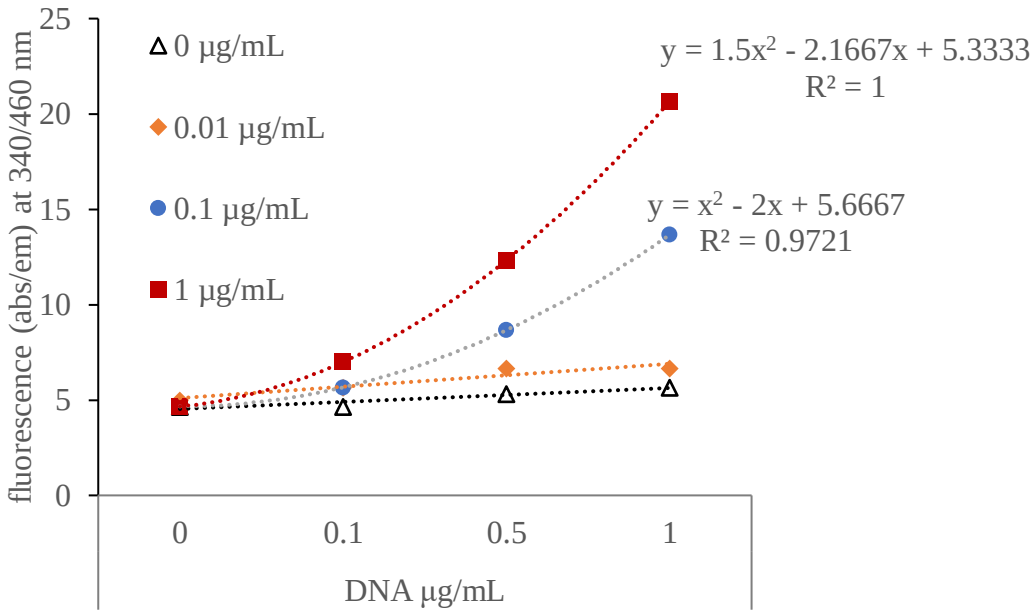


Figure 2.17 Standard curve for validation of the Hoechst dye (0-1 ug/ml) assay to measure DNA Fluorescence emission of 0-1 µg/mL of DNA suspension treated with different concentrations of Hoechst (0-1 ug/ml)was measured at λ(ex/em) 340/460 nm using a Synergy HT fluorescence plate reader. The graph represents the mean and standard deviation of octuplicates in one experiment.

2.2.7.5. E-screen assay endpoint

The end-point of the E-screen assay is proliferation relative to hormone-free control proliferation. The proliferative effect (PE) is the percentage proliferation of the sample normalized to the solvent negative control (100%).

$$PE = \frac{\text{proliferation value of the tested sample}}{proliferation\ value\ of\ the\ negative\ control} \times 100$$

Equation 2.2 Proliferative effect

If MCF-7 proliferation by standards or extracted sample is suppressed by co-incubation with ICI (10^{-7} M), the estrogenic activity of the standard or extract is confirmed. Otherwise, the estrogenic activity (EA) of the standard or extract was not confirmed and MCF-7 cell proliferation is considered not mediated via an ER mechanism or via the genomic pathway.

If the sample was shown to be estrogenic, its activity relative to 17β-estradiol (E2) was calculated.

Relative proliferative effect (RPE) (ie proliferative effect relative to E2) was calculated as follows:

$$RPE = \frac{(\text{proliferation value of tested sample} - \text{proliferation value of negative control})}{(\text{proliferation value of E2} - \text{proliferation value of negative control})} \times 100$$

Equation 2.3 Relative proliferative effect index % E2

Sometimes negative values could be observed when the sample triggered lower proliferative response than the negative control, in those cases, it was considered that the sample did not have estrogenic activity. In some other cases, RPE values could be over 100% when the sample triggered proliferative response higher than the positive control.

Total agonists of E2 were defined by Kuch et al. (Kuch *et al.*, 2010) as samples having RPE between 80 % and 100 % relative proliferation. Partial agonists were defined as samples having RPE between 25 % and 80 % relative proliferation while weak agonists were defined as samples having RPE between 10 % and 25 % respectively. Non-estrogenic substances have RPE values between 0-10 %.

2.2.7.6. Estradiol equivalence

Estradiol equivalents (EEQs) indicate the amount or concentration of estradiol needed to trigger a similar proliferative response in the E-screen assay to the response triggered by the sample.

Estradiol equivalents (EEQ) were calculated by interpolation from a dose-response curve of 17 β estradiol in the range 0.01 pM to 1 nM E2. For experimental samples, EEQs were corrected for the final concentration/dilution factor and reported as ng/L of the original sample.

Since E2 doesn't generate a liner or monotonic dose-response curve, concentrations values from 0.01 pM to 1 nM were fitted to a power trending line with the following equation:

$$y = 8500.6x^{0.2096}$$

Equation 2.4 Equation for the interpolation of values from the dose-response curve of 17 β estradiol

From that equation nonlinear interpolations of the positive RPE values of each sample were determined by calculating the x value:

$$\text{EEQ [M]} = \left(\frac{\text{tested sample RPE}}{8500.6}\right)^{\frac{1}{0.2096}}$$

Equation 2.5 Nonlinear interpolation of Estradiol Equivalents in [M]

EEQs were corrected for the final concentration/dilution factor and reported as ng/L of the original water sample.

The EEQ values obtained were compared with the Acceptable Daily Intake (ADI) of estradiol of 0-50 ng/kg of body weight per day recommended by the WHO ("Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2000)"), by adjusting to the average water consumption and average body weight of each age group.

2.2.7.7. Statistical analysis

Graphs were plotted as the mean ± SD of the Proliferative Effect (PE) using the Excel Microsoft office software (Microsoft professional plus 2019). Student's t-test was used to compare the proliferative effects of MCF-7cells co-incubated with the antagonist ICI against cells without ICI to assess the antagonism effect. Statistical data analysis was performed using IBM SPSS statistics 25, all the data points that fell more than 1.5 times the interquartile range above the third quartile or below the first quartile (1.5IQR) were considered outliers and removed. Analysis of variance (single factor ANOVA) was used to compare the effects of the different treatments on the MCF-7 proliferative effect; followed by a Tukey and Game-Howell Post Hoc analysis. All differences were considered statistically significant when $p<0.05$.

2.2.8. The Ames Test

2.2.8.1. Components

The Ames test was carried out using the Ames II kit by Xenometrix AG (Allschwil, Switzerland). The kit components and storage conditions for each component are summarised in Table 2.2

Table 2.2 The components and storage conditions for the Ames II kit by Xenometrix AG

Component	Storage Temperature
Vials containing *Salmonella* strains (TA98 and TAMix)	-70 °C to -80 °C
Vial(s) containing; - Ampicillin (50 mg/ml) -Lyophilized liver S9 fraction -Co-factors of the S9 30% mix: • NADP 0.04M • G-6-P 0.2M	-20 °C:
- Positive controls before reconstitution: • 20 µg of 2-nitrofluorene (2-NF) • 50 µg of 4-nitroquinoline N-oxide (4-NQO) • 100 µg of 2-aminoanthracene (2-AA) - Buffer-Salts for the S9 30% mix	4 °C:
Growth Medium Exposure Medium Indicator Medium	20 – 25 °C (room temperature) (protected from light)

Salmonella typhimurium strains

The kit contains *Salmonella typhimurium* strains TA98 and TAMix.

TA98 is used for the detection of frameshift mutations.

TAMix contains a mixture of equal proportions of the Ames II TA7001-TA7006 strains. Individually, these strains are designed to revert by only one specific base-pair substitution out of six possible changes. Thus, when mixed, all six base substitution mutations can be represented in one culture.

The genotypes of the strains are summarised in Table 2.3

Table 2.3 Genotypes of the TA98 and TAMix Salmonella typhimurium strains

Strain	Mutation	Type	Target	Cell Wall	Repair	pKM101
TA98	hisD3052	Frameshifts	GCGCGCGC	rfa	uvrB	yes
TAMix contains						
TA7001	hisG1775	Base-pair subst	A:T>G:C	rfa	uvrB	yes
TA7002	hisC9138	Base-pair subst	T:A>A:T	rfa	uvrB	yes
TA7003	hisG9074	Base-pair subst	T:A>G:C	rfa	uvrB	yes
TA7004	hisG9133	Base-pair subst	G:C>A:T	rfa	uvrB	yes
TA7005	hisG9130	Base-pair subst	C:G>A:T	rfa	uvrB	yes
TA7006	hisC9070	Base-pair subst	C:G>G:C	rfa	uvrB	yes

- rfa: This mutation leads to a defective lipopolysaccharide (LPS) layer that coats the cell surface, making the bacteria more permeable to bulky chemicals and non-pathogenic (Mortelsmans and Zeiger, 2000).

uvrB: The uvrB deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by error-prone DNA repair mechanisms. The deletion through the biotin gene makes the bacteria biotin dependent.

pKM101: This R factor plasmid enhances chemical and UV-induced mutagenesis via an error-prone recombinational DNA repair pathway. The plasmid also confers ampicillin resistance.

2.2.8.2. Overview of the method

Freshly prepared overnight cultures of TA98 and TAMix are exposed to the test sample as well as to a positive and negative control for 90 min in exposure medium containing sufficient histidine to support a few cell divisions. After 90 min exposure, cultures are diluted in pH indicator medium lacking histidine and aliquoted into 48 wells of a 384-well plate. Within 2 days, cells that have undergone the reversion to histidine prototrophy—either spontaneously, or as a result of the exposure to a mutagen—will grow into colonies. Bacterial metabolism reduces the pH of the medium, changing the colour of that well from purple to yellow. The number of wells containing revertant colonies is counted for each dose and compared to a solvent (negative) control. Each dose is tested in triplicate to allow for statistical analysis of the data. An increase in the number of revertant wells relative to the solvent controls indicates that the chemical is mutagenic in the Ames II assay. The mutagenic potential of substances is assessed directly and in the presence of liver S9. An overview of the method is described in Fig. 2.18.

2.2.8.3. Procedure

Preparation of cultures

Overnight cultures of TA98 and TAMix were prepared. 10 µL of freshly thawed and carefully mixed bacterial stocks were added to 50 mL culture tubes containing 10 mL growth medium and 10 µL of 50 mg/mL ampicillin. The culture tubes were capped loosely to allow for sufficient aeration. The cultures were grown for 12–15 h at 37 °C, 250 rpm in an environmental shaker to the late exponential phase to achieve OD_{600} ≥2

Preparation of controls

The positive controls were prepared in a 25X stock in DMSO. The negative control was DMSO. The final assay concentrations for the positive controls are described in Table 2.4

Table 2.4 Final concentrations of positive controls used in the Ames test

Strain	*+S9*	*-S9*
TA98	*2-aminoanthracene (5ug/ml)*	*2-Nitroflourine (2ug/ml)*
TAMix	*2-aminoanthracene(5ug/ml)*	*4-Nitroquinalone N-oxide (0.5ug/ml)*

Preparation of 30% S9- Mix

A 30% S9- Mix was prepared by combining lyophilised S9 liver fraction reconstituted in distilled water, S9- Buffer-Salts, NADP and G-6-P as described in Table 2.5

Table 2.5 Composition of 30% S9- Mix

Solution	Percentage
S9- Buffer-Salts*	57.5%
S9- G-6-P	2.5%
S9-NADP	10%
S9 liver fraction**	30%

*A ready to use phosphate buffer pH 7.4 was provided by Xenometrix and consisted of equal amounts of $MgCl_2 \cdot 6H_2O$ (0.25M) and KCl (1M).

Preparation of 24-well exposure plate

An exposure plate was set up for each strain (TA98 and TAMix) with and without S9 – 4 plates.

10ul of test sample/positive control/negative control was plated in triplicate in the labelled 24 well plate. An overnight culture was diluted 1/10 in exposure medium with or without S9 and 240 ul aliquots were added to each well of the relevant plate. The final concentration of S9 in the culture was 4.5%. The plates were incubated at 37 °C, 250rpm in an environmental shaker for 90 minutes. Following incubation, 2.6 – 2.8 ml indicator medium was added to each well.

Setting up 384-well plates

Three 384 well plates were set up for each 24 well plate by transferring 50ul aliquots from the 24 well plate to appropriately labelled 384 well plates. The 384 well plates were put in a sealable plastic incubation bag and incubated at 37 ^{o}C for 2 days. Following incubation, the number of positive wells (revertants), those that turned yellow or had visible growth, were recorded.

2.2.8.4. Calculation of response

The number of revertants was determined by calculating the mean value for triplicate samples. Data analysis was performed using Excel office 2019 software.

The results were expressed as **Fold induction over the baseline**

The fold induction over the baseline is the ratio of the mean number of positive wells (revertants) for each sample divided by the baseline value.

The Baseline value = Mean number of positive wells(revertants) for the negative control plus the standard deviation.

e.g. Mean +/- SD of negative control = 1.2 +/- 0.7

Baseline (1.2 + 0.7) = 1.9

A sample that shows a clear fold induction ≥2 above the baseline is classified as a mutagen.

Solvent negative controls values for TA98 and TAmix should be ≤8 positive wells (mean of replicas). While positive control values for TA98 and TAmix should be ≥20 positive wells (mean of replicas or ≥3-fold of the baseline.

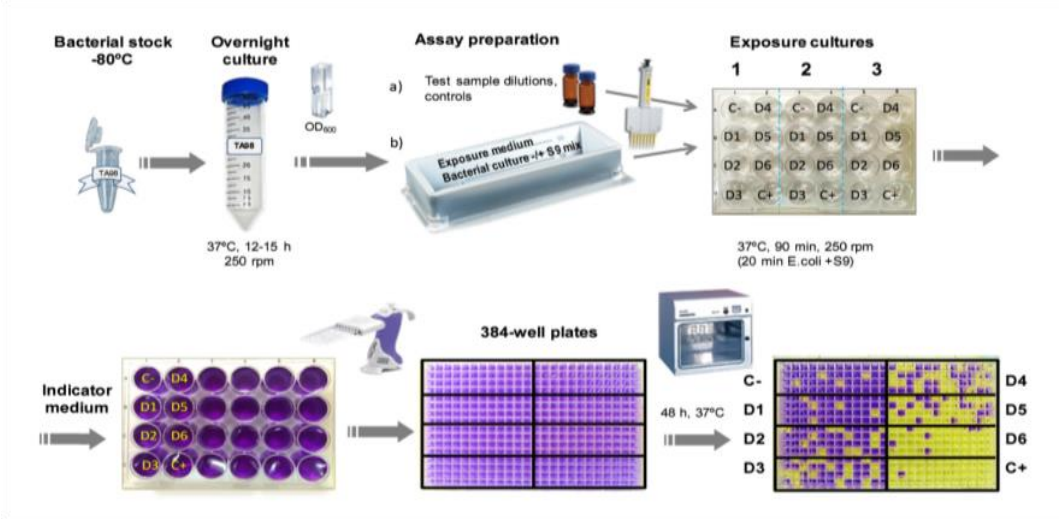


Figure 2.18 Schematic representation of the Ames II test kit procedure

Chapter III

3. Validation and Optimization of the E-screen assay

The E-screen assay was used to assess the estrogenic activity of extractables and leachates from the three novel SODIS reactors developed for the *Waterspoutt* project. The E-screen assay, based on the ability of human breast cancer cells (MCF-7) to increase their proliferation when cultured in an estrogenic environment, was carried out in accordance with the method described by Soto *et al.* (1995) with minor modifications. The E-screen bioassay and a solid phase extraction (SPE) protocol for preparation of water extracts were validated prior to testing.

3.1. E-screen validation

An overview of the E-screen assay is described in Fig 2.14 in Chapter 2. Samples were cultured in the presence of MCF-7 cells for a period of 6 days. Cell proliferation was used as a measure of estrogenic activity. The E-screen bioassay was validated and optimized to ensure it is capable of measuring cell proliferation, the hallmark of estrogenic activity testing, before being used to assess the estrogenic activity of test samples. The assay was optimised in terms of the cell line used, choice of assay to measure cell proliferation, optimum cell seeding density, time of incubation and evaluation of anti-estrogenic properties of ICI.

3.1.1. Selection of cell line

The cell line used in this project was MCF-7 cell line that was originally derived from a pleural effusion of a patient with metastatic mammary carcinoma. Two different stocks of this cell line were investigated for use in the assay. One cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC #86012803) and the other one MCF-7 BUS was generously gifted by the group C. Sonnenschein and A. Soto (Tufts University, Boston), who cloned the cells (MCF-7) from passage 173 of the original MCF-7 cells, received from C. McGrath of the Michigan Cancer Foundation; they were at post-cloning passages 70-103 at the time of our study. Three methods for measurement of MCF-7 proliferation were investigated - two indirect methods, the acid phosphatase (AP) assay and the MTS method and a direct method for DNA quantification.

3.1.1.1. Selection of assay to measure cell proliferation of MCF-7 cells ECACC stock

MCF-7 ECACC cells were the first cell line investigated for use in the E-screen assay. To determine which of the three methods (acid phosphatase assay, MTS assay or Hoechst fluorescence) was optimal for measuring proliferation of estrogenic compounds, MCF-7 ECACC cells were seeded at 4000 cells/well and treated with E2 (10^{-9} M) and BPA (10^{-7} M). Proliferation

was assessed by calculating the proliferative effect of each standard relative to the negative control. The reasoning behind the selected concentrations of the standards was that 10^{-9} M represents a typical midcycle premenopausal serum concentration of estradiol in women (Teoh *et al.*, 1999) and because 10^{-7} M BPA represents the maximum concentration of BPA leached from aged epoxy resin-coated pipes into drinking water (Rajasärkkä *et al.*, 2016) and within the EC50 range established in the literature (Khetan, 2014). Parameters for assessing the performance of the E-screen were selected based on previous literature references that used the E-screen bioassay (Soto *et al.*, 1995).

E2 and BPA-induced proliferation of MCF-7 ECACC cells were higher using the MTS assay relative to the Hoechst fluorescence or AP assays (**Fig 3.1**). MTS assay yielded a proliferative effect of 152 ± 8% for E2 and 158 ± 10% for BPA. The DNA quantification with Hoechst dye assay yielded a proliferative effect of 136 ± 5.5% for E2 and 134 ± 7% for BPA, while the AP assay yielded a proliferative effect of around 113 % for both standards.

Since the proliferative effects of E2 and BPA measured with the AP assay were not significantly different from the proliferative effect of the negative control, the AP assay was considered not suitable to use with the E-screen as a tool to measure proliferation.

Moreover, neither BPA nor E2 were able to induce a 2-fold increase in the proliferative effect over the negative control, which is one of the requirements to validate the E-screen assay.

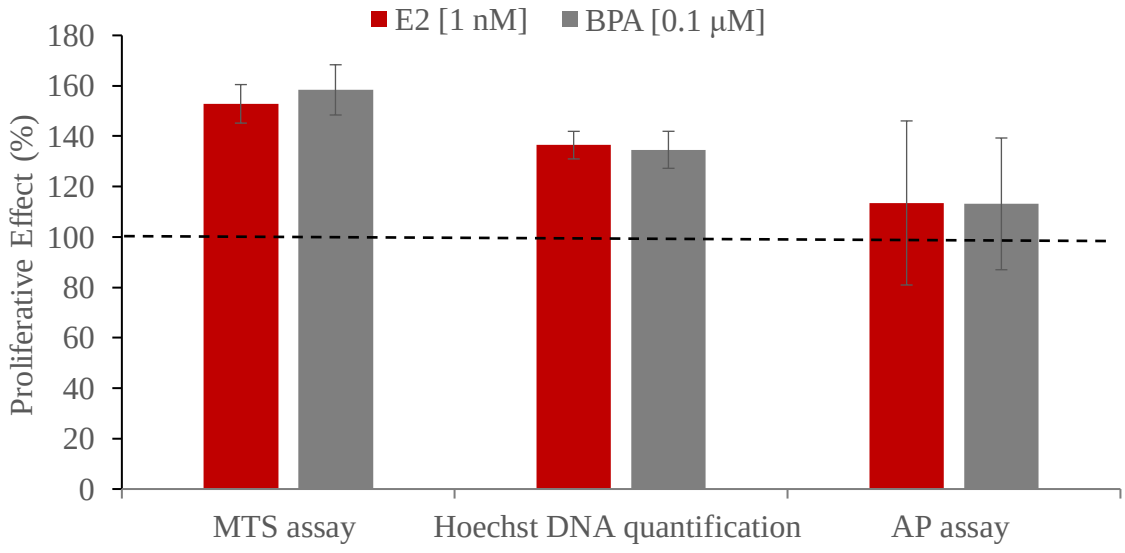


Figure 3.1 Comparison of the MTS assay, DNA quantification assay and AP assay to measure proliferation of MCF-7 ECACC cells during the E-screen. *The black dotted line represents proliferation by the negative control. The bars represent the mean of triplicates of three independent experiments (n=9).*

3.1.1.2. Selection of cell line stock and assay to measure proliferation

Since the proliferative effect measured with MCF-7 ECACC stock did not reach 200%, the cell line was deemed not sensitive enough for use in the assay. The proliferative responses of MCF-7 ECACC and MCF-7 BUS to E2 were compared by measuring proliferation with two different assays to determine if MCF-7 BUS was more sensitive to measure estrogenic activity and to test which of the methods (MTS assay or Hoechst fluorescence) was optimal for measuring proliferation.

E2 [1 nM] exerted a proliferative effect < 200% in MCF-7 (ECACC) cells (**Fig 3.2**). Treatment of cells with 1 nM E2 increased proliferation by 152 ± 15% and 136 ± 5.5% as measured with the MTS and DNA quantification assays respectively. No significant difference was observed between the assays. Since a proliferative effect of 200 % was not attained, the cells were deemed not sensitive enough to assess estrogenic activity of samples during the E-screen bioassay.

By contrast, it is apparent from **Fig 3.2** that E2 (1 nM) exerted a proliferative effect of 205.7 ± 24.8 % in MCF-7 BUS cells when proliferation was measured using DNA quantification, while when the proliferation was indirectly assessed by the MTS assay there was a proliferative effect of 148.9 ± 26.3%. This indicated that there is a significant difference in performance between methods used to assess proliferation. It is apparent that MCF-7BUS cells were more sensitive to estrogen than MCF-7 (ECACC). All subsequent parameters for validation and optimisation of E-screen with MCF-7 BUS cells were measured by determining direct cell number using the Hoechst DNA assay.

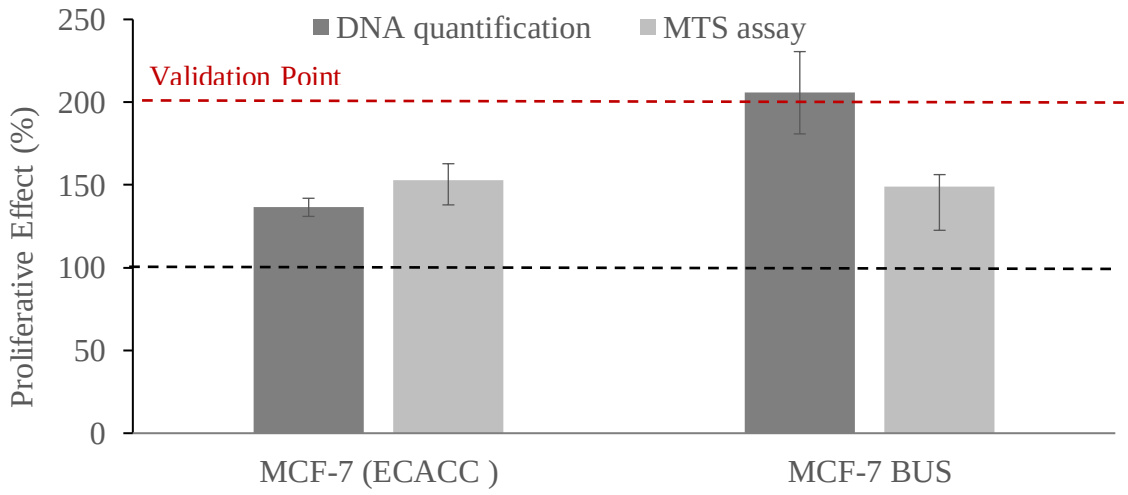


Figure 3.2 A comparison of the DNA quantification method and the MTS assay to measure proliferation of MCF-7 (ECACC) and MCF-7 BUS cells stocks exposed to E2 [1 nM]. *The black dotted line represents proliferation by the negative control. Red dotted represents the minimum desired proliferation exerted by E2 for assay validation. The bars represent the mean of triplicates of three independent experiments (n=9).*

3.1.2. Optimization of E-screen parameters with MCF-7 (BUS) cell line

3.1.2.1. DNA quantification validation and limit of detection

To establish the limit of detection of the Hoechst DNA assay two 96-well plates were seeded with 100 µl MCF-7 BUS cells in the range 100-40000 cells / well.

After allowing the cells to attach for one day, the cells from the wells of one of the plates were lysed and treated with Hoechst dye. The cells of the second plate were quantified after 6 days of growth. Fluorescence measured at $\lambda_{(ex/em)}$ 340/460 nm was directly proportional to cell number in the range $4x10^3$ – $4x10^4$ cells after 6 days of growth (**Fig 3.3**). Both fitting curves have a good correlation (R^2) factor higher than 0.9. Fluorescence did not respond to cell number in the range 100-2000 cells/well. Fluorescence of 6th day 4000 cells/well was almost 2-fold higher (40,7±10,0) than cells in the range 100-2000 cells/well (12.5 ± 0.5 – 19.5 ± 2.6) and increased 3.2-fold in the range 4000 cells/well to 40000 cells/well. Since cell density less than 2000 cells /well was not detected the limit of detection was set at 4000 cells/well.

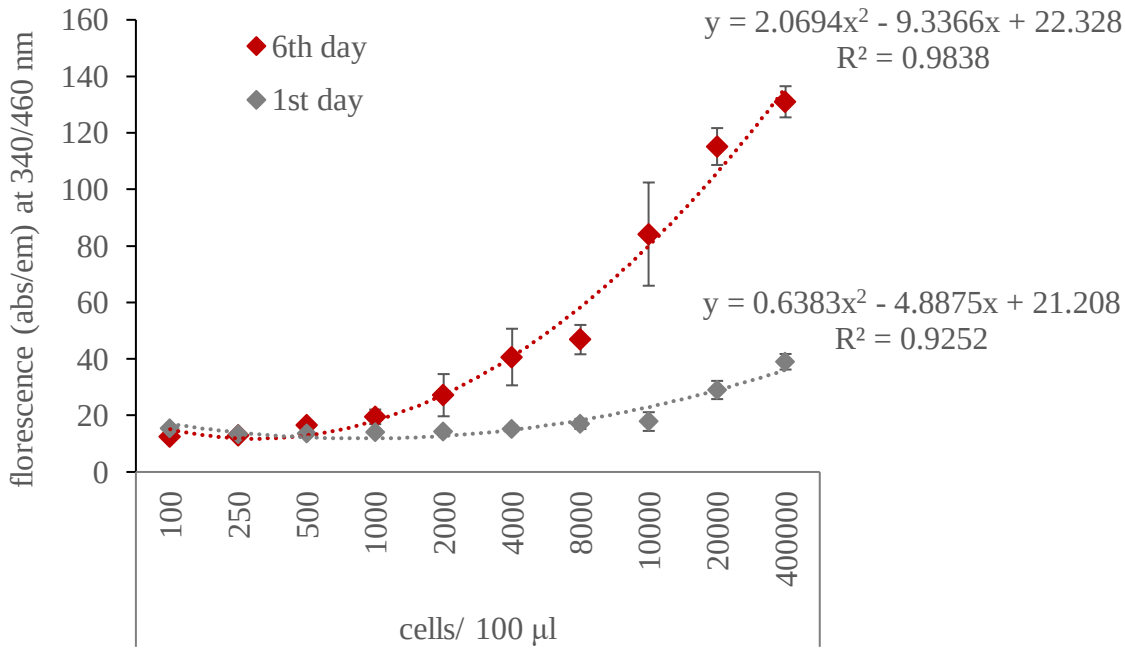


Figure 3.3 Fluorescence of Hoechst 33258 as a function of MCF-7 (BUS) cell number after 1-day and 6-days of growth. *Each point represents the mean of octuplicates of one experiment (n=8).*

3.1.2.2. Optimal seeding density

To determine the seeding density at which MCF-7 cells showed an optimal proliferative response to estrogen, cells were seeded at varying cell densities and exposed to E2 and BPA at concentrations of 10^{-14} to 10^{-6} M. The DNA quantification assay was then used to assess cell proliferation following a 6-day exposure period. Cell proliferation was expressed as the mean proliferative effect relative to the negative control. Dose-response curves were generated by plotting proliferative effects against the log standards [M], E2 and BPA. Each point in the graphs represent the mean of triplicates of three independent experiments (n=9).

The seeding density has a marked effect on E2-induced proliferation as shown in **Fig 3.4**. An E2 dose-response was not detected at cell density $\leq$ 1000 cells /well. At a density of 2000 cells / well a suboptimal dose-response was observed in which proliferation increased 140-225 % in the range 10^{-14} M to 10^{-9} M. However, as E2 concentration increased to 10^{-6} M proliferation decreased to a level similar to the negative control. A linear E2 dose-response was observed when cells were seeded at 4000 cells/well. All concentrations of E2 between 10^{-14} M and 10^{-8} M enhanced proliferation to 183-269 %. Maximum proliferation (269 %) was obtained following treatment of cells with 0.01 μM E2. As E2 concentration increased to 10^{-6} M proliferation decreased to a level similar to the negative control.

Seeding density also had a marked effect on BPA-induced proliferation since BPA dose-response was not detected at cell density $\leq$1000 cells /well (**Fig 3.5**). At a density of 2000 cells / well a suboptimal dose-response was observed in which proliferation increased 0-49 % in the range 10^{-14} M to 10^{-8} M. However, as BPA concentration increased to 10^{-6} M proliferation decreased. A linear BPA dose-response was observed when cells were seeded at 4000 cells/well. It is apparent all concentrations of BPA between 10^{-14} M and 10^{-7} M enhanced proliferation to 202-289 %. Maximum proliferation (289 %) was obtained following treatment of cells with 0.1 μM BPA. Cell density of 4000 cells/well was chosen as optimum for the E-screen bioassay

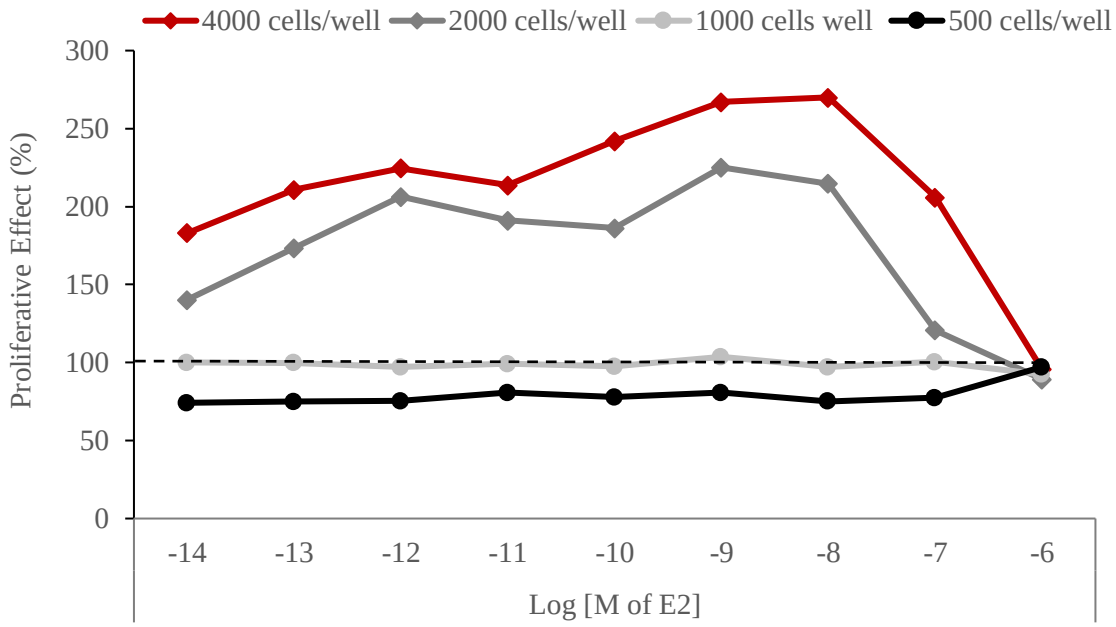


Figure 3.4 Dose-response curve of MCF-7 BUS cells seeded at different densities and exposed to E2 (0.01 pM to 1 μM). *The dotted line represents the proliferative effect exerted by the negative control. Data are mean of three experiments, completed in triplicate.*

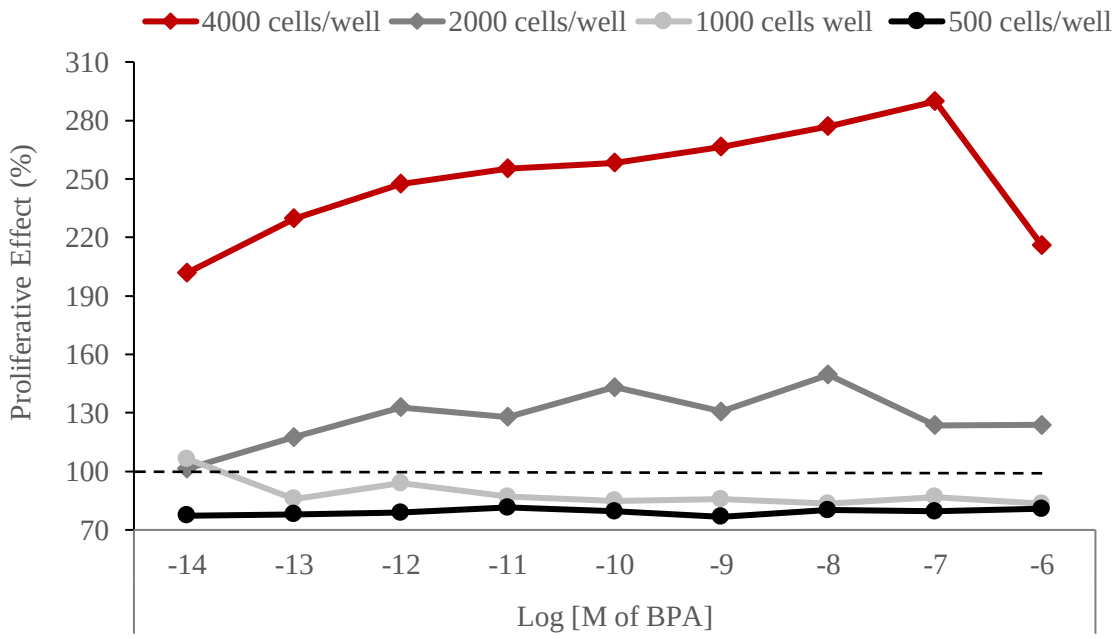


Figure 3.5 Dose-response curve of MCF-7 BUS cells seeded at different densities and exposed to BPA (0.01 pM to 1 μM). *The dotted line represents the proliferative effect exerted by the negative control. Data are mean of three experiments, completed in triplicate.*

3.1.2.3. Optimal duration for the E-screen assay

To determine the optimum period of treatment of MCF-7 BUS cells for E-screen bioassay, cells were seeded at 4000 cells/well in 6 plates and exposed to E2 and BPA at a concentration of 1 nM and 0.1 µM respectively for 1, 2, 3, 4, 5 and 6 days. DNA quantification with Hoechst was used to assess cell proliferation following the different exposure periods. The proliferative effect of each sample was plotted against time (days) of incubation (**Fig 3.6**).

As shown in **Fig 3.6** after 5 days of incubation the proliferative effect of the two standards rose above 200% necessary to validate the E-screen assay. Proliferation was 213.1 ± 6 % for E2 and 207.8 ± 3.9 % for BPA. By the 6th day of incubation mean proliferative effects of 280 % and 294 % were observed for E2 and BPA respectively. A six-day incubation period was selected for use in the assay

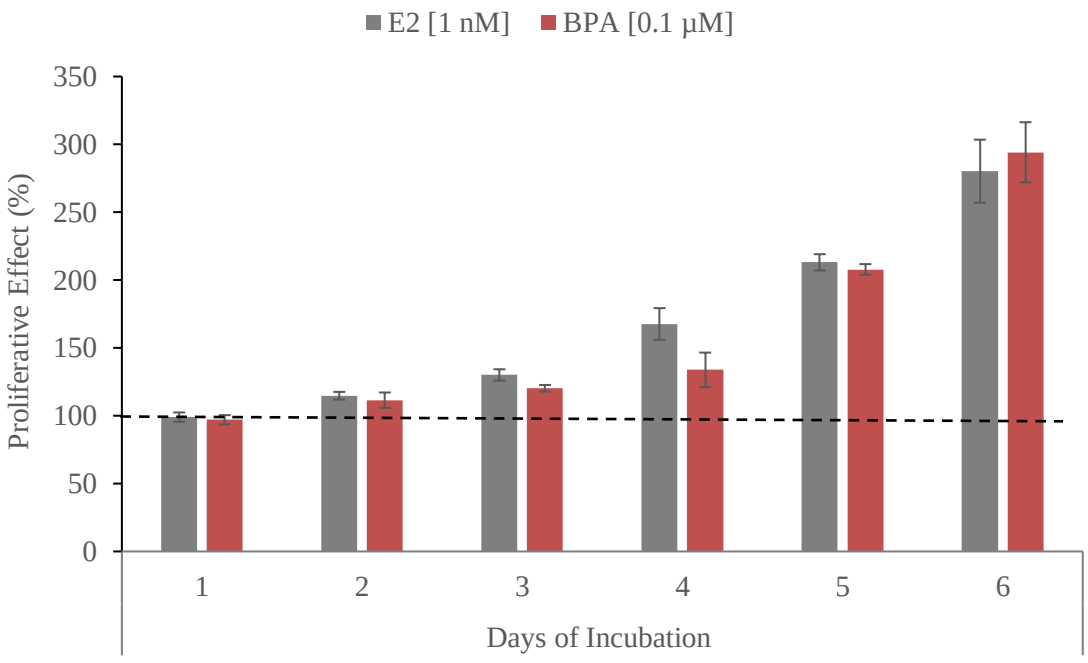


Figure 3.6 Time dependence of E2 and BPA-induced MCF-7 BUS cell proliferation. The dotted line represents the proliferative effect exerted by the negative control. *The bars represent the mean of triplicates of one experiment (n=3).*

3.1.2.4. Evaluation of anti-estrogenic properties of ICI

As stated previously, the E-screen bioassay was developed to assess the estrogenicity of environmental chemicals using the proliferative effects of E2 on MCF-7 cells as an endpoint. Assessment of the anti-estrogenic properties and potency of ICI in the bioassay was deemed necessary for confirmation of estrogenicity. Cells were exposed to a range of concentrations of the antagonist ICI (0.01 nM to 1 µM) with and without physiological levels of estradiol E2 [1 nM]. The assay was able to detect both the magnitude of the antagonistic properties of ICI and possible cytotoxic effect of ICI on MCF-7 proliferation during E-screen, as well as the dose of ICI required to cancel the proliferative effect of E2 (**Fig 3.7**).

ICI [1 µM] significantly reduced the proliferative to 71.4 ± 5.2% when compare to the negative control (100%) suggesting a possible cytotoxic effect at that specific concentration (**Fig 3.7**). Lower concentrations of ICI yielded proliferative effects (94-116%) similar to the negative solvent control indicating that by itself ICI does not have a significant effect on the proliferation of MCF-7 BUS cells. It is apparent that the antagonistic effects of ICI on the estrogenic activity of E2 is dose-dependent. Maximum inhibition of E2-induced proliferation was observed at 1 µM and 0.1 µM, restoring proliferative effects of MCF-7 to 103.6±9.8% and 109.6±11.2% respectively, which is not significantly different from the proliferative effects exerted by negative solvent control. Since ICI at 1 µM seems to be cytotoxic, ICI at 0.1 µM was selected for co-incubation with samples in order to corroborate estrogenic activity in future experiments.

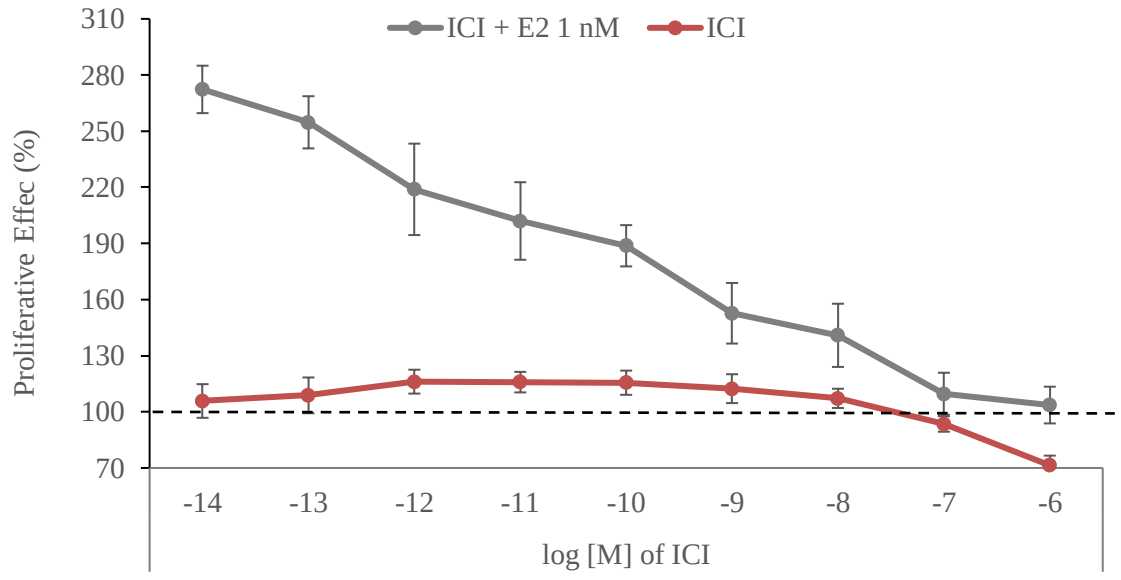


Figure 3.7 Dose-response curve of MCF-7 BUS cells to ICI (0.01 pM-1µM) in the presence and absence of E2 [1 nM]. *The dotted line represents the proliferative effect exerted by the negative solvent control. Each point represents the mean of triplicates of three independent experiments (n=9).*

3.1.2.5. Dose-response curve of E2

As stated previously, the E-screen bioassay was developed to assess the estrogenicity of environmental chemicals using the proliferative effects of E2 on MCF-7 cells as an endpoint.

To further validate the reliability and sensitivity of this assay for the detection of xenestrogens, MCF-7 cells were exposed to the endogenous estrogen E2 (17 beta-estradiol) in the concentration range of 10^{-14} M to 10^{-6} M in the presence and absence of estrogen antagonist ICI [10^{-7} M]. The estrogenic response of MCF-7 BUS cells to E2 (10^{-14} M -10^{-9} M) was abolished when cells were co-incubated with ICI [0.1 µM] as shown in **Fig 3.8**. Proliferation of MCF-7 BUS cells increased progressively with E2 concentration reaching a peak of 212 ±10.6 % when exposed to E2 at 1 nM and decreased to 97.8±0.7% when co-incubated with ICI at 0.1 µM (**Fig 3.8**). Proliferation of cells co-incubated with E2 and ICI fluctuated between 82-97% relative to the negative control thus validating the E-screen test and corroborating the estrogenic effects of estrogen.

In the case of test extracts having a proliferative effect, a quantitative assessment of estrogenicity relative to E2 [1nM] may be made. **Fig 3.9** represents a standard curve of proliferation relative to E2 [1 nM] as a function of E2 concentration. It was fitted to an equation that was used to calculate EEQ (estradiol equivalents) of samples. From this dose-response curve, an EC50 for E2 equal to 2.1 pM was calculated.

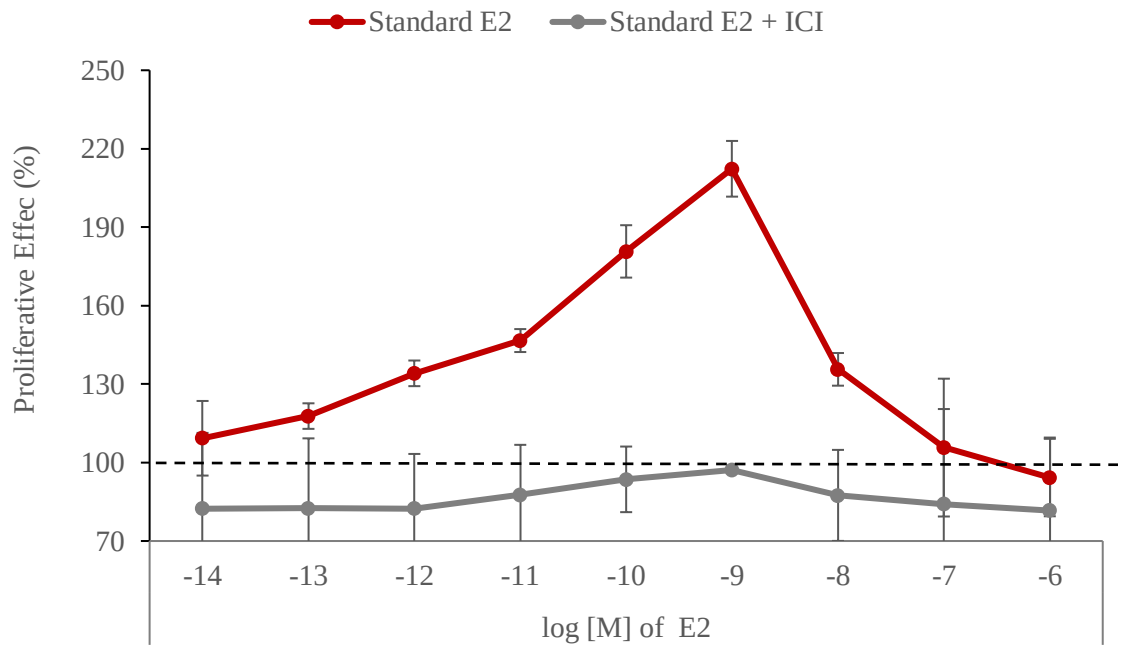


Figure 3.8 Proliferative effects of E2 incubated with and without ICI [0.1 µM] on MCF-7 BUS cells. *The dotted line represents the proliferative effect exerted by the negative control. Each point represents the mean of triplicates of three independent experiments (n=9).*

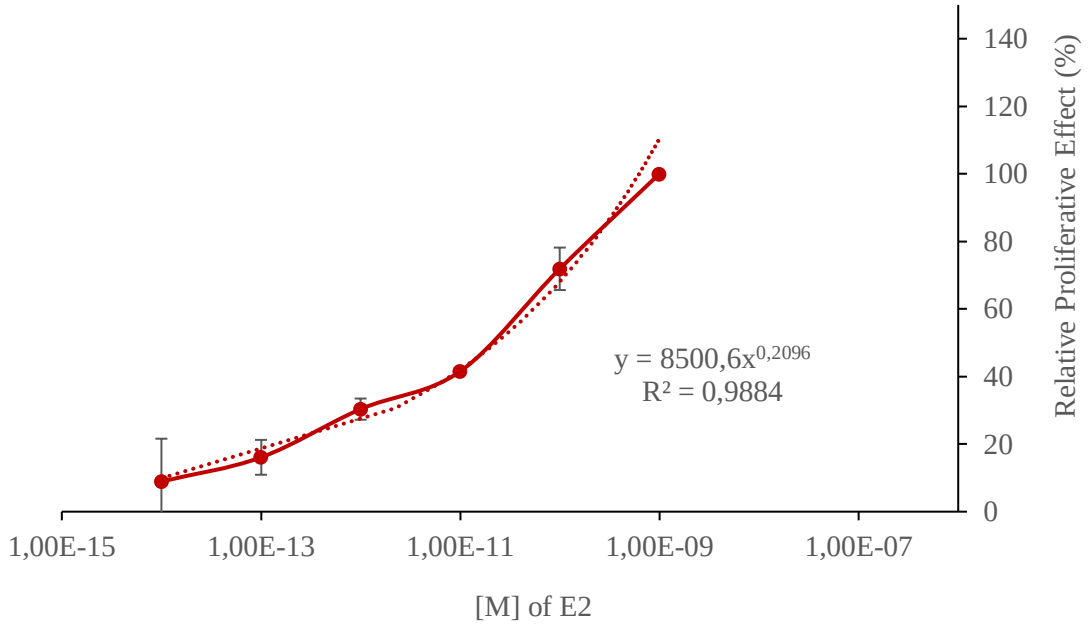


Figure 3.9 Dose-response of MCF-7 BUS cells to E2 expressed as Relative Proliferative Effect (% of E2 [1 nM]). *The generated curve was fitted to a potential equation.*

3.2. Validation of the Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) is a frequently used sample preparation method for chemical analysis. It is increasingly being used for bioassays, especially for the detection of estrogenic and mutagenic activities of unknown compounds and mixtures (Fang *et al.*, 2016; Severin *et al.*, 2017). The SPE method for preparing water extracts for E-screen was optimized in terms of sorbent selection and usage protocol procedure.

3.2.1. Optimization of procedure

Water samples were extracted using a modification of the solid phase extraction method described by Wagner and Oehlmann (Wagner and Oehlmann, 2011). Distilled water (1L) was spiked with either E2 or BPA at 10^{-9} M and 10^{-7} M respectively, while distilled water was used as a negative control. The samples were extracted with two different SPE cartridges: Oasis HLB and Perkin Elmer (PE) N9306613. Following elution, the samples were dried down under nitrogen in the absence (protocol A) or presence of 100 µL DMSO (protocol B). The samples of the method A were resuspended in 100 µL of DMSO after the drying step

These 10 000X concentrated samples were diluted 100 times in cell media prior to E-screen assay.

Optimal extraction of estrogenic compounds from the water was achieved using Oasis HLB cartridges and protocol B in which DMSO was added before the drying step, as shown in **Fig 3.10**.

BPA [0.1 μM] and E2 [1 nM] induced proliferation to 241% and 221% respectively. Samples extracted with protocol A showed lower proliferative responses (between 111-115 %) regardless of sorbent type indicating a probability of loss of volatile estrogenic activity during the drying step. SPE procedure with Perkin Elmer cartridges following protocol B yielded proliferative effects of 168 % and 142 % for BPA [0.1 μM] and E2 [1 nM] respectively. Being < 200 % (the desired threshold) the SPE procedure with Oasis HLB glass cartridges following protocol B in which DMSO was added before the drying step was selected for future use in this project.

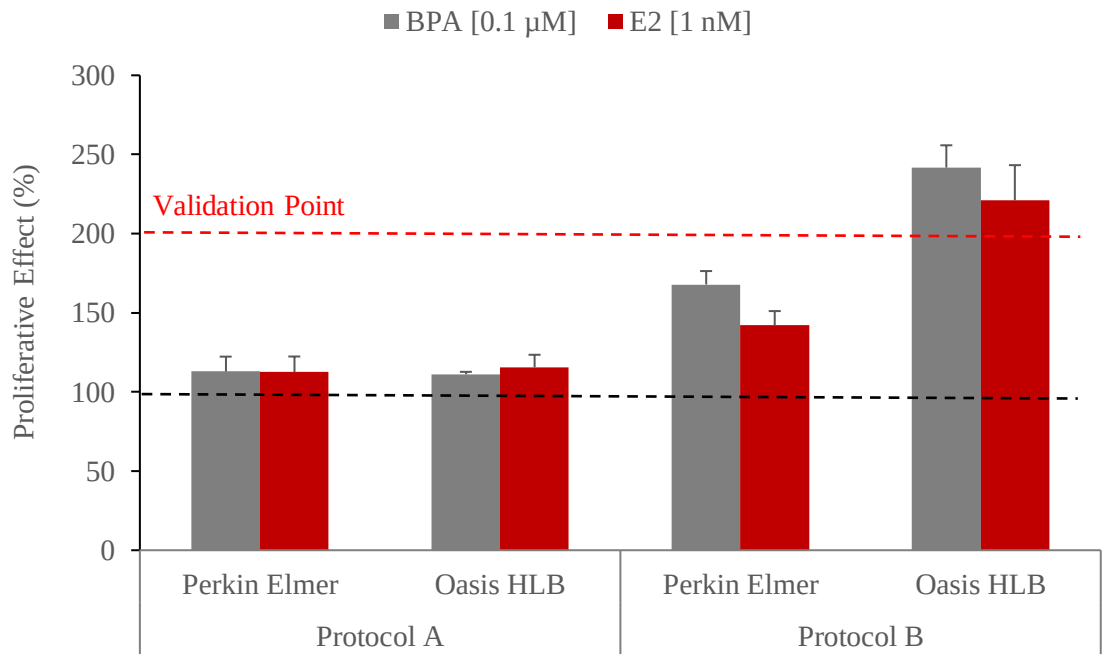


Figure 3.10 Proliferative effects of solid phase extracted samples of BPA and E2 using two different cartridges and two different variations of the SPE protocol. *The black dotted line represents the proliferative effect exerted by the negative control, while the red dotted line represents minimum desired proliferation by E2 or BPA for assay validation.*

3.2.2. Efficiency of Oasis HLB cartridges

To assess the efficiency of SPE cartridges in selection, purification and concentration of estrogenic compounds, water spiked with a range of concentrations (10^{-14} M to 10^{-6} M) of the synthetic xenestrogen BPA or endogenous estrogen E2 was extracted with the optimised SPE protocol described in Chapter 2. The SPE samples were diluted in cell media to a concentration factor of 100X. E-screen was conducted to assess the proliferative effect of the solid phase extracted samples and to compare with equivalent concentrations of BPA and E2 (10^{-14} M to 10^{-6} M) that did not undergo extraction. The proliferative effects were determined by measuring cell proliferation with DNA Hoechst dye.

Water extracts containing E2 exerted a dose-dependent proliferative effect in the range 10^{-14} M-10^{-9} M (**Fig 3.11**). Maximum proliferation (204%) was achieved in extracts of water that were spiked with 1 nM E2 and was comparable to non-solid phase extracted E2 at 1 nM (212%).

On the other hand, water extracts containing BPA exerted a dose-dependent proliferative effect in the range 10^{-14} M-10^{-7} M (**Fig 3.12**). Maximum proliferation (217%) was achieved in extracts of water that were spiked with 0.1 μM BPA and was comparable to non-solid phase extracted samples (281%). Therefore, the cartridges were determined suitable to extract estrogenic compounds, of diverse nature and structure, at various concentrations from the water with minimal loss due to extraction process.

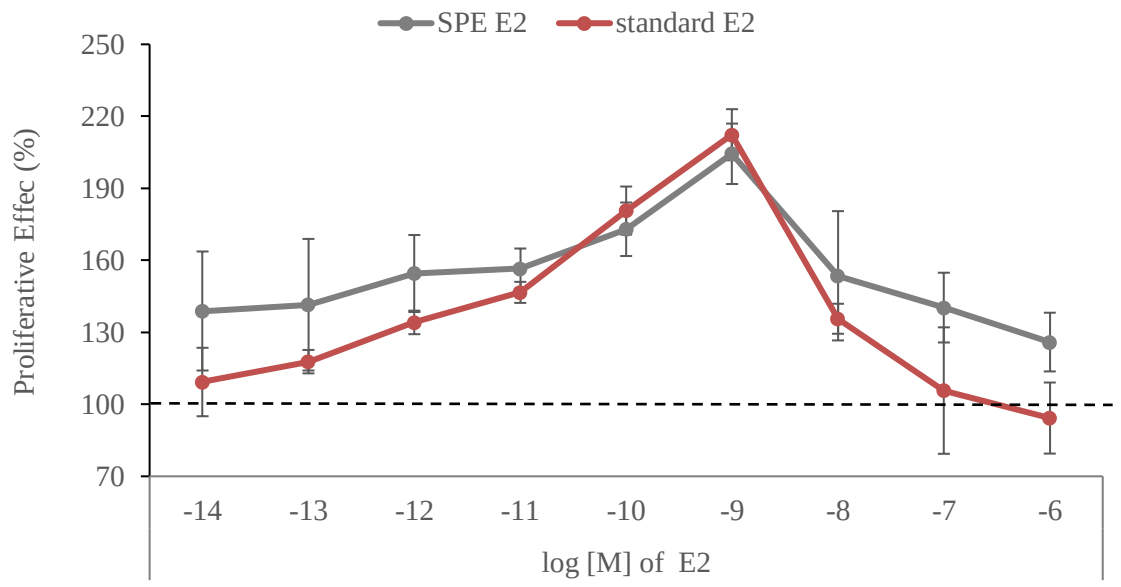


Figure 3.11 Proliferative effect of E2 (0.01 pM- 1 µM) and water extracts containing E2 on MCF-7 BUS. *The dotted line represents the proliferative effects of the negative control. Each point represents the mean of triplicates of three independent experiments (n=9).*

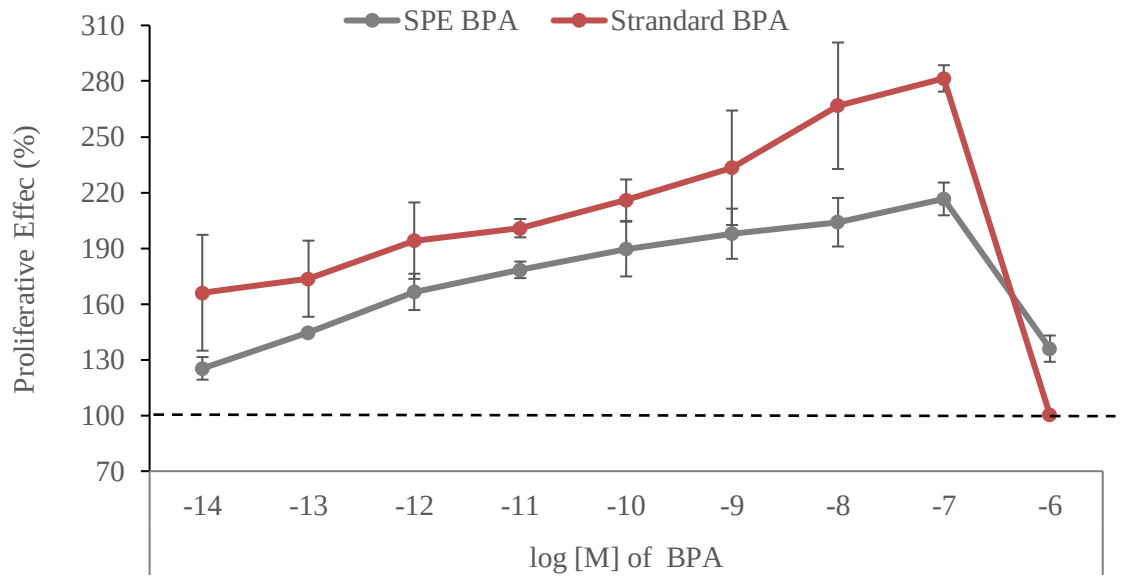


Figure 3.12 Proliferative effects of BPA (0.01 pM- 1 µM) and water extracts containing BPA on MCF-7 BUS. *The dotted line represents the proliferative effects of the negative control. Each point represents the mean of triplicates of three independent experiments (n=9).*

3.3. Summary

The E-screen assay was optimised in terms of:

- the cell line used,
- choice of assay to measure cell proliferation,
- optimum cell seeding density,
- time of incubation and
- evaluation of anti-estrogenic properties of ICI.

The findings of the validation study are summarised in **Table 3.1**. These conditions were used in all further studies.

Table 3.1 Critical parameters for optimization of the E-screen bioassay

Parameter	Optimal condition
Cell line	MCF-7 (BUS) stock
Proliferation assay	DNA quantification
Cell seeding density	4000 cells/well
Time of incubation	6 days
Use of anti-estrogenic ICI	[0.1 μM]

Oasis HLB cartridges were chosen for use in the solid phase extraction protocol and the DMSO was added before the drying step of the extraction

Chapter IV

4. Toxicological studies on the novel PMMA reactors.

Novel large-volume batch solar reactors [Prototype I (140 L) and II (88 L)] for treating harvested rainwater were developed for use in South Africa and Uganda. The reactor tubes were made from polymethylmethacrylate (PMMA). Toxicity was investigated using the E-screen assay to investigate estrogenic activity and the Ames test to investigate mutagenicity. The raw and aged PMMA used in the construction of the reactors was tested for extractables (Section 4.1) and the water produced by the reactors was tested for leachates (Section 4.2).

4.1. Testing the PMMA plastic polymer for extractables

In carrying out toxicity studies for the PMMA tubular SODIS reactors, raw and weathered/aged pieces of polymethylmethacrylate (PMMA) used in the construction of the reactors were tested for extractables. The plastic was subjected to a range of stress conditions as described in Section 2.2.1 and tested for toxicity using the E-screen (4.1.1 - 4.1.2) and the Ames test (4.1.3).

4.1.1. Testing raw PMMA plastic polymer with the E-screen bioassay

The E-screen was carried out as described in Section 2.2.7 Chapter 2 and with the optimised conditions summarised in Section 3.3 Chapter 3. PMMA extractables into cell culture medium and into ethanol were screened for estrogenic activity. Media extracts from PMMA pieces were diluted and presented to cells at two different concentrations (10- and 100-fold dilutions). Ethanolic extracts were diluted and tested at one concentration (100-fold dilution) so the final concentration of EtOH was 1% v/v (which is the maximum concentration of solvent at which no cytotoxic effects were observed). The positive control was 1 nM estradiol (E2) and the negative control was DMEM7/F12 hormone-free medium supplemented with dilution vehicle 1% (v/v). Half of the plate was reserved for cells that were co-treated with ICI at 10^{-7} M. The proliferative effect of the samples was assessed using the DNA quantification assay as described in Section 2.2.7.4 Chapter 2. All extracts and experimental controls were run in triplicate per plate and each plate was repeated 3 times.

The extractant solvent used had a profound effect on the release of estrogenic extracts from raw PMMA plastic (**Fig 4.1)**. Extraction of raw PMMA into DMEM/F12 culture medium, showed estrogenic activity of the 100-fold diluted extracts (**Fig 4.1**). However, ethanolic extracts of raw PMMA exerted insignificant effects on cell proliferation relative to the negative control (**Fig 4.1**). The estrogenic activity detected in some of the samples is correlated to time and temperature of extraction (**Fig 4.1**). Raw PMMA subjected to 20 °C for 24 h and 72 h or to 37 °C for 5 hours within a 24 h period yielded extracts in DMEM/F12 medium (100-fold diluted) that showed no significant increase on proliferation. Longer extraction periods at 37 °C in DMEM/F12 yielded extracts showing significant ($p < 0.05$) proliferative activity. Raw PMMA plastic exposed in DMEM/F12 medium solvent to 37 °C for more than 15 h increased proliferation relative to the negative control between 34 ± 18% and 63 ± 8.3%. Increasing the extraction temperature to 50 °C also yielded significant ($p < 0.05$) proliferative activity relative to the negative control. Exposure of raw PMMA in DMEM/F12 medium solvent to 50 °C for periods of 5 h over a 15-h period and 15 h over a 72-h period yielded extracts that increased proliferation by 71 ± 14% and 74 ± 23% respectively. Co-incubation of raw PMMA extracts with ICI [0.1 μM], an ER antagonist, reduced the proliferation of the extracts when compared to the negative control by 12% on average.

Interestingly, analysis of more concentrated extracts (10-fold diluted) of raw PMMA in DMEM/F12 medium showed a trend towards proliferation but it was not deemed statistically significant (**Fig 4.2**). This suggested that extracts obtained after prolonged exposure at 37 °C and 50 °C exerted a non-monotonic response, *i.e.* that estrogenic activity of the extracts may not be linearly related to concentration.

Relative to E2, the estrogenic effects of the raw PMMA extracts in DMEM/F12 amounted to approximately 27-65 % RPE (**Table 4.1**). Thus, extracts of raw PMMA obtained after 15, 24 or 72 h at 37 °C or after 5 or 15 h at 50 °C contained estrogenic components showing partial agonistic activity. Statistically significant estradiol equivalents of raw PMMA extracts obtained by extrapolation from the standard curve relating E2 concentration to % RPE (**Fig 3.9** Section 3.1.2.5) approximately amounted to values between 32- 2114.2 ng/L

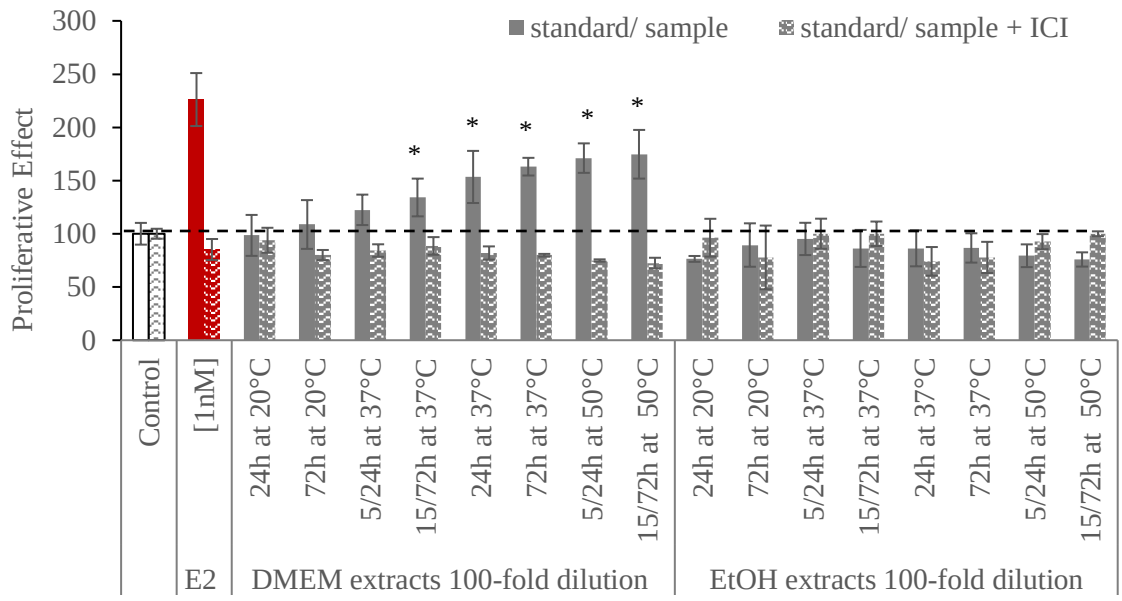


Figure 4.1 Proliferative effects of raw PMMA extracts (100-fold dilution). *Raw PMMA was submerged in DMEM /F12 media solvent or in EtOH solvent and exposed to different temperature conditions. Extractables were diluted 100x and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor ANOVA indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

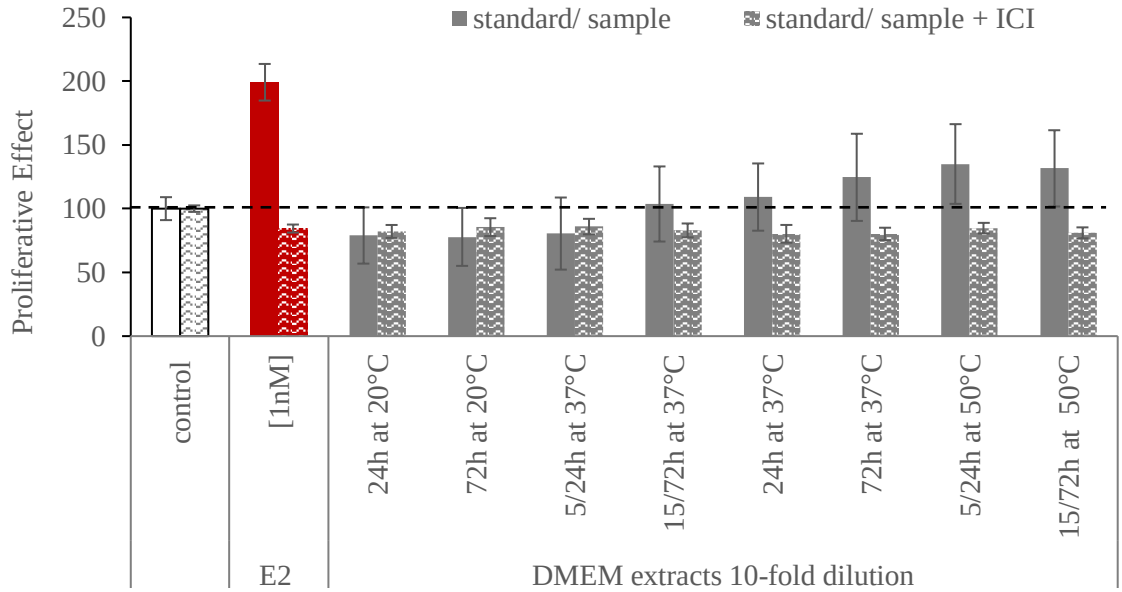


Figure 4.2 Proliferative effects of raw PMMA extracts (10-fold dilution). *Raw PMMA was submerged in DMEM/F12 media solvent and exposed to different temperature conditions. Extractables were diluted 10x and screened for estrogenic activity in the presence and absence of ICI [0.1 µM].*

Table 4.1 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of exudates leached from raw PMMA polymer into two solvents (DMEM/F12 and ethanol) during thermal stress. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

	DMEM/F12 media solvent				EtOH solvent	
Dilution →	1:10		1:100		1:100	
Temperature condition ↓	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
24h at 20 °C	-22.9±24.2	nd	-1.7±14.5	nd	-28.2±7.6	nd
72h at 20 °C	-24.1±25	nd	6.4±16.8	3.2E-02	-10.8±28.3	nd
5/24h at 37 °C	-21.1±31.2	nd	15.8±7.9	11.0	-3.7±19.7	nd
15/72h at 37 °C	4.3±32.5	4.8E-04	27.3±12.6	32.1	-16.6±21.3	nd
24h at 37 °C	10.2±29.2	2.9E-02	43.2±19.4	286.4	-15.1±21.4	nd
72h at 37 °C	27.5±38.2	3.3	50±12.4	581.7	-15.2±16.1	nd
5/24h at 50 °C	39±34.8	17.6	58.5±16.8	1228.9	-26.4±9.5	nd
15/72h at 50 °C	35.1±33.2	10.7	65.5±13.6	2114.2	-32±3.6	nd

4.1.2. Testing the aged PMMA with the E-screen bioassay

The use of the SODIS reactors and their constant exposure to sunlight could have an effect on the chemical stability and robustness of the plastic components of the reactor. This implies that the aging of the plastic could have a critical effect on the rate of release of substances with estrogenic activity.

In order to mimic the aging process of the plastic, plastic pieces of PMMA were exposed to the weather conditions in Plataforma Solar of Almeria, Spain for 3, 6 and 9 months. Extractables of the plastic pieces were sampled as indicated in Chapter 2 (**Fig 2.6**) with various temperature stress conditions (20 °C for 24 hours and 72 hours, 37 °C for 5 hours in one day, 5 hours each day for 3 consecutive days, 24 hours and 72 hours and 50 °C for 5 hours in one day and 5 hours each day for 3 consecutive days). Aged PMMA plastic extracts were diluted 10-fold and 100-fold as previously stated prior to E screen analysis.

4.1.2.1. **Aged PMMA (100-fold dilution)**

An inverse relationship between the age of PMMA plastics and proliferation can be observed in **Fig 4.3**. PMMA plastic aged for 3 months and subjected to extraction in DMEM/F12 under varying time and temperature conditions yielded extracts that were more proliferative than PMMA plastic aged for longer periods of 6 and 9 months.

Aged PMMA subjected to 20 °C for 24 h and 72 h or to 37 °C for any period below 72 h yielded extracts that had negligible effects on proliferation. Continuous exposure of aged PMMA to 37 °C for 72 h increased proliferation by 22 ± 4% relative to the negative control. Increasing the extraction temperature to 50 °C also yielded significant ($p < 0.05$) proliferative activity relative to the negative control. It is apparent that exposure of raw PMMA to 50 °C for periods of 5 h over a 24-h period and 15 h over a 72-h period yielded extracts that increased proliferation by 38 ± 16% and 43 ± 6% respectively. Co-incubation of raw PMMA extracts with ICI [0.1 μM], an ER antagonist mitigated against proliferation confirming estrogenic activity of aged PMMA plastic extracts obtained under thermal stress.

Extraction of 6 and 9-month aged PMMA pieces into DMEM/F12 or ethanol for varying times and temperatures yielded extracts showing negligible proliferation relative to the negative control. The absence of estrogenic activity from thermally stressed 6 and 9-month PMMA may be a reflection of possible photodegradation.

Interestingly, ethanolic extracts of aged PMMA also showed an inverse relationship between the age of PMMA plastic and proliferation (**Fig 4.3**). PMMA plastic aged for 3 months and subjected to extraction in ethanol under conditions varying in time and temperature yielded extracts that were more proliferative than extracts from PMMA plastic aged for 6 and 9 months. Aged PMMA (3 months) subjected to 20 °C for 24 h and 72 h or to 37 °C for 5, 15 or 24 h yielded extracts in ethanol that had negligible effects on proliferation. Continuous exposure of 3-months-aged PMMA to 37 °C for 72 h in ethanol increased proliferation by approximately 50% relative to the negative control. Increasing the extraction temperature to 50 °C also yielded significant ($p < 0.05$) proliferative activity relative to the negative control. Exposure of 3-month-aged PMMA to 50 °C for periods of 5 h and 15 h period yielded extracts in ethanol that increased proliferation by 82 ± 17% and 92 ± 19% respectively. Co-incubation of aged PMMA ethanolic extracts with ICI [0.1 μM], an ER antagonist reduced proliferation of extracts when compared to the negative control by 21% on average.

4.1.2.2. **Aged PMMA (10-fold dilution)**

Thermally stressed 10-fold diluted DMEM/F12 medium solvent extracts showed proliferation at 3 and 6 months but not at 9 months (**Fig 4.4**). The absence of estrogenic activity from thermally stressed 9-month PMMA is consistent with the observation made with the corresponding 100-fold dilution extract and maybe a reflection of possible photodegradation.

Thermally stressed 10-fold diluted DMEM/F12 medium solvent extracts showed higher proliferation at 3 months than that observed by corresponding 100-fold diluted extracts. Extraction temperatures of 37 °C for 24 h and 72 h and 50 °C for 24 h and 72 h yielded significant ($p < 0.05$) proliferative activity relative to the negative control. Exposure of 3-months-aged PMMA to 50 °C for periods of 5 h and 15 h yielded extracts that increased proliferation by 47 ± 8% and 51 ± 9%, respectively. Co-incubation of aged PMMA extracts with ICI [0.1 μM], an ER antagonist mitigated against proliferation confirming estrogenic activity of 3-month aged PMMA plastic extracts obtained under thermal stress.

Thermally stressed 10-fold diluted DMEM/F12 extracts from 6-months-aged PMMA showed proliferative activity. Continuous exposure at 37 °C for 72 h increased proliferation by 36 ± 18 % relative to the negative control. Increasing the extraction temperature to 5 0°C also yielded significant ($p < 0.05$) proliferative activity relative to the negative control. Exposure of aged PMMA to 50 °C for periods of 5 h over a 24-h period and 15 h over a 72-h period yielded extracts that increased proliferation by 47 ± 11% and 59 ± 15%, respectively. Co-incubation of aged PMMA extracts with ICI [0.1 μM], an ER antagonist mitigated against proliferation confirming estrogenic activity of 6-month aged PMMA plastic extracts obtained under thermal stress.

Thermally stressed 10-fold diluted DMEM/F12 extracts of 9-month aged PMMA showed a similar pattern to corresponding 100-fold extracts. Extraction of 9-month aged PMMA into DMEM/F12 for varying times and temperatures yielded extracts showing negligible proliferation relative to the negative control. The lack of effect of ICI when co-incubated with 9-month extracts further suggests the absence of estrogenic components in 9-month aged PMMA.

Relative to E2, the maximum estrogenic effect of the 100-fold diluted aged PMMA extract in DMEM/F12 amounted to 27 ± 6% RPE. Thus, extracts of aged PMMA obtained after 15 h at 50 °C contained estrogenic components showing partial agonistic activity (**Table 4.2**). Estradiol equivalent of aged PMMA extract in DMEM/F12 obtained by extrapolation from the standard curve relating E2 concentration to % RPE (**Fig 3.9** Section 3.1.2.5) amounted to 32 ng/L. Similarly, the maximum estrogenic effect of the 100-fold diluted aged ethanolic PMMA extract amounted to approximately 31 ± 2% RPE and to 57 ng EEQ / L. Estradiol equivalents of the 10-fold diluted extracts in DMEM/F12 amounted to approximately 1098 ng/L after 3 months, 326 ng/L after 6 months and 4 ng/L after 9 months.

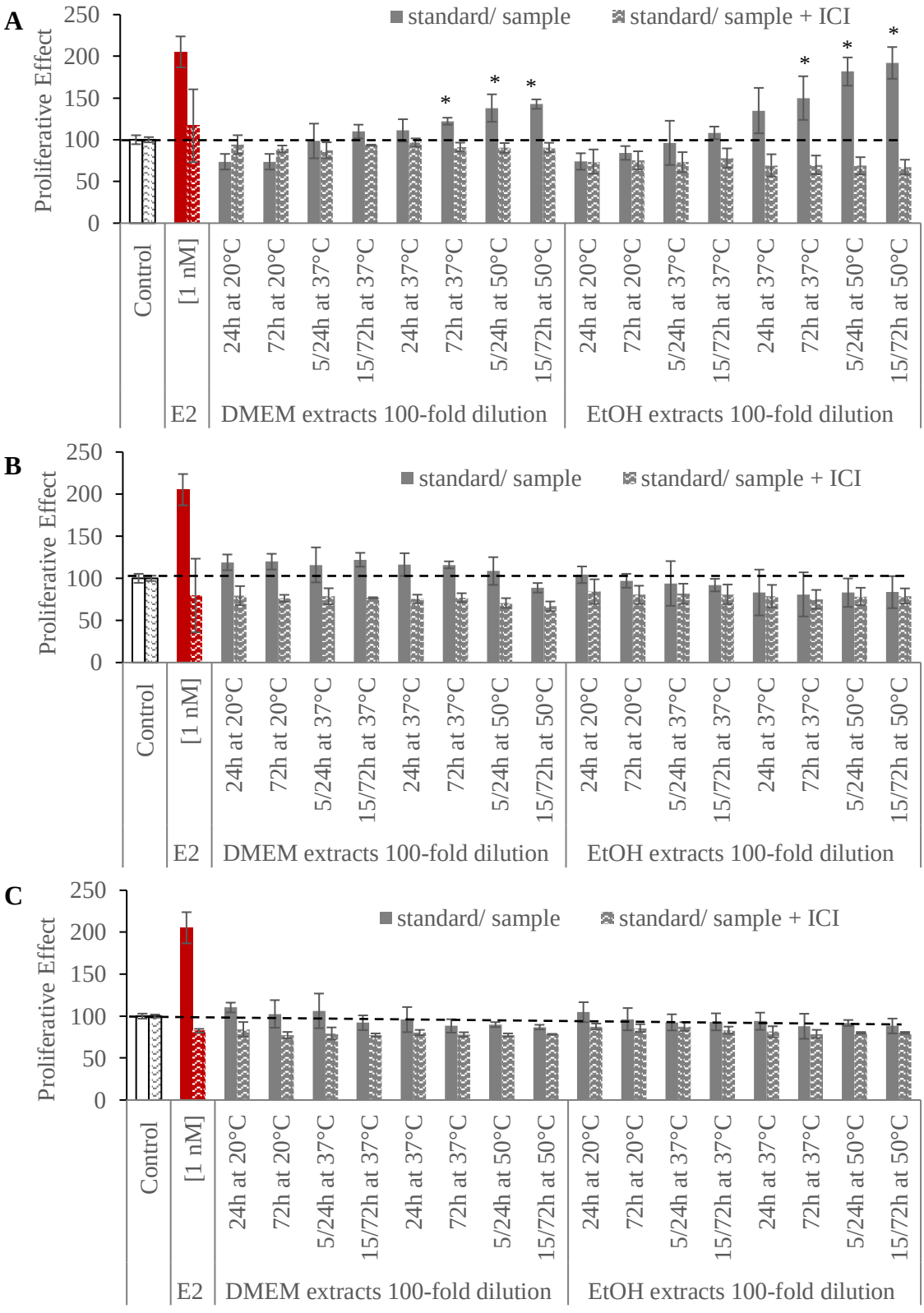


Figure 4.3 Proliferative effects of aged PMMA extracts (100-fold diluted) *3-months aged (A), 6-months aged (B) and 9-months aged (C) PMMA was submerged in DMEM/F12 media or EtOH solvent and exposed to different temperature conditions. Extractables were diluted 100 times and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

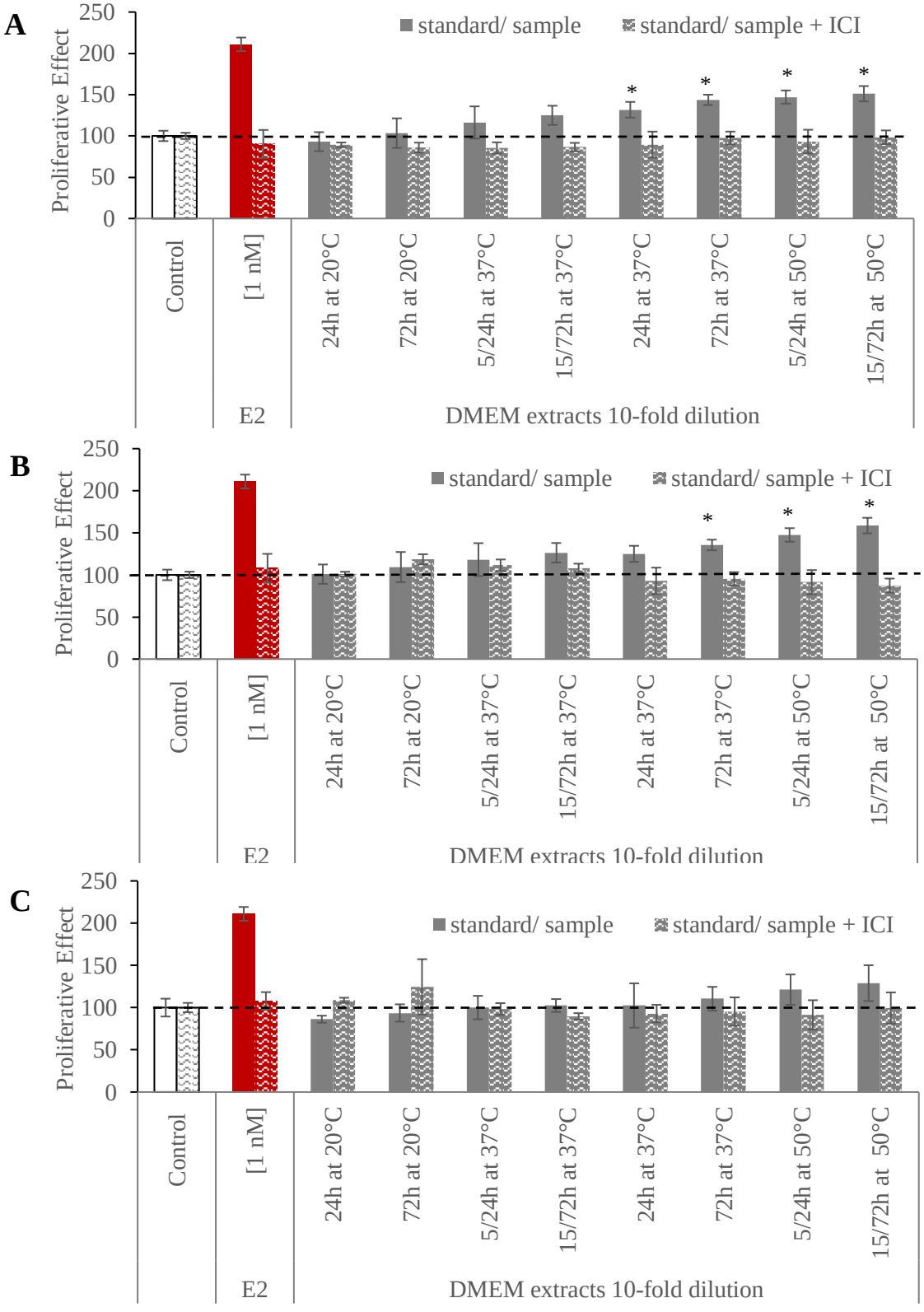


Figure 4.4 Proliferative effects of Aged PMMA polymer (10-fold diluted). *3-months aged (A), 6-months aged (B) and 9-months aged (C) PMMA was submerged in DMEM/f12 media solvent and exposed to different temperature conditions. Extractables were diluted 10 times and screened for estrogenic activity in the presence and absence of ICI [0.1 μM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

Table 4.2 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of exudates leached from 3-month, 6-month and 9-month-aged PMMA plastic polymer into two solvents (DMEM/F12 and ethanol) during thermal stress. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

		DMEM media solvent				EtOH solvent	
	Dilution →	1:10		1:100		1:100	
	Temperature condition ↓	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
3-months-age	24h at 20 °C	-11.7±19.8	Nd	-10.1±4.0	nd	-8.5±4.6	nd
	72h at 20 °C	6.2±30.7	2.6E-03	-10.1±4.0	nd	-5.8±3.4	nd
	5/24h at 37 °C	30.8±34.7	5.7	-0.7±8.4	nd	-1.7±9.0	nd
	15/72h at 37 °C	46.0±21.6	38.8	3.8±3.4	2.5E-03	4.2±3.5	4.2E-03
	24h at 37 °C	58.3±19.3	121.1	4.3±5.4	4.6E-03	14.6±6.9	1.6
	72h at 37 °C	79.0±8.2	516.5	8.4±1.9	0.1	17.4±7.1	8.4
	5/24h at 50 °C	85.0±9.9	732.0	17.1±8.0	3.4	27.4±2.4	32.4
	15/72h at 50 °C	92.5±13.3	1098.2	27.2±5.9	31.7	30.8±1.8	56.8
6-months-age	24h at 20 °C	-4.7±21.4	nd	7.8±10.4	0.1	6.5±13.4	3.4E-02
	72h at 20 °C	6.4±23.8	3.1E-03	3.3±12.6	1.3E-03	-4.0±5.5	nd
	5/24h at 37 °C	14.9±32.4	0.2	11.3±30.0	0.5	-8.0±4.0	nd
	15/72h at 37 °C	24.1±27.1	1.8	15.0±16.9	1.9	-11.2±7.3	nd
	24h at 37 °C	24.8±17.9	2.0	4.3±11.6	4.6E-03	-27.6±11.4	nd
	72h at 37 °C	40.6±9.9	21.5	4.3±18.0	4.6E-03	-21.9±8.9	nd
	5/24h at 50 °C	57.9±7.9	117.2	0.6±48.1	3.1E-07	-25.0±5.6	nd
	15/72h at 50 °C	71.7±13.4	326.2	-13.7±16.4	nd	-23.7±6.0	nd
9-months-age	24h at 20 °C	-25.1±7.4	Nd	13.8±7.5	1.2	9.6±21.7	0.2
	72h at 20 °C	-11.9±18.1	Nd	3.9±26.9	2.8E-03	1.3±13.5	nd
	5/24h at 37 °C	0.5±24.2	1.8E-08	9.9±33.6	0.2	-0.1±6.5	nd
	15/72h at 37 °C	4.7±13.5	6.8E-04	-1.2±12.7	nd	-0.4±4.5	nd
	24h at 37 °C	-3.3±43.7	Nd	-0.4±20.1	nd	0.2±14.2	nd
	72h at 37 °C	17.9±24.9	0.4	-8.2±6.1	nd	-1.3±3.9	nd
	5/24h at 50 °C	25.0±17.1	2.1	-1.8±5.2	nd	-0.1±6.5	nd
	15/72h at 50 °C	28.7±15.7	4.1	-7.1±9.0	nd	-0.1±6.5	nd

4.1.3. Testing raw and aged PMMA plastic polymer with the Ames test

During solar disinfection, the components of the reactors are exposed to a lot of stress factors, such as high temperatures and continuous reuse of the plastics (aging). To better understand how these stress factors, affect the migration of substances from the plastic components during SODIS, analysis of how duration of exposure to different thermal stresses influenced the migration of mutagenic and genotoxic extractables from raw PMMA SODIS reactor plastic components was performed.

All extractables samples (sampled as stated in the methodology) were assessed for mutagenicity with the Ames test performed with and without metabolic activator S9 by following the procedure described in the methodology section. The extractables were sampled from both raw and aged PMMA plastic. In order to simulate the aging process of the PMMA plastic, raw PMMA plastic pieces were exposed to weather conditions for 3, 6 and 9 months in the Plataforma Solar of Almeria. Spain.

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 4.3**)

Table 4.3 Ames test results of extractables released from PMMA plastic polymer after different temperature extraction treatments.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as the mean ± standard deviation of the fold induction over the baseline. A fold induction over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Age	Extraction solvent	Treatment	TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	3.86±1.53	2.49±1.15	1.24±0.58	2.24±0.58
		Positive control	21.24±0.58	18.76±2.31	34.83±1.53	26.3538291
Raw	PBS	24h at 20 °C	0.70±1.00	0.27±0.58	0.00±0.00	0.70±0.58
		72h at 20°C	0.23±0.58	0.54±1.53	0.54±0.58	0.35±1.15
		5/24h at 37 °C	0.47±1.53	0.27±0.58	0.54±0.58	0.52±1.00
		15/72h at 37 °C	0.47±1.15	0.80±1.00	0.54±0.58	1.40±1.53
		24h at 37 °C	0.47±0.58	1.07±0.58	0.54±0.58	1.22±0.58
		24h at 37 °C	0.35±1.00	0.54±0.58	0.27±0.58	0.70±1.15
	EtOH	24h at 20 °C	0.35±0.58	0.40±1.00	0.27±0.58	0.45±1.00
		72h at 20 °C	0.17±0.58	0.67±1.15	0.00±0.00	0.45±1.00
		5/24h at 37 °C	0.35±1.53	0.40±1.00	0.27±0.58	0.59±1.15
		15/72h at 37 °C	0.35±0.58	0.67±1.53	0.27±0.58	1.04±2.08
		24h at 37 °C	0.69±1.53	0.54±1.53	0.54±0.58	0.45±1.00
		24h at 37 °C	0.35±0.58	0.54±0.58	0.27±0.58	0.74±0.58
3 months	PBS	24h at 20 °C	0.33±0.58	0.21±0.58	0.17±0.58	0.67±1.53
		72h at 20 °C	0.42±0.58	0.08±1.15	0.00±0.00	0.17±0.58
	EtOH	24h at 20 °C	0.32±0.58	0.32±0.58	0.12±0.58	0.56±1.15
		72h at 20 °C	0.48±1.00	0.18±0.58	0.00±0.00	0.56±0.58
6 months	PBS	24h at 20 °C	0.31±1.00	0.17±1.00	0.00±0.00	0.67±1.15
		72h at 20 °C	0.42±2.08	0.17±1.00	0.17±0.58	0.67±0.58
	EtOH	24h at 20 °C	0.61±0.58	0.29±1.00	0.23±0.58	0.78±0.58
		72h at 20 °C	0.61±1.53	0.24±0.58	0.35±1.00	0.44±0.58
9 months	PBS	24h at 20 °C	0.21±0.58	0.04±0.58	0.21±0.58	0.44±2.08
		72h at 20 °C	0.08±1.15	0.00±0.00	0.08±1.15	0.53±-0
	EtOH	24h at 20 °C	0.41±1.73	0.08±1.15	0.33±0.58	0.83±2.08
		72h at 20 °C	0.55±2.52	0.13±1.73	0.33±0.58	1.00±-0

4.2. Testing the PMMA reactors for leachates

Leachates are substances that migrate from plastic into its surroundings during regular use of the plastic. Since the SODIS process involves the continuous re-exposure of the reactor to sunlight and other weather conditions, and since the PMMA plastic extractables released into DMEM/F12 medium when exposed to extreme temperature conditions showed estrogenic activity, this study assessed if sunlight conditions promoted the release of leachates from the plastic components of the reactor into the water. While waiting for the PMMA reactors to be constructed, pieces of PMMA were submerged in water and tested for leachates (4.2.1) as described in Section 2.2.3. When the reactors were installed in South Africa and Uganda the water produced by the reactors was tested for leachates (4.2.2).

4.2.1. Testing pieces of PMMA for leachates

Pieces of PMMA submerged in water were tested for leachates as described in Section 2.2.3. To determine if differences in weather conditions due to latitude and location have an effect on leaching from the PMMA plastic a similar set of experiments was performed at PSA, Spain, Stellenbosch, South Africa, rural Uganda close to the equator and at Dublin City University in Dublin, Ireland. The E-screen was used to test for estrogenic activity (4.2.1.2) and the Ames test was used to test for mutagenicity (4.2.1.3)

4.2.1.1. **Salinity and conductivity of the water in contact with the PMMA pieces at PSA**

As already stated, two different types of water were used for these evaluations. Well water and distilled water collected at 3, 6 and 9 months were analyzed with respect to mineral composition and conductivity as described in Chapter 2. Well water contained higher amounts of chloride, sulphate, sodium, calcium, potassium and magnesium than distilled water (**Table 4.4**). Exposure of well water to sunshine for 9 months increased sulphate and ammonium concentrations compared to 3 months and 6 months. Sulphate ion concentration of well water stored for 9 months was double that of well water stored for 3 months. Ammonium ion concentration increased 15-fold between 3 and 9 months. There was no change in the salinity and conductivity of the distilled water over the course of the 9 months incubation.

Table 4.4 Salinity and conductivity measurements of well and distilled water with submerged PMMA pieces exposed to sunshine at PSA, Almería, Spain.

		Well water			Distilled water		
		3 months	6 months	9 months	3 months	6 months	9 months
Salinity (mg/L)	Chloride	31.7±0.1	40.2±0.5	31.2±0.3	0.3±0.2	0.1±0.0	0.1±0.0
	Nitrite	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Nitrate	0.5±0.0	1.3±0.0	0.5±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Phosphate	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Sulfate	24.1±0.1	34.7±0.4	47.4±33.5	0.4±0.1	0.3±0.0	0.3±0.0
	Sodium	46.6±0.2	43.2±0.6	47.1±0.5	0.6±0.8	0.0±0.0	0.0±0.0
	Ammonium	0.1±0.0	0.1±0.0	1.5±2.2	0.1±0.1	0.0±0.0	0.0±0.0
	Calcium	0.7±0.1	0.7±0.1	0.7±0.1	0.0±0.0	0.1±0.1	0.1±0.0
	Potassium	0.6±0.0	0.5±0.1	0.9±0.5	0.2±0.1	0.2±0.0	0.1±0.1
	Magnesium	6.6±0.3	8.1±0.1	6.5±0.7	0.2±0.0	0.2±0.0	0.3±0.0
Conductivity		2.5±0.0 mS/cm	2.5±0.1 mS/cm	2.6±0.0 mS/cm	5.0±1.9 µS/cm	4.0±0.0 µS/cm	4.2±0.2 µS/cm

4.2.1.2. **E-screen**

PSA, Spain

At PSA, the pieces of PMMA were immersed in distilled water and in local well water and exposed to local sunshine for 3,6 and 9 months. After the exposure period, 1L of water sample was solid phase extracted as described in Section 2.2.6. DMSO extract (100 µl) was diluted in DMEM/F12 hormone-free medium to yield final concentration factors of 100X, 50X and 10X when compared to the original water sample.

The concentrated extracted samples of leachates were then screened for estrogenic activity in the presence and absence of ICI as described in Chapter 2. The proliferative effects and the RPE of each sample were calculated by measuring cell proliferation with the DNA quantification method. EEQs of each concentrated extracted sample of leachates were assessed in order to determine if the samples were in compliance with the ADI recommended by the WHO.

Exposure of PMMA pieces in distilled water to sunlight for 3, 6 and 9 months had no significant effect on cell proliferation (**Fig 4.5**). Distilled water extracts that were 10X, 50X and 100X concentrated showed proliferative effects similar to the negative control irrespective of exposure to sunlight or darkness.

Exposure of PMMA pieces in well water to sunlight for 3 and 6 months had no significant effect on cell proliferation (**Fig 4.6**). Well water extracts that were 10X, 50X and 100X concentrated

and exposed to the sun showed proliferative effects similar to the negative control. Interestingly, the concentration factor of the extracts appeared to impact on proliferation after prolonged exposure to sun. Though the 50X water extract had negligible effect on growth after 9 months exposure (**Fig 4.6B**) both 10X and 100X concentrated samples showed a decreased proliferative effect relative to the negative control after 9 months in the sun. Relative to the negative control, the 10X and the 100X extracts inhibited growth by 21.5 % and 24.6 % respectively after 9 months of exposure to sun.

Storage of well water in the dark accentuated the decrease in the proliferative effects of PMMA pieces leachates. Relative to the negative control (100%), all 10X extracts prepared from well water samples kept in darkness for 3, 6 and 9 months showed a significantly ($p<0.05$) decreased proliferation of approximately 67.5-71.7% (**Fig 4.6A**). The 100X samples kept in darkness for 9 months significantly ($p<0.05$) inhibited cell growth by 42% relative to control (**Fig 4.6C**). However, 50X extracts of well water kept in darkness had negligible effects on growth after 3-, 6- and 9-months exposure (**Fig 4.6B**). Similarly, 100x extracts of well water kept in darkness for 3 months (110 ± 10.4%) and 6 months (119 ± 17%) had negligible effects on growth relative to the control.

Overall, it is apparent that sunlight had no effect on estrogenic activity of well water extracts exposed to PMMA pieces. RPE values of the 50X extract after 3, 6 and 9 months were approximately 4.5 ± 20 %, 15 ± 14% and 12 ± 18% respectively **(Table 4.5).** RPE values of the 50X extracts of well water kept in darkness for 3, 6 and 9 months exhibited negligible estrogenic activity with RPE values of approximately 8.5 ± 20 %, 13 ± 7% and 7.5 ± 6% respectively. Coincubation of 50X water extracts with ICI had negligible effects on growth (**Fig 4.6B**) confirming the absence of estrogenic activity in extracts prepared from well water exposed to PMMA pieces in the sun or darkness for 3, 6 and 9 months.

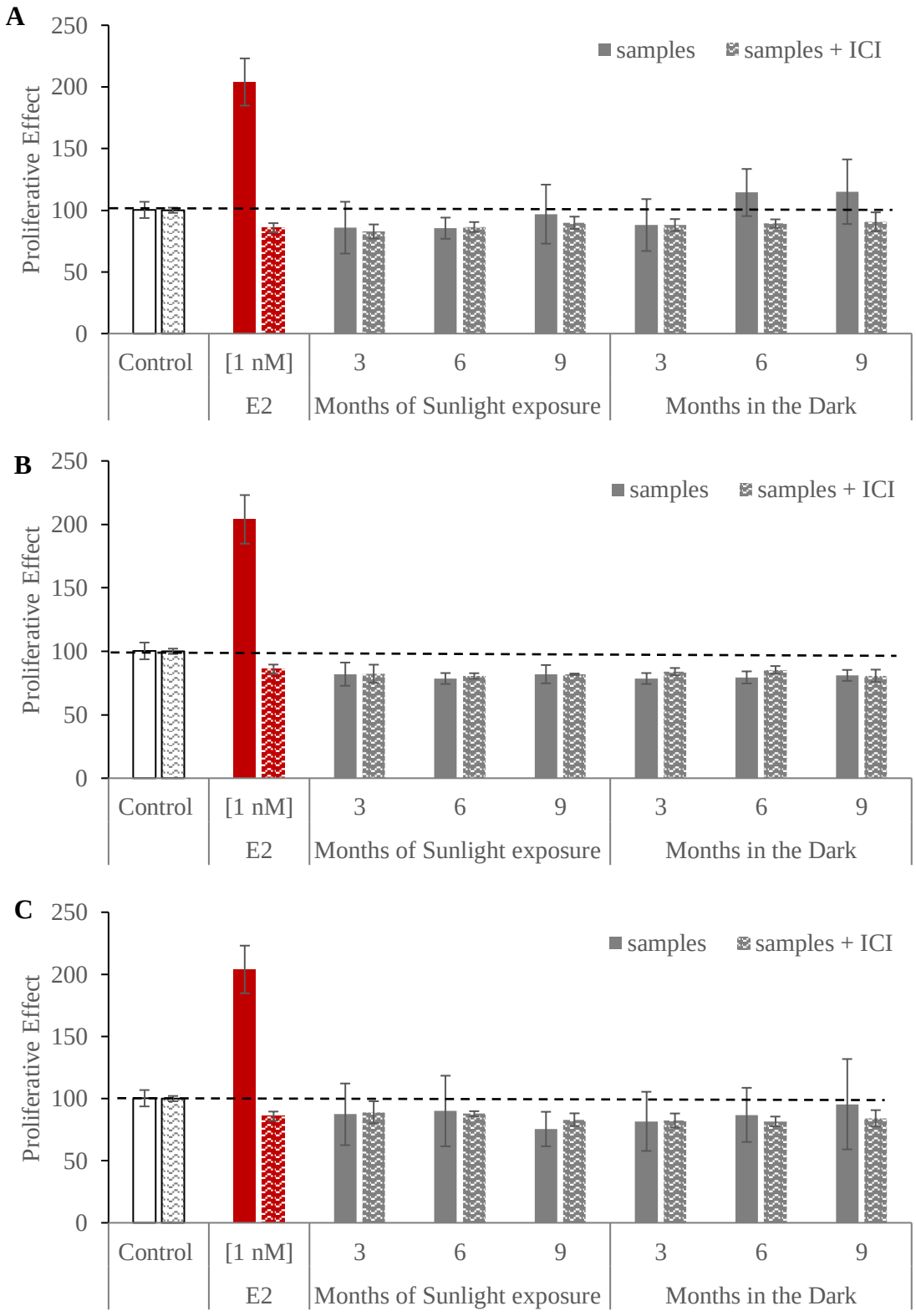


Figure 4.5 Proliferative effects of PMMA leachates in distilled water at Almeria, Spain. *Leachates released into distilled water after 3, 6 and 9 months of exposure of PMMA pieces to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 μM].*

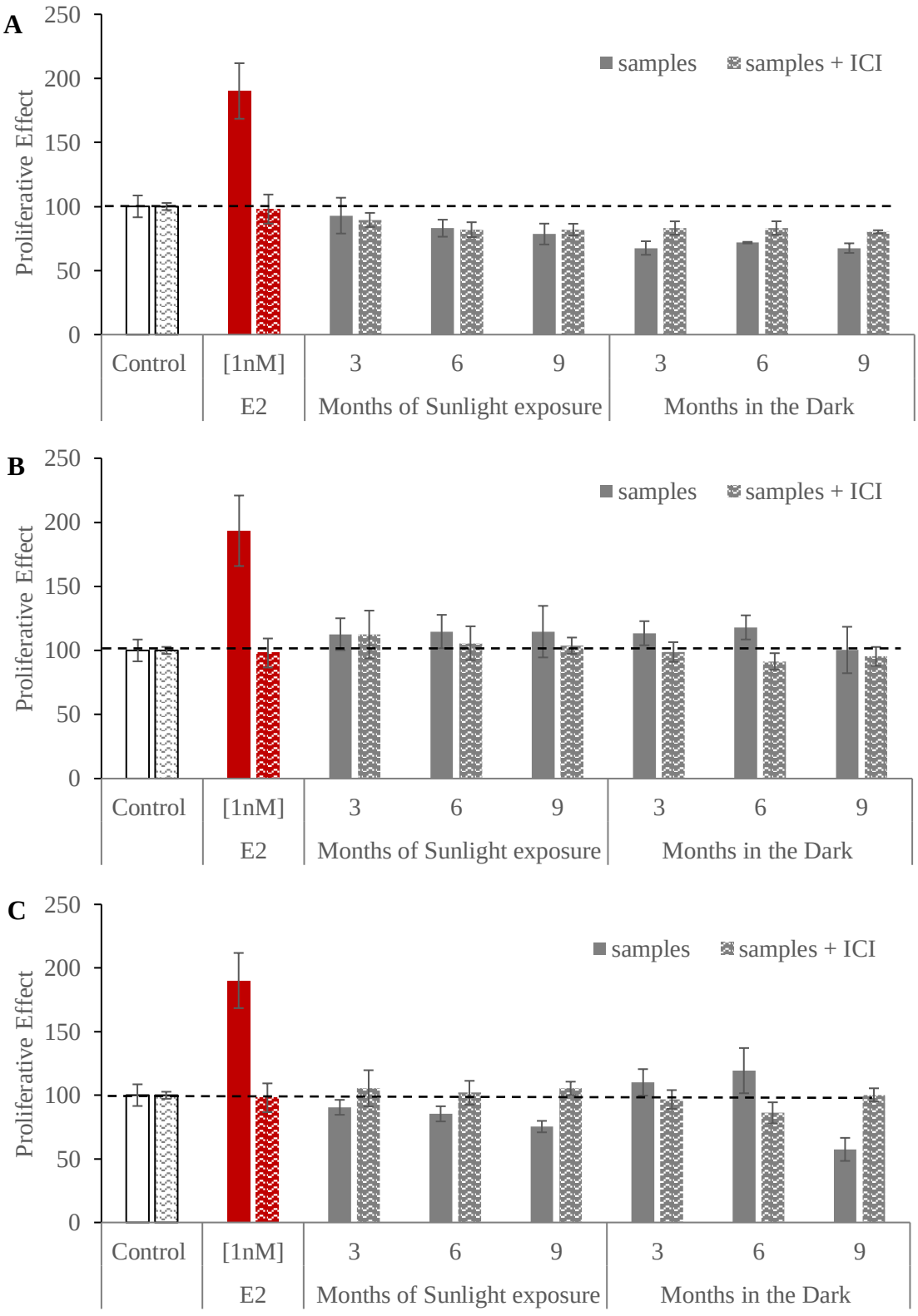


Figure 4.6 Proliferative effects of PMMA leachates in well water at Almería, Spain. *Leachates released into well water after 3, 6 and 9 months of exposure of PMMA pieces to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 μM].*

Table 4.5 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of leachates released from PMMA pieces into distilled water or well water after 3-,6- and 9-months exposure to sunlight and darkness at Almeria, Spain. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

			Concentration factor					
			10X		50X		100X	
			RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
Distilled water	Months of exposure to Sunlight	3	-12.5±20.8	nd	-3.5±10.3	nd	-20.1±22.6	nd
		6	-14±8.9	nd	-5.9±6.5	nd	-26.9±38.1	nd
		9	-2.8±24.2	nd	-3.5±8.8	nd	-47.7±8.9	nd
	Months in the Dark	3	-10.8±21	nd	-5.9±6.5	nd	-34.9±17.3	nd
		6	15.6±18.2	2.2E-03	-5.3±7	nd	-38.7±33.1	nd
		9	16.4±26.3	2.8E-03	-4.1±5.4	nd	-13.9±35.8	nd
Well water	Months of exposure to Sunlight	3	-13.4±27.6	nd	4.5±19.9	1.2E-06	-14.7±10.7	nd
		6	-17.8±17.4	nd	14.6±13.6	3.2E-04	-21.7±11.3	nd
		9	-26.5±14.5	nd	11.7±17.6	1.1E-04	-36.7±15.8	nd
	Months in the Dark	3	-19.5±17.6	nd	8.5±3.0	2.5E-05	10.7±12.9	3.7E-05
		6	-13.9±11.9	nd	12.7±6.7	1.6E-04	10.1±11	2.8E-05
		9	-5.6±8.8	nd	7.5±6	1.4E-05	-62.8±27.3	nd

DCU, Dublin, Ireland

PMMA plastic pieces were submerged in distilled water and exposed to weather conditions in Dublin for 3, 6 and 9 months. PMMA pieces in distilled water which were stored in darkness for 3,6 and 9 months served as controls. Water extracts were prepared from 1 L aliquots and analyzed for estrogenic activity using the E screen bioassay as described in Chapter 2. Extracts of distilled water stored in Dublin behaved similarly to water extracts stored in Spain (**Figure 4.7**). Nor 10x, 50X nor 100X water extracts (Dublin) showed proliferative activity relative to the negative control (**Figure 4.7**). Estimation of corresponding RPE values shown in Table 6 indicated that all extracts irrespective of concentration factor were not estrogenic.

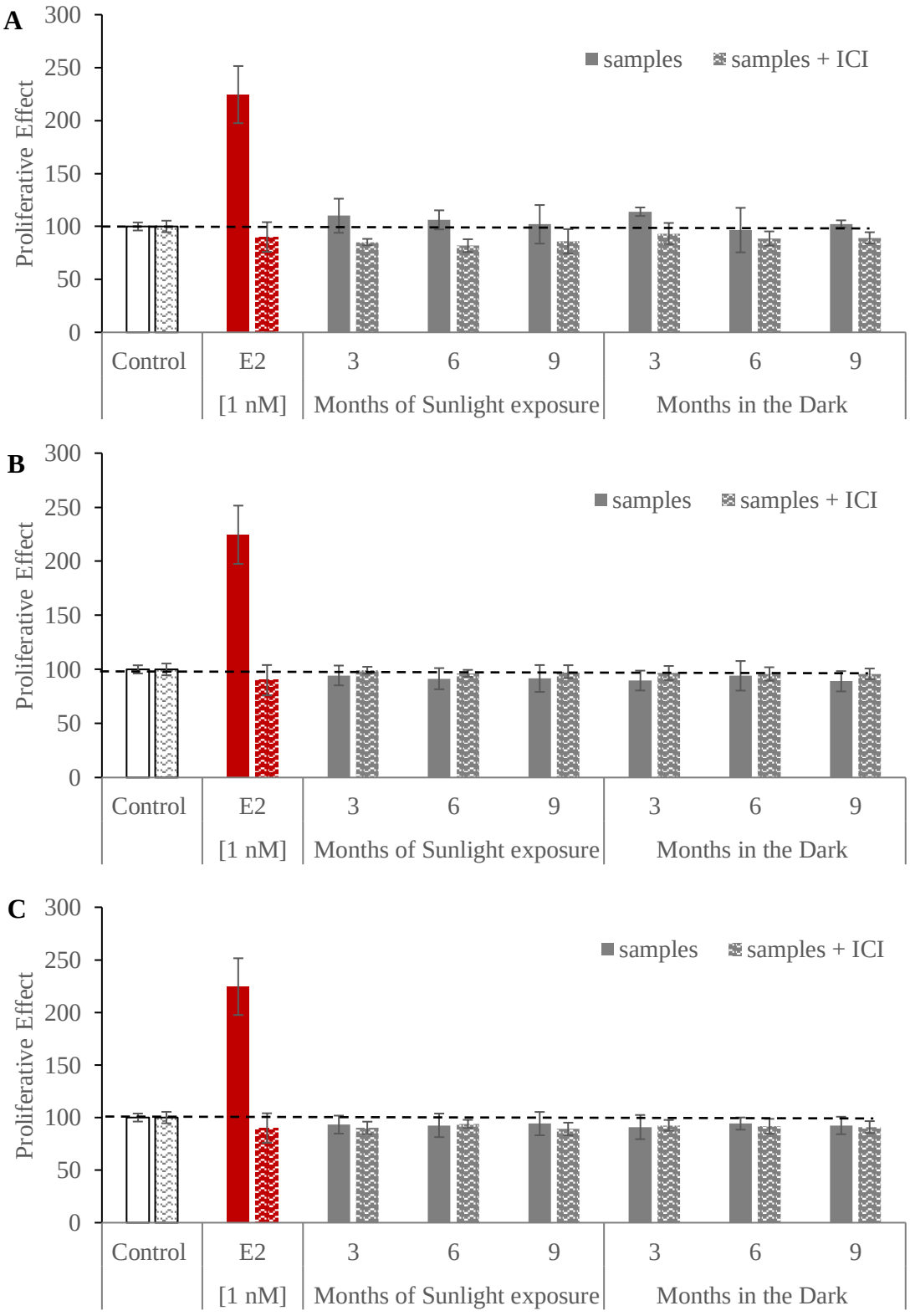


Figure 4.7 Proliferative effects of PMMA leachates into distilled water after sunlight exposure at Dublin, Ireland. *Leachates released into distilled water after 3, 6 and 9 months of exposure of PMMA pieces to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 µM].*

Table 4.6 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng/L) of leachates released from PMMA pieces into distilled after 3-, 6- and 9-months exposure to sunlight and darkness at Dublin, Ireland. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

			Concentration factor					
			10X		50X		100X	
			RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
Distilled water	Months of exposure to Sunlight	3	9.2±13.5	1.8E-04	-0.2±4.4	nd	-1.2±4.8	nd
		6	4.6±15.3	6.4E-06	-3.6±4.4	nd	-3.8±4.6	nd
		9	0.8±20.2	1.4E-09	-3.3±6.7	nd	-2.8±6.1	nd
	Months in the Dark	3	9.7±6.9	2.2E-04	-4.9±4.7	nd	-4.3±4.9	nd
		6	0.6±5.3	4.2E-10	-0.2±6.7	nd	-1.6±9.7	nd
		9	-0.9±26.3	nd	-5.8±3.6	nd	-4.6±4.2	nd

African countries (South Africa and Uganda)

As reported above leachates in water from exposed PMMA pieces to the sun in Spain and Ireland had no estrogenic effect on MCF-7 BUS cell growth after 3, 6, or 9 months. Once again, PMMA pieces were submerged in water and exposed to the sunlight or kept in the dark for 3, 6 and 9 months in South Africa, while PMMA pieces were submerged in water and exposed to the sunlight and kept in the dark for 1 and 3 weeks in Uganda. Water samples were extracted and concentrated as described in Chapter 2. Distilled water extracts from South Africa were screened for estrogenic activity at three concentration levels (10X, 50X and 100X), while water extracts from Uganda were screened for estrogenic activity at only one concentration (100X)

Extracts of distilled water prepared in South Africa behaved similarly to water extracts stored in Spain and Dublin (**Fig 4.8**). The 10X, 50X or 100X distilled water extracts (South Africa) showed no increased proliferative activity relative to the negative control. Water extracts from Uganda (100X) showed no estrogenic effects on MCF-7 BUS proliferation (data not shown). Estimation of the corresponding RPE values shown in **Table 4.7** indicated that all extracts irrespective of concentration factor showed % RPE <10% and were not estrogenic.

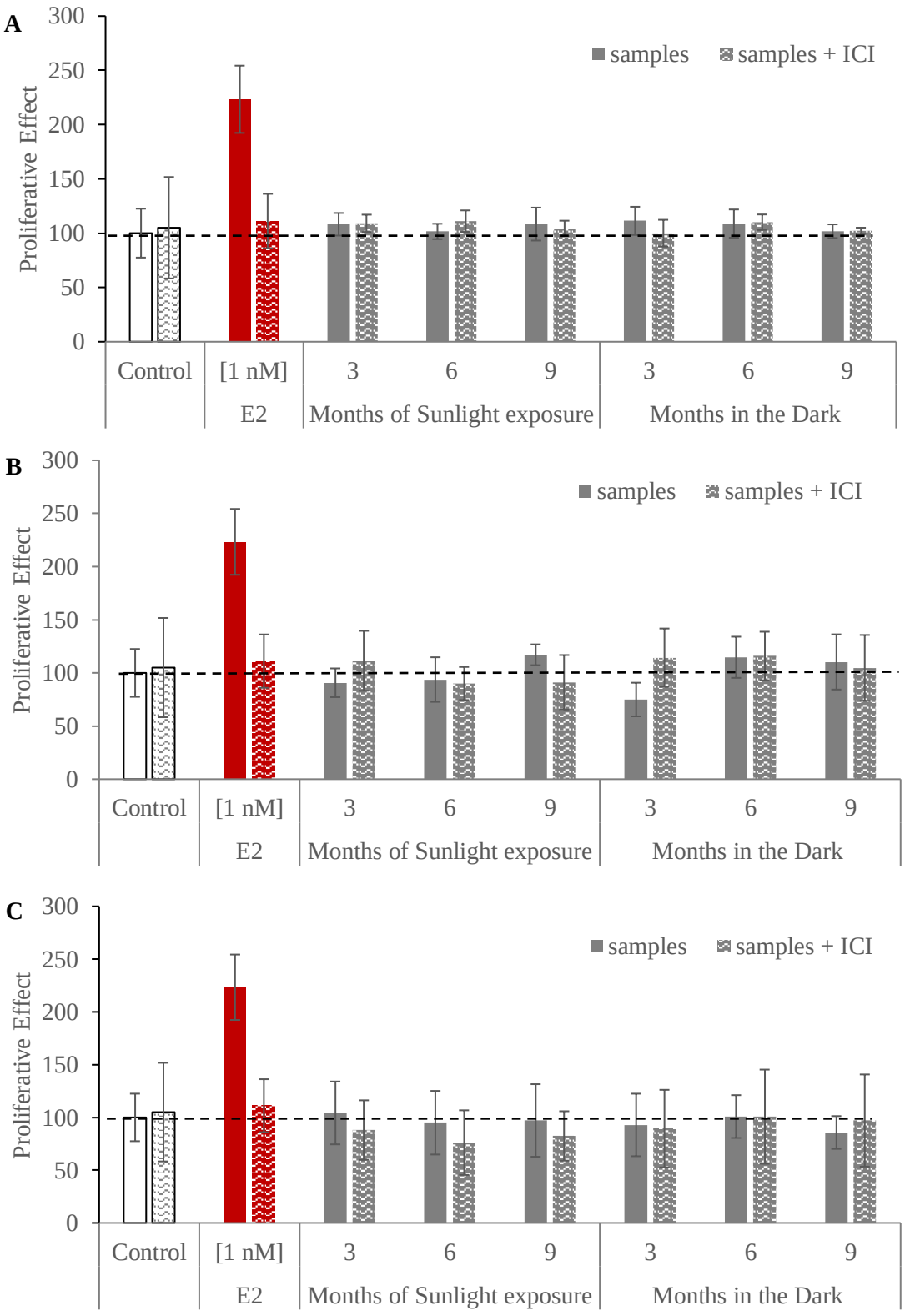


Figure 4.8 Proliferative effects of PMMA leachates into distilled water after sunlight exposure at Stellenbosch, South Africa. *Leachates released into distilled water after 3, 6 and 9 months of exposure of PMMA pieces to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 µM].*

Table 4.7 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng/L) of leachates released from PMMA pieces into distilled or well water after 3-,* 6- and 9-months *exposure to sunlight and darkness at South Africa. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined*

			Concentration factor					
			10X		50X		100X	
			RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
South Africa water	Months of exposure to Sunlight	3	6.0±7.7	2.3E-05	-7.6±11.0	nd	2.4±17.9	3.0E-08
		6	1.2±5.2	9.2E-09	-7.0±19.0	nd	0.1±22.8	1.2E-14
		9	6.2±11.2	2.6E-05	9.6±16.6	4.4E-05	8.9±23.9	1.5E-05
	Months in the Dark	3	8.4±9.6	1.1E-04	-17.4±14.9	nd	-5.5±19.1	nd
		6	6.5±9.6	3.4E-05	10.3±18.1	5.9E-05	4.2±20.4	4.0E-07
		9	3.1±6.6	9.9E-07	3.3±19.0	2.8E-07	-3.4±13.7	nd

4.2.1.3. **Ames test**

All the water samples were solid phase extracted and were assessed for mutagenicity at a concentration factor of 1000X with the Ames test performed with and without metabolic activator S9 by following the procedure described in Section 2.2.8

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 4.8**)

Table 4.8 Ames test results of leachates released from PMMA plastic polymer after 3, 6 and 9 months of exposure at different locations to sunlight or darkness.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as the mean ± standard deviation of the induction fold over the baseline. An induction fold over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Location	Exposure conditions		TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	2.00±1.00	3.00±1.00	1.91±0.58	2.24±0.58
		Positive control	22.67±1.53	22.83±1.53	24.95±0.58	21.39±2.00
Distilled water PSA at Almeria. Spain	Months of Sunlight exposure	3	0.41±2.65	0.98±0.58	0.42±2.65	0.50±0.00
		6	0.62±1.00	1.60±1.73	0.21±1.00	0.33±0.58
		9	1.03±1.00	1.60±2.65	0.21±0.00	0.17±0.58
	Months in the Dark	3	0.62±1.00	1.24±3.21	0.07±0.58	0.17±0.58
		6	0.62±1.00	1.78±2.52	0.00±0.00	0.33±1.15
		9	0.21±1.00	1.16±1.53	0.42±1.00	0.33±0.58
Well water PSA at Almeria. Spain	Months of Sunlight exposure	3	0.55±0.58	0.87±3.00	0.50±1.00	1.22±1.53
		6	1.04±3.46	1.86±3.21	0.08±0.58	0.70±0.58
		9	0.28±1.53	0.76±2.52	0.58±2.08	0.52±1.00
	Months in the Dark	3	0.48±2.31	0.93±1.15	0.25±1.00	0.35±0.58
		6	0.35±0.58	1.75±1.73	0.17±0.58	0.52±1.00
		9	0.69±3.21	2.04±2.52	0.17±0.58	0.35±0.58
DCU at Dublin. Ireland	Months of Sunlight exposure	3	0.56±2.08	0.09±0.58	0.87±1.15	0.54±0.58
		6	0.56±2.52	0.80±1.00	1.57±2.65	0.00±0.00
		9	0.34±1.00	1.42±1.15	0.35±1.15	1.61±1.73
	Months in the Dark	3	0.39±2.31	1.87±3.46	0.52±1.00	0.54±0.58
		6	0.68±1.00	1.60±3.00	0.35±1.15	1.34±1.53
		9	0.79±2.08	1.96±1.53	0.17±0.58	2.14±2.08
South Africa	Months of Sunlight exposure	3	1.28±0.58	1.28±0.58	0.59±1.15	0.59±1.15
		6	0.58±0.58	0.58±0.58	1.18±3.21	1.18±3.21
		9	1.05±2.00	1.05±2.00	1.06±2.00	1.06±2.00
	Months in the Dark	3	0.82±1.15	0.82±1.15	0.95±0.58	0.95±0.58
		6	0.47±0.58	0.47±0.58	0.71±2.65	0.71±2.65
		9	1.51±0.58	1.51±0.58	0.59±0.58	0.59±0.58
Uganda	Weeks of sunlight	3	0.42±1.53	0.56±1.53	0.40±1.73	0.27±1.00
	Weeks in the dark	3	0.42±1.53	0.22±0.58	0.35±2.08	0.63±1.15

4.2.2. Testing the PMMA reactors for leachates

The PMMA reactors were installed in South Africa and Uganda as described in Section 2.2.3 and tested for leachates between August 2018 and April 2019 using the E-screen (4.2.2.1) and the Ames test (4.2.2.2).

4.2.2.1. **E-screen**

Harvested rainwater from two different locations in South Africa (Bonfoi and Enkanini) serving 2 target rural communities were examined to ensure that local water and weather conditions in South Africa did not influence estrogenic activity. Monthly samples were taken from the harvested rainwater tank prior to disinfection and from the PMMA tubular reactor after the SODIS disinfection process. Harvested rainwater was collected from two different locations in Uganda (Arise and Shine School and Kabuyoga Primary School in rural Uganda). Samples were taken once from the collector tank prior to disinfection and once from the PMMA tubular reactor after the SODIS disinfection process.

Water samples were extracted and concentrated as described in Chapter 2. Extracts of water samples from the harvested rainwater tank and the SODIS reactor were only tested at 100X concentration factor. Proliferative effects, % RPE and EEQs were assessed for each sample in the presence and absence of ICI, an E2 receptor antagonist.

Extracts (100X) of water processed by the PMMA SODIS reactors at Bonfoi and Enkanini did not show proliferative activity relative to the negative control (**Fig 4.9**), neither did samples of water processed by the PMMA SODIS reactors at Uganda (data not shown)

Proliferative effects of extracts collected over 9 months from Aug 2018 to April 2019 in Bonfoi ranged between 81 and 105% of the negative control (100%). Proliferative effects of extracts from the harvested rainwater tank feeding the tubular PMMA SODIS reactor ranged between 76 and 98% of the negative control (100%). Estimation of corresponding RPE values shown in **Table 4.8** indicated that all extracts showed % RPE <10% and were not estrogenic. Coincubation of cells with extracts and ICI had no effect consistent with an absence of estrogenic leachates.

Proliferative Effects of extracts collected over 7 months between Aug 2018 and February 2019 in Enkanini ranged between 74 and 103% of the negative control (100%). Proliferative effects of extracts from harvested rainwater tank feeding the tubular PMMA SODIS reactor ranged between 71 and 88% of the negative control. Estimation of corresponding RPE values shown in **Table 4.8** indicates that all extracts showed % RPE <10% and were not estrogenic.

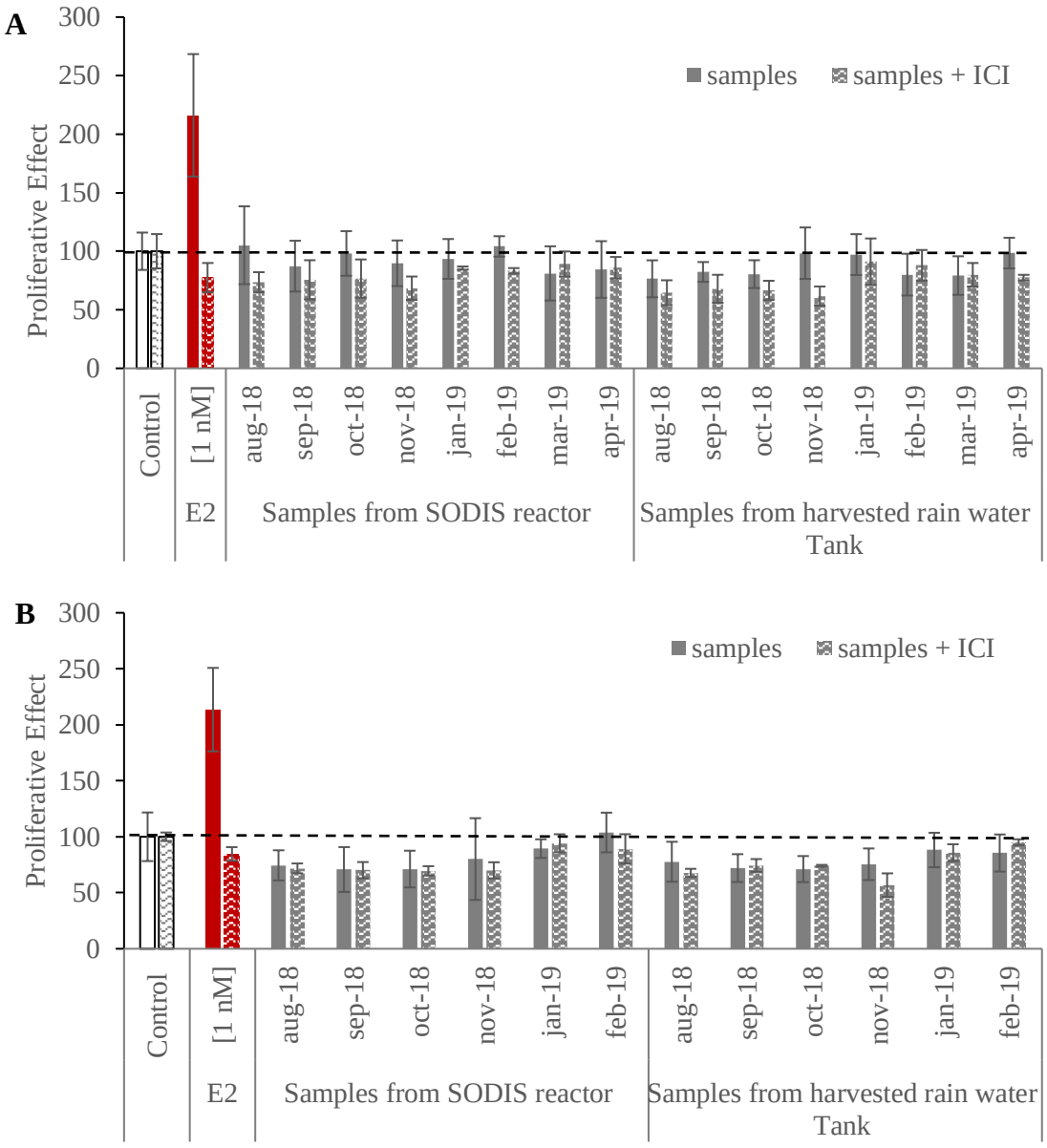


Figure 4.9 Proliferative effects of leachates from tubular PMMA SODIS reactor and its associated Harvested Rain Water Tank located at Stellenbosch, South Africa. *Seasonal water samples were collected after treatment from the tubular PMMA SODIS reactor and before treatment from its associated Harvested Rain Water Tank at two different locations at South Africa; Bonfoi (A), and Enkanini (B). The collected samples were concentrated 100 times with SPE and assessed for estrogenic activity in the presence and absence of ICI [0.1 μM].*

Table 4.9 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng/L) of leachates released from tubular PMMA SODIS reactor after treatment or released from the Harvested Rain Water Tank before treatment at Bonfoi and Enkanini, two different locations in South Africa. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

	Timepoint of collection ↓	Location			
		Bonfoi		Enkanini	
		RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
Samples from SODIS reactor (after treatment)	aug-18	6.1±30.6	2.4E-06	-15.5±8.6	nd
	sep-18	-7.6±22.6	nd	-12.8±15.3	nd
	oct-18	0.2±21.9	5.0E-13	-13.3±16.7	nd
	nov-18	-6.5±17.3	1.3E-08	-6.8±26.1	nd
	jan-19	-6.5±17.3	nd	-7.9±12.1	nd
	feb-19	6.5±11.4	3.3E-06	5.0±17.5	9.7E-07
	mar-19	-18.6±22.7	nd	-	-
	apr-19	-15.3±23.6	nd	-	-
Samples from harvested rain water Tank (before treatment)	aug-18	-14.9±20.0	nd	-11.6±12.5	nd
	sep-18	-2.5±28.7	nd	-10.1±17.6	nd
	oct-18	-8.3±18.6	nd	-12.8±19.4	nd
	nov-18	7.1±30.9	nd	-16.0±10.2	nd
	jan-19	-3.3±17.0	nd	-11.3±13.9	nd
	feb-19	-19.9±18.4	nd	-14.3±15.8	nd
	mar-19	-15.1±22.8	nd	-	-
	apr-19	-6.5±17.8	nd	-	-

4.2.2.2. **Ames test**

All the water samples were solid phase extracted and were assessed for mutagenicity at concentration factor of 1000X with the Ames test performed with and without metabolic activator S9 by following the procedure described in the Section 2.2.8

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 4.10**)

Table 4.10 Ames test results of leachates released from PMMA tubular SODIS reactor or its associated Tank for harvested rainwater collected at different seasonal timepoints at two locations of South Africa and two different locations at Uganda.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as the mean ± standard deviation of the fold induction over the baseline. A fold induction over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Location	Time point of collection ↓		TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	2.82±1.15	2.00±1.00	2.86±1.53	2.82±1.15
		Positive control	15.24±2.65	24.00±1.00	16.31±1.15	23.33±1.53
Bonfoi	Samples from PMMA SODIS reactor	aug-18	0.59±2.08	0.59±2.08	0.73±0.58	0.17±0.58
		sep-18	1.18±3.21	1.89±1.53	1.46±1.15	0.35±1.15
		oct-18	1.06±3.00	1.77±1.73	0.37±0.58	0.09±0.58
		nov-18	0.95±0.58	1.65±3.79	0.00±0.00	0.78±0.00
		jan-19	0.95±1.53	1.06±2.00	0.00±0.00	0.69±2.08
		feb-19	1.18±0.58	0.95±0.58	0.00±0.00	0.60±1.53
		mar-19	0.59±0.58	1.54±4.16	0.00±0.00	1.04±2.65
		apr-19	0.59±0.58	1.54±1.15	0.73±1.15	0.78±1.73
	Samples from harvested rainwater Tank	aug-18	0.35±1.73	1.18±3.51	1.46±1.15	0.35±1.15
		sep-18	1.18±1.53	1.77±2.00	1.83±1.15	0.43±1.15
		oct-18	0.47±1.53	1.89±2.08	1.10±0.00	0.26±0.00
		nov-18	0.95±0.58	0.47±1.53	0.37±0.58	0.52±1.00
		jan-19	0.95±0.58	1.54±3.51	0.37±0.58	0.78±1.00
		feb-19	0.95±0.58	1.30±1.53	0.37±0.58	1.04±1.00
		mar-19	0.47±0.58	1.30±1.53	0.37±0.58	1.12±2.08
		apr-19	0.83±0.58	0.83±2.31	0.37±0.58	0.78±1.00
Enkanini	Samples from PMMA SODIS reactor	aug-18	1.17±0.58	1.12±1.53	0.47±1.15	0.24±1.15
		sep-18	0.93±1.53	0.95±2.52	0.24±0.58	0.12±0.58
		oct-18	1.17±1.15	1.12±0.58	0.47±0.58	0.24±0.58
		nov-18	0.35±1.00	0.86±0.58	0.00±0.00	0.77±2.52
		jan-19	0.93±0.58	0.52±1.73	0.12±0.58	0.71±2.00
		feb-19	0.70±1.00	0.52±1.00	0.47±0.58	0.47±2.08
	Samples from harvested rain water Tank	aug-18	0.70±2.00	0.95±1.53	0.59±1.15	0.30±1.15
		sep-18	0.93±2.31	0.86±1.15	0.71±1.00	0.59±1.53
		oct-18	0.58±1.15	0.43±1.15	0.12±0.58	0.06±0.58
		nov-18	1.05±1.73	0.43±1.15	0.24±0.58	0.59±1.15
		jan-19	0.82±2.31	0.69±1.53	0.00±0.00	0.53±0.00
		feb-19	0.70±2.00	0.78±3.00	0.12±0.58	0.89±0.00
Uganda	Arise School SODIS reactor		0.26±1.53	0.67±1.73	0.09±1.15	0.80±2.00
	Arise School Tank		0.42±1.53	0.56±0.58	0.18±0.58	0.63±1.53
	Kabuyoga School SODIS reactor		0.52±1.53	0.78±1.53	0.31±1.53	0.45±1.53
	Kabuyoga School Tank		0.47±1.73	0.89±1.53	0.44±2.08	0.27±0.00

4.3. Summary of the toxicological studies on the novel PMMA reactors

Testing of PMMA plastic polymer extractables:

- There was a direct correlation between the increase in temperature and time of incubation with the release of estrogenic substances (**Table 4.11**). No estrogenic activity was detected when samples were extracted at room temperature however estrogenic activity was detected when samples were extracted for extended time at 37 °C and when extracted at 50 °C.
- Detection of estrogenic activity in the extracts was affected by the age of the plastic and the solvent used. In the case of raw PMMA estrogenic activity was only detected when 100-fold DMEM was used. At elevated temperatures and times of extraction all solvents extracted estrogenic activity in 3-month aged PMMA. Only 100-fold DMEM extracted estrogenic activity in 6-month aged PMMA and no estrogenic activity was detected in 9-month aged PMMA.
- No mutagenicity was detected in any sample

The findings of the toxicological assessments of estrogenic activity for extractables from the novel PMMA extractables are summarised in **Table 4.11**.

Table 4. 11. Summary of the statistically significant extractables from the plastics PMMA samples for assessments of estrogenic activity.

Plastic stage		solvent	Temperature extraction conditions							
			20 °C 24 h	20 °C 72 h	37 °C 5 h	37 °C 15 h	37 °C 24 h	37 °C 72 h	50 °C 5 h	50 °C 15 h
PMMA	raw	Ethanol								
		DMEM (100-fold)				+	+	+	+	+
		DMEM (10-fold)								
	3-months-aged	Ethanol						+	+	+
		DMEM (100-fold)						+	+	+
		DMEM (10-fold)					+	+	+	+
	6-months-aged	Ethanol								
		DMEM (100-fold)					+	+	+	+
		DMEM (10-fold)								
	9-months-aged	Ethanol								
		DMEM (100-fold)								
		DMEM (10-fold)								

Testing the water from the PMMA reactor for leachates:

- When pieces of PMMA were immersed in water for 3-, 6- and 9-month periods of time in distinct geographic locations no oestrogenic activity or mutagenicity was detected in the water.
- Water sampled from the PMMA tubular SODIS reactors used in field studies over a calendar year showed no estrogenic or mutagenic activity.

Chapter V

5. Toxicological assessments of the transparent PET jerrycan SODIS reactor.

PET has been one of the main plastic polymers used in SODIS reactors; however, evidence that this polymer is usually synthesised using additives that have the ability to influence the endocrine system of vertebrates by mimicking or interfering with endogenous hormones has sparked concern. During SODIS plastics are exposed to a variety of weather and environmental conditions (temperature, UV, reuse, etc) which may induce the release of these plastic additives and monomers into water. To better understand this process, this investigation aimed to analyse a variety of normal and worst-case scenarios and conditions to assess which of them might mediate release of toxic compounds. The PET plastic was tested for extractables (5.1) and the water from the reactors was tested for leachates (5.2).

5.1. Testing the PET plastic for extractables

PET plastic used in the construction of the PET reactors was tested for extractables as outlined in section 2.2.2. Samples of both raw plastic (5.1.1) and aged plastic (5.1.2) were tested using the E-screen assay for estrogenic activity and the Ames test for mutagenicity (5.1.3).

5.1.1. Testing raw PET for extractables with the E-screen bioassay

PET was extracted into two different solvents. Media extracts were diluted 10- and 100-fold and applied to the cells. Ethanolic extracts were diluted and tested only at one concentration (100-fold dilution) so the final concentration of EtOH was 1% (which is the maximum concentration of solvent at which no cytotoxic effects were observed). During the E-Screen assay (carried out as described in the methodology) the proliferative effects of each extract in the presence and absence of the estrogen antagonist ICI [0.1 μM] were assessed by measuring cell proliferation with the DNA quantification method described in the methodology section. The corresponding RPE values of each sample and the EEQs of all the samples were calculated. EEQs lower than 0.01 ng/L and/or corresponding to RPE values lower than 10% are not considered estrogenic samples. Each sample was measured in triplicate wells and each experimental plate was replicated three times.

In some cases, due to variabilities in weather conditions, such as cloudiness, SODIS treatment had to be held for more than 6 h in order to achieve inactivation of pathogens.

Therefore, this investigation aimed to analyse how the duration of exposure to different thermal stresses influenced the migration of estrogenic leachates from raw PET plastic cut pieces from the transparent jerrycan SODIS reactor.

The extractant solvent used had a profound effect on the release of extracts with estrogenic activity from raw PET plastic. Extraction of raw PET into DMEM/F12 culture medium solvent yielded extracts (100-fold diluted) that were not estrogenic (**Fig 5.1**). Interestingly, analysis of more concentrated extracts of raw PET in DMEM/F12 medium (10-fold dilution) showed a trend towards proliferation but, except for one condition, it was not deemed statistically significant (**Fig 5.2**). Only exposure of raw PET to culture medium at 50 °C for 15 h of a 72-h period induced a proliferative response (155 ± 43%) that was significantly higher ($p < 0.05$) than the negative control. These two results together suggested a concentration-dependent dose-response relationship of the proliferative effects exerted by the DMEM/F12 culture medium PET extracts.

On the other hand, ethanolic extracts of raw PET (100-foold diluted) exerted significant effects on cell proliferation relative to the negative control (**Fig 5.1**). Estrogenic activity was directly related to time and temperature of extraction for ethanolic extracts. Raw PET subjected to 20 °C for 24 h and 72 h or to 37 °C for 5 h within a 24 h period yielded ethanolic extracts that had negligible effects on proliferation. Longer extraction periods in ethanol solvent at 37 °C yielded extracts showing significant ($p < 0.05$) increase on the proliferative activity. Raw PET plastic in ethanol exposed to 37 °C for 15 h and 24 h period released extracts that increased proliferation by 20% and 30% respectively relative to the negative control. Continuous exposure of raw PET in ethanol solvent to 37 °C for 72 h increased proliferation by 44 % relative to the negative control. Increasing the extraction temperature to 50 °C also yielded a significant ($p < 0.05$) increment on the proliferative activity relative to the negative control. Exposure of raw PET in ethanol solvent to 50 °C for periods of 5 h over a 24 h period and 15 h over a 72-h period yielded extracts that increased proliferation by 46 % and 56 % respectively.

Co-incubation of raw PET extracts with ICI [0.1 μM], an ER antagonist significantly inhibited the proliferation of all extracts.

The statistically significant estrogenic effects of the raw PET extract in DMEM/F12 (10-fold dilution) relative to E2 (%E2 or RPE) amounted to 51.4 % and in ethanol ranged approximately between 24-52 %. Thus, extracts of raw PET obtained after 15 h at 50 °C contained estrogenic components showing partial agonistic activity (**Table 5.1**). Corresponding estradiol equivalents (EEQs) of the raw PET extracts in culture medium and ethanol that reached maximum proliferative effects were approximately 66 ng/L and 685 ng/L respectively.

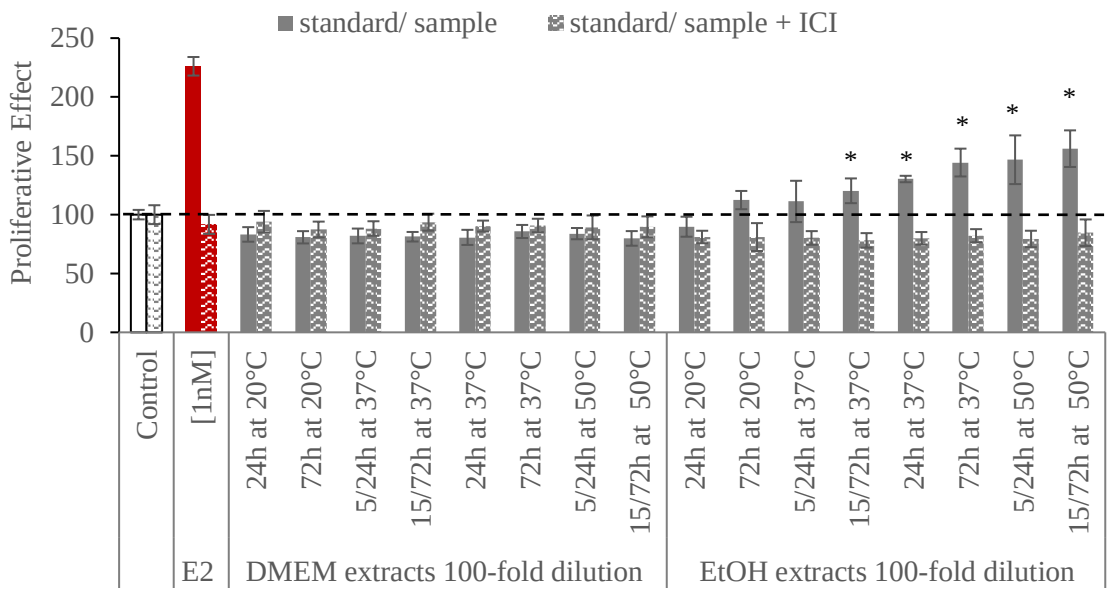


Figure 5.1 Proliferative effects of raw PET extracts (100-fold dilution). *Raw PET was submerged in DMEM /F12 media solvent or in EtOH solvent and exposed to different temperature conditions. Extractables were diluted 100x and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

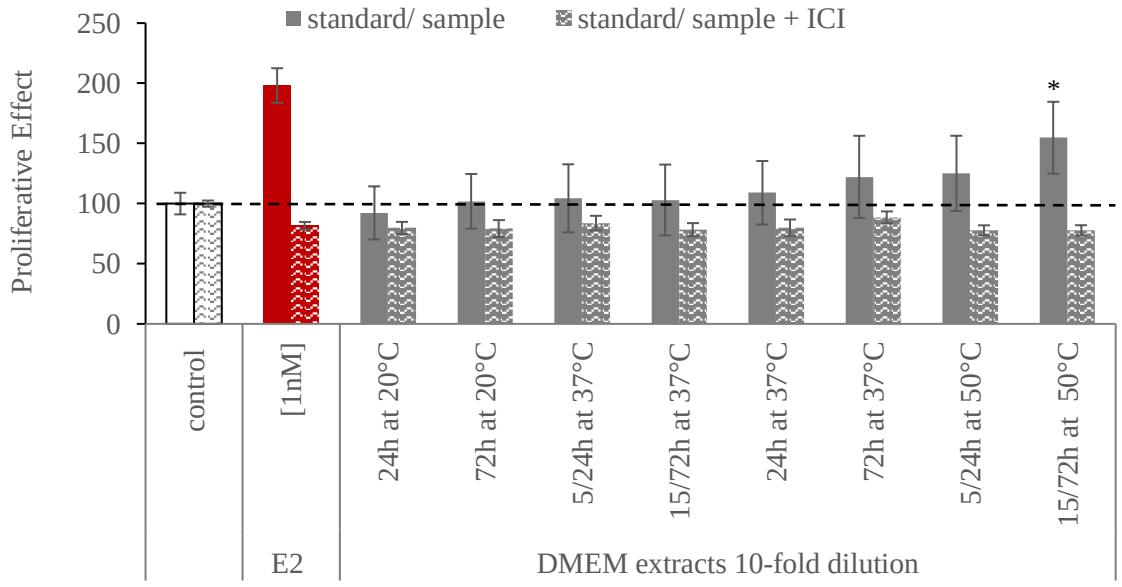


Figure 5.2 Proliferative effects of raw PET extracts (10-fold dilution). *Raw PET was submerged in DMEM/F12 media solvent and exposed to different temperature conditions. Extractables were diluted 10x and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

Table 5.1 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of exudates leached from raw PET polymer into two solvents (DMEM/F12 and ethanol) during thermal stress. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

	DMEM media solvent				EtOH solvent	
Dilution →	1:10		1:100		1:100	
Temperature condition ↓	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
24h at 20 °C	-8.1±12.0	nd	-20.7±7.5	nd	-8.1±19.1	Nd
72h at 20 °C	2.3±15.6	2.4E-05	-23.6±5.8	nd	8.5±20.4	0.1
5/24h at 37 °C	7.7±11.5	7.4E-03	-22.3±7.6	nd	24.0±12.8	17.4
15/72h at 37 °C	3.4±14.9	1.5E-04	-23.1±4.3	nd	34.4±27.4	96.3
24h at 37 °C	1.0±13.3	4.6E-07	-23.5±6.7	nd	33.4±19.8	84.2
72h at 37 °C	18.6±19.8	0.5	-17.6±6.7	nd	29.0±28.0	42.7
5/24h at 50 °C	27.2±14.6	3.1	-20.1±6.6	nd	38.1±27.7	157.9
15/72h at 50 °C	51.4±28.6	66.3	-25.2±8.4	nd	51.8±11.3	684.8

5.1.2. Testing aged PET for extractables with the E-screen bioassay

The constant use of the SODIS reactors and their constant exposure to sunlight could have an effect on the chemical stability and robustness of the plastic components of the reactor. This implies that the aging of the plastic could have a critical effect on the rate of release of substances with estrogenic activity.

In order to mimic the aging process, plastic pieces of PET were exposed to the weather conditions in Plataforma Solar of Almeria, Spain for 3, 6 and 9 months. Extractables of the plastic pieces were sampled as indicated in Chapter 2 with various temperature stress conditions. Aged PET plastic extracts were diluted 10-fold and 100-fold as previously stated prior to E screen analysis.

PET plastic aged for 3 months, 6 months and 9 months and subjected to extraction in either DMEM/F12 or ethanol under varying time and temperature conditions yielded 100-fold diluted extracts that were non-estrogenic (**Fig 5.3**). Co-incubation of 100-fold diluted extracts with ICI had negligible effects on proliferation confirming the absence of estrogenic effects in 100- fold diluted aged PET extracts.

However, analysis of more concentrated PET extracts (10-fold dilution) did reveal estrogenic activity when aged PET was subjected to thermal stress (**Fig 5.4**). Results from **Fig 5.3** and **Fig 5.4** suggest a concentration-dependent relationship between the induced proliferative effects and

the concentrations of the extracts from aged PET plastic. This linear dose-response relationship was also observed on the extracts from raw PET plastic (**Figs 5.1-5.3** Section 5.1.1).

Three-month aged PET subjected to 20 °C for 24 h and 72 h or to 37 °C for up to 72h yielded extracts (10- fold diluted) that had negligible effects on proliferation (**Fig 5.4A**). Increasing the extraction temperature to 50 °C yielded significant ($p < 0.05$) increased proliferative activity relative to the negative control. Exposure of 3-months-aged PET to 50 °C for 5h over 24 h period and 15 h over a 72-h period yielded extracts (10- fold diluted) that increased proliferation by 46 % and 60 % respectively. Co-incubation of aged PET extracts with ICI [0.1 μM] confirmed estrogenic activity of 3-months aged PET plastic extracts obtained under thermal stress. A similar pattern was observed in 6-months and 9-month aged PET plastics (**Fig 5.4B and C**) with a trend towards estrogenic activity at 37 °C. Increasing the extraction temperature to 50 °C yielded significant ($p < 0.05$) increased proliferative activity relative to the negative control. Exposure of 6-month aged PET to 50 °C for 5h over 24-h period and 15 h over a 72-h period yielded extracts (10- fold diluted) that significantly increased proliferation by 48 % and 70 % respectively. Exposure of 9-month aged PET to 50 °C for 5h over 24-h period and 15 h over a 72-h period yielded extracts (10- fold diluted) that significantly ($p<0.05$) increased proliferation by 44 % and 78 %.

The maximum relative estrogenic effect of the 10-fold diluted aged PET extracts in DMEM/F12 amounted to 60 %, 54 % and 53 % after 3-, 6- and 9-months-age respectively. Thus, extracts of aged PET obtained after 15 h at 50 °C contained estrogenic components showing partial agonistic activity (**Table 5.2**). Corresponding estradiol equivalents (EEQs) of aged PET extracts amounted to 140, 84 and 79 ng/L respectively.

Results from **Fig 5.3** and **Fig 5.4** suggested that when the PET plastic from the SODIS Jerrycan ages and the chemical bonds between the plastic polymer and the additives used in the synthesis of the plastic has become weaker, polar solvents such as media, or water are able to extract substances with estrogenic activity under less harsh conditions than other solvents such as ethanol. Results from **Fig 5.1** and **Fig 5.2** suggested that ethanol was a more efficient solvent to get extractables with estrogenic activity from raw PET plastic from the SODIS jerrycan reactor than from the aged PET plastic from the SODIS jerrycan reactor.

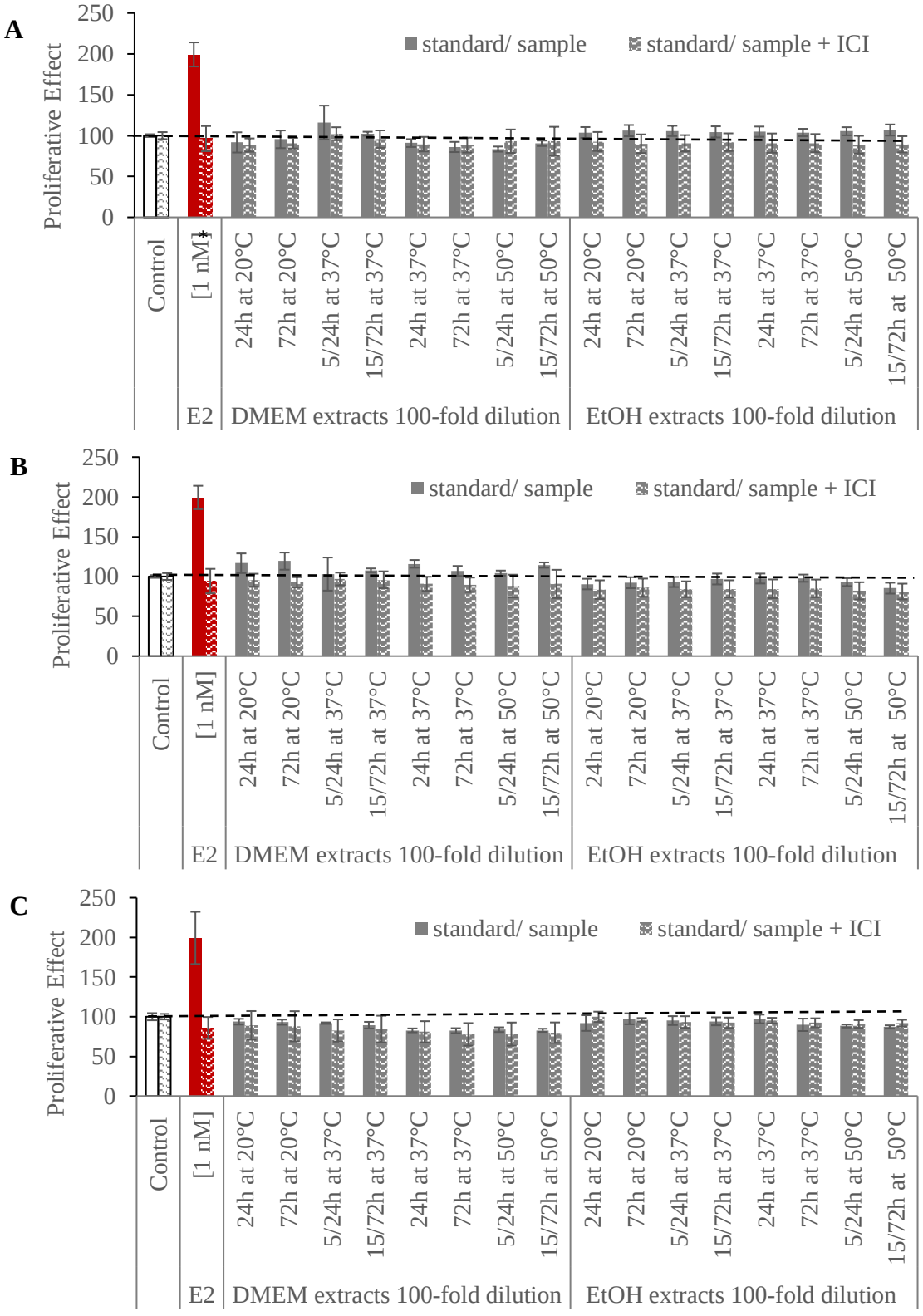


Figure 5.3 Proliferative effects of aged PET extracts (100-fold diluted) *3-months aged (A), 6-months aged (B) and 9-months aged (C) PET was submerged in DMEM/F12 media or EtOH solvent and exposed to different temperature conditions. Extractables were diluted 100 times and screened for estrogenic activity in the presence and absence of ICI [0.1 µM].*

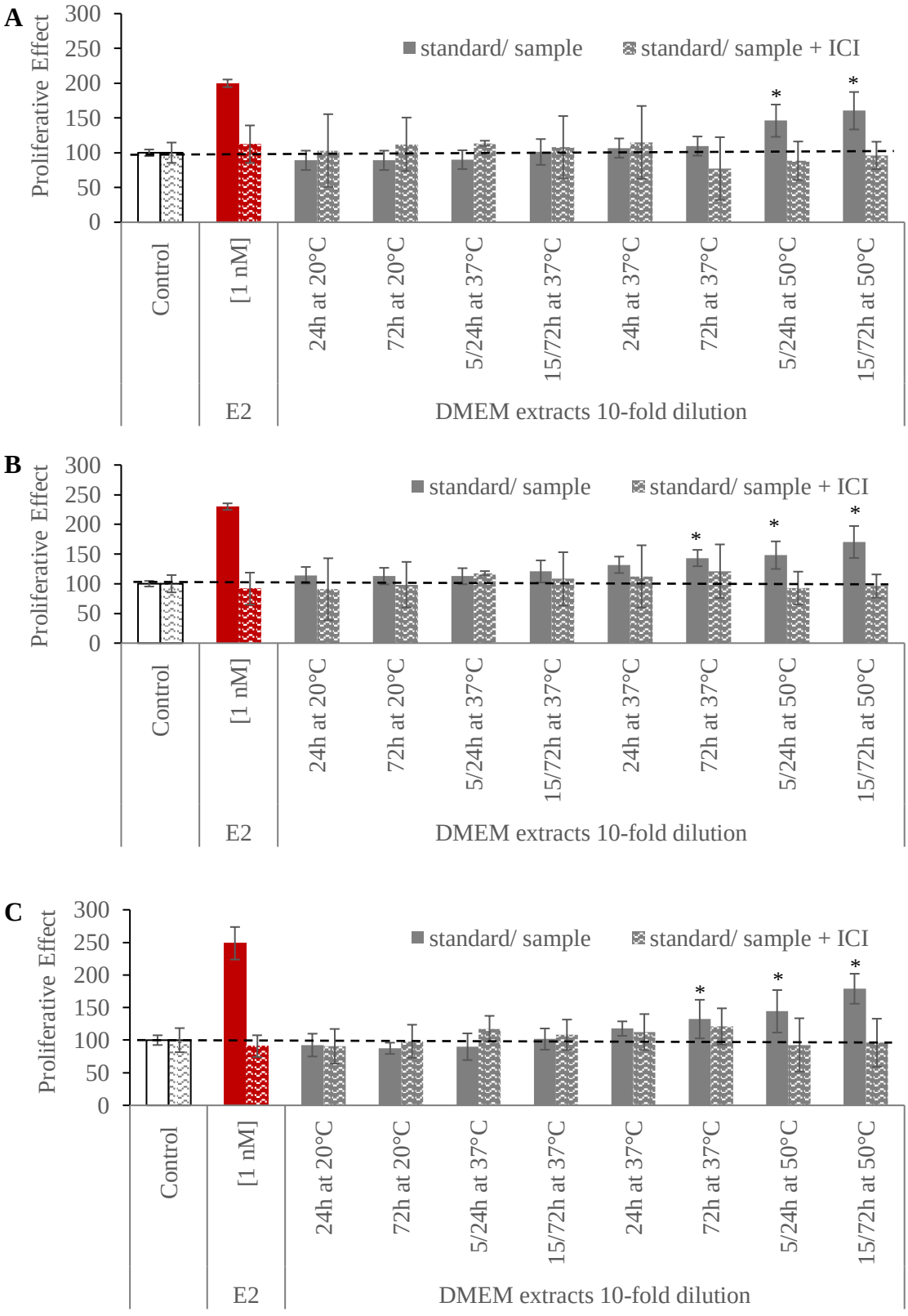


Figure 5.4 Proliferative effects of Aged PET polymer (10-fold diluted). *3-months aged (A), 6-months aged (B) and 9-months aged (C) PET was submerged in DMEM/f12 media solvent and exposed to different temperature conditions. Extractables were diluted 10 times and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

Table 5.2 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of exudates leached from 3-month, 6-month and 9-month-aged PET plastic polymer into two solvents (DMEM/F12 and ethanol) during thermal stress. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

		DMEM media solvent				EtOH solvent	
	Dilution →	1:10		1:100		1:100	
	Temperature condition ↓	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
3-months-age	24h at 20 °C	-10.3±15.3	nd	-10.3±15.3	nd	4.7±8.2	7.0E-03
	72h at 20 °C	-10.8±13.8	nd	-5.6±13.4	nd	7.5±8.6	0.1
	5/24h at 37 °C	-10.0±13.8	nd	11.3±18.4	0.5	4.7±10.2	7.0E-03
	15/72h at 37 °C	1.2±18.9	1.1E-06	1.9±6.6	8.8E-05	5.6±8.5	1.7E-02
	24h at 37 °C	6.9±14.1	4.4E-03	-11.3±6.0	nd	8.5±10.3	0.1
	72h at 37 °C	9.8±14.1	2.4E-02	-15.0±9.9	nd	4.7±5.6	7.0E-03
	5/24h at 50 °C	45.8±22.3	38.0	-17.8±8.6	nd	6.6±6.1	3.5E-02
	15/72h at 50 °C	60.1±25.9	139.5	-14.1±6.0	nd	8.5±8.5	0.1
6-months-age	24h at 20 °C	5.1±0.3	1.0E-03	7.7±8.0	0.1	-11.6±10.3	nd
	72h at 20 °C	9.8±3.2	2.4E-02	7.3±12.0	0.1	-10.1±4.2	nd
	5/24h at 37 °C	9.4±10	2.0E-02	-0.2±16.3	nd	-12.8±9.2	nd
	15/72h at 37 °C	10.4±7.2	3.1E-02	0.4±7.9	6.9E-08	-3.7±12.4	nd
	24h at 37 °C	25.5±2.8	2.3	7.2±17.0	0.1	-3.0±14.2	nd
	72h at 37 °C	33.4±8.9	8.4	-1.3±28.4	nd	-1.0±15.3	nd
	5/24h at 50 °C	36.8±16.8	13.3	-4.6±16.0	nd	-7.7±13.4	nd
	15/72h at 50 °C	54.1±9.5	84.1	5.0±18.5	9.7E-03	-19.2±6.8	nd
9-months-age	24h at 20 °C	-16.8±8.1	nd	-4.1±11.3	nd	-10.1±7.7	nd
	72h at 20 °C	-13.5±6.0	nd	-5.1±7.1	nd	-3.7±7.5	nd
	5/24h at 37 °C	-5.5±13.2	nd	-9.4±3.5	nd	-7.0±7.2	nd
	15/72h at 37 °C	1.5±10.8	2.8E-06	-9.9±8.5	nd	-2.3±19.4	nd
	24h at 37 °C	9.7±4.7	2.3E-02	-17.5±6.6	nd	-4.5±10.0	nd
	72h at 37 °C	23.1±19.9	1.4	-18.6±5.8	nd	-14.1±3.3	nd
	5/24h at 50 °C	30.7±20.9	5.6	-17.5±6.8	nd	-16.1±3.5	nd
	15/72h at 50 °C	53.3±15.6	78.7	-18.7±5.9	nd	-17.6±5.8	nd

5.1.3. Testing raw and aged PET with the Ames test

During solar disinfection, the components of the reactors are exposed to a lot of stress factors, such as high temperatures and continuous reuse of the plastics (aging). To better understand how these stress factors, affect the migration of substances from the plastic components during SODIS, analysis of how duration of exposure to different thermal stresses influenced the migration of mutagenic and genotoxic extractables from raw PET SODIS reactor plastic components was performed.

All extractables were assessed for mutagenicity with the Ames test performed with and without metabolic activator S9 by following the procedure described in Section 2.2.8. The extractables were sampled from both raw and aged PET plastic as described in Section 2.2.2. In order to simulate the aging process of the PET plastic, raw PET plastic pieces were exposed to weather conditions for 3, 6 and 9 months in the Plataforma Solar of Almeria. Spain.

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 5.3**).

Table 5.3 Ames test results of extractables released from PET plastic polymer at different aged states after different temperature extraction treatments.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as fold induction over the baseline which is the mean of revertant relative to the baseline ± standard deviation. A fold induction over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Age	Extraction solvent	Treatment	TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	1.82±1.15	1.24±0.58	2.86±1.53	2.73±1.73
		Positive control	38.58±0.00	36.17±0.00	26.35±0.00	16.23±1.15
Raw	PBS	24h at 20 °C	0.54±0.58	0.18±0.58	0.12±0.58	0.00±0.00
		72h at 20 °C	0.54±0.58	0.37±0.58	0.23±1.15	0.00±0.00
		5/24h at 37 °C	1.07±1.53	0.92±1.15	0.00±0.00	0.00±0.00
		15/72h at 37 °C	0.27±0.58	1.10±0.00	0.23±0.58	0.00±0.00
		24h at 37 °C	1.34±0.58	0.73±0.58	0.47±0.58	0.24±0.58
		24h at 37 °C	1.88±0.58	1.46±1.15	0.23±0.58	0.12±0.58
	EtOH	24h at 20 °C	0.27±0.58	0.12±0.58	0.22±0.58	0.21±0.58
		72h at 20 °C	0.00±0.00	0.12±0.58	0.22±1.15	0.21±1.15
		5/24h at 37 °C	1.07±1.15	0.47±1.15	0.56±1.15	0.63±1.00
		15/72h at 37 °C	0.00±0.00	0.24±1.15	0.33±1.73	0.31±1.73
		24h at 37 °C	0.54±0.58	0.12±0.58	0.67±1.00	0.52±1.15
		24h at 37 °C	0.54±0.58	0.24±0.58	0.00±0.00	0.00±0.00
3 months	PBS	24h at 20 °C	0.42±0.58	0.29±0.58	0.00±0.00	0.33±1.15
		72h at 20 °C	1.08±3.21	0.21±0.58	0.17±0.58	0.50±0.00
	EtOH	24h at 20 °C	0.40±0.58	0.32±0.58	0.00±0.00	0.22±1.15
		72h at 20 °C	0.40±1.53	0.23±0.58	0.12±0.58	0.33±0.00
6 months	PBS	24h at 20 °C	0.42±1.15	0.22±1.15	0.00±0.00	0.50±1.00
		72h at 20 °C	0.52±1.53	0.06±0.58	0.50±0.00	1.00±1.00
	EtOH	24h at 20 °C	0.70±1.15	0.19±1.15	0.00±0.00	0.33±1.00
		72h at 20 °C	0.87±1.53	0.05±0.58	0.35±0.00	0.67±1.00
9 months	PBS	24h at 20 °C	0.18±0.00	0.08±0.58	0.17±0.58	0.53±1.00
		72h at 20 °C	0.12±0.58	0.33±1.53	0.17±0.58	0.53±2.00
	EtOH	24h at 20 °C	0.21±0.00	0.08±0.58	0.17±0.58	1.00±1.00
		72h at 20 °C	0.14±0.58	0.34±1.53	0.17±0.58	1.00±2.00

5.2. Testing water from the transparent jerrycan for leachates

Unlike extractables, which are all the chemical substances that have been released from plastic under extreme conditions, leachates are substances that migrate from the plastic to its surroundings during regular use. Since, PET plastic released into DMEM/F12 medium (10-fold dilution) substances with estrogenic activity after exposure to extreme temperature extraction conditions, since the SODIS process involves continuous re-exposure of the reactor to sunlight and other weather conditions, this study assessed if weather conditions promoted the release of leachates from the plastic reactor into water.

To test the PET reactors for leachates, 25 L transparent jerrycans were filled with well water and exposed to the weather conditions of PSA in Spain for 3, 6 and 9 months. Reactors filled with well water and stored in the dark for the same period of time served as controls. The behavior of the novel 25 L PET jerrycan reactor was compared to that of 2 L PET bottles which are the most commonly used reactor for the SODIS process. The water was tested using the E-screen assay (5.2.2) and the Ames test (5.2.3).

5.2.1. Salinity and conductivity of the water in the PET reactors

Well water collected at 3, 6 and 9 months from the 25 L PET jerrycan and 2 L PET bottle was analyzed with respect to mineral composition and conductivity as described in Chapter 2. The composition of well water collected from both SODIS reactors was not significantly altered over the course of the 9 months. There was a trend towards a decrease in chloride ions, sulfate and potassium in water collected from 25 L PET jerrycan at 9 months relative to 3 months. A similar trend towards a decrease in chloride and sulfate in water collected from 2 L PET bottle at 9 months relative to 3 months was noted (**Table 5.4**). There were no significant differences between the conductivity of the water from different reactors after different periods of exposure.

Table 5.4 Salinity and conductivity measurements of the well water exposed to sunshine in PET 25 L transparent jerrycan at PSA, Almería, Spain.

		PET Jerrycan SODIS reactor			PET Bottle SODIS reactor		
		3 months	6 months	9 months	3 months	6 months	9 months
Salinity (mg/L)	Chloride	31.6±0.4	31.0±0.1	29.7±0.7	33.7±0.2	41.4±0.6	29.7±1.6
	Nitrite	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Nitrate	0.6±0.0	0.5±0.0	0.5±0.0	0.5±0.0	1.4±0.0	0.5±0.0
	Phosphate	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Sulfate	24.0±0.4	22.9±0.4	21.3±1.1	25.6±0.1	35.4±0.5	21.9±1.4
	Sodium	46.6±0.6	47.3±0.2	48.0±0.6	49.6±0.2	44.6±0.5	46.7±1.8
	Ammonium	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0
	Calcium	0.7±0.1	0.9±0.1	0.9±0.2	0.9±0.0	0.7±0.1	0.7±0.1
	Potassium	0.5±0.2	0.3±0.2	0.2±0.1	0.4±0.1	0.8±0.0	0.3±0.3
	Magnesium	6.9±0.2	6.8±0.1	6.5±0.3	6.8±0.1	8.4±0.1	6.3±0.4
Conductivity mS/cm		2.4±0.0	2.4±0.1	2.4±0.0	2.6±0.0	2.6±0.0	2.5±0.0

5.2.2. Testing the water using the E-screen bioassay

After the exposure period, 1L of water sample was solid phase extracted as described in Chapter 2. The DMSO extract (100 µl) was diluted in DMEM/F12 hormone-free medium to yield final concentration factors of 100X, 50X and 10X when compared to the original water sample.

The concentrated extracted samples of leachates were then screened for estrogenic activity in the presence and absence of ICI [0.1 µM] as described in Chapter 2. The proliferative effects and the RPE of each sample were calculated by measuring cell proliferation with the DNA quantification method. EEQs of each concentrated extracted sample of leachates were assessed in order to determine if the samples were in compliance with the ADI recommended by the (JEFCA, 2000).

Exposure of 25 L PET jerrycans containing well water to sunlight did not yield significant estrogenic activity in extracts that were concentrated 10X and 50X times concentrated. Well water extracts that were 10X and 50X concentrated showed proliferative effects similar to the negative control irrespective of the duration of exposure to sunlight or darkness (**Fig 5.5**). Coincubation with ICI had negligible effect. However, well water extracts that were 100X concentrated did show a time-dependent estrogenic activity. Extracts prepared from well water stored in 25 L PET jerrycan for 3, 6 and 9 months in the sun showed proliferative activity that was 74 %, 115 % and 162 % relative to the negative control (100%). Corresponding values for water stored in the dark were 75 %, 101 % and 95 % relative to the negative control (100%). Co-incubation of 9-month extracts with ICI [0.1 µM] mitigated the proliferative response confirming estrogenic activity of the 9-month 100X concentrated extracts.

A similar pattern was observed with 2 L PET SODIS bottles. Estrogenic activity was not detected in well water extracts that were 10X and 50X concentrated (**Fig 5.5**). Proliferation of 10X and 50X extracts obtained after 3, 6 and 9 months was similar to the negative control irrespective of exposure to sunlight or darkness. Coincubation with ICI had negligible effect on the sample proliferation. However, well water extracts that were 100X concentrated did show a time-dependent estrogenic activity. Extracts prepared from well water stored in 2 L PET bottles for 3,6 and 9 months in the sun showed proliferative activity that was 57 %, 67 % and 138 % relative to the negative control (100%). Corresponding values for water stored in the dark were 62 %, 67 % and 95 % relative to the negative control (100%). Coincubation of 9 month extracts with ICI [0.1 μM] mitigated proliferation confirming estrogenic activity of the 9 month 100X extracts.

Of note, proliferative effects lower than the negative control (100%) exerted by 100X well water extracts from 2 L PET bottles exposed to sun for 3 and 6 months can be observed (**Fig 5.5B and C**) and in 100X well water extracts from 25 L jerrycans exposed to sun for 3 months (**Fig 5.5C**).

The maximum relative estrogenic effects of the water extracts from the 25 L PET jerrycan and 2 L PET bottle reactors after 9 months amounted to 69.7 % and 48.7 % respectively. Thus, water extracts from both PET SODIS reactors contained estrogenic components showing partial agonistic activity (**Table 5.5**). Corresponding eestradiol equivalents (EEQs) of water extracts amounted to 0.3 and 0.05 ng/L respectively.

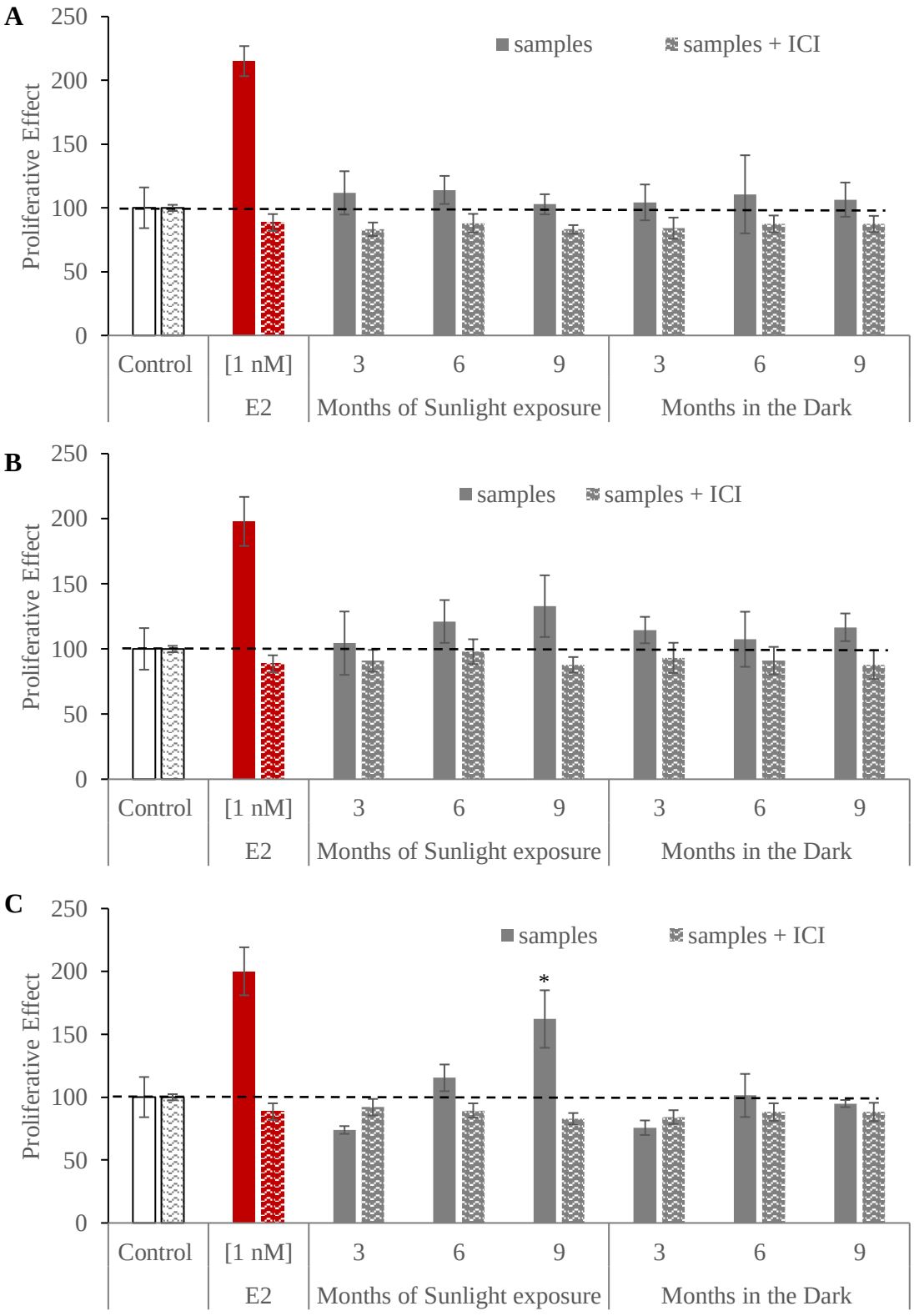


Figure 5.5 Proliferative effects of 25 L PET jerrycan reactor leachates in well water at Almería, Spain. *Leachates released into well water after 3, 6 and 9 months of exposure of 25 L PET jerrycan reactors to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

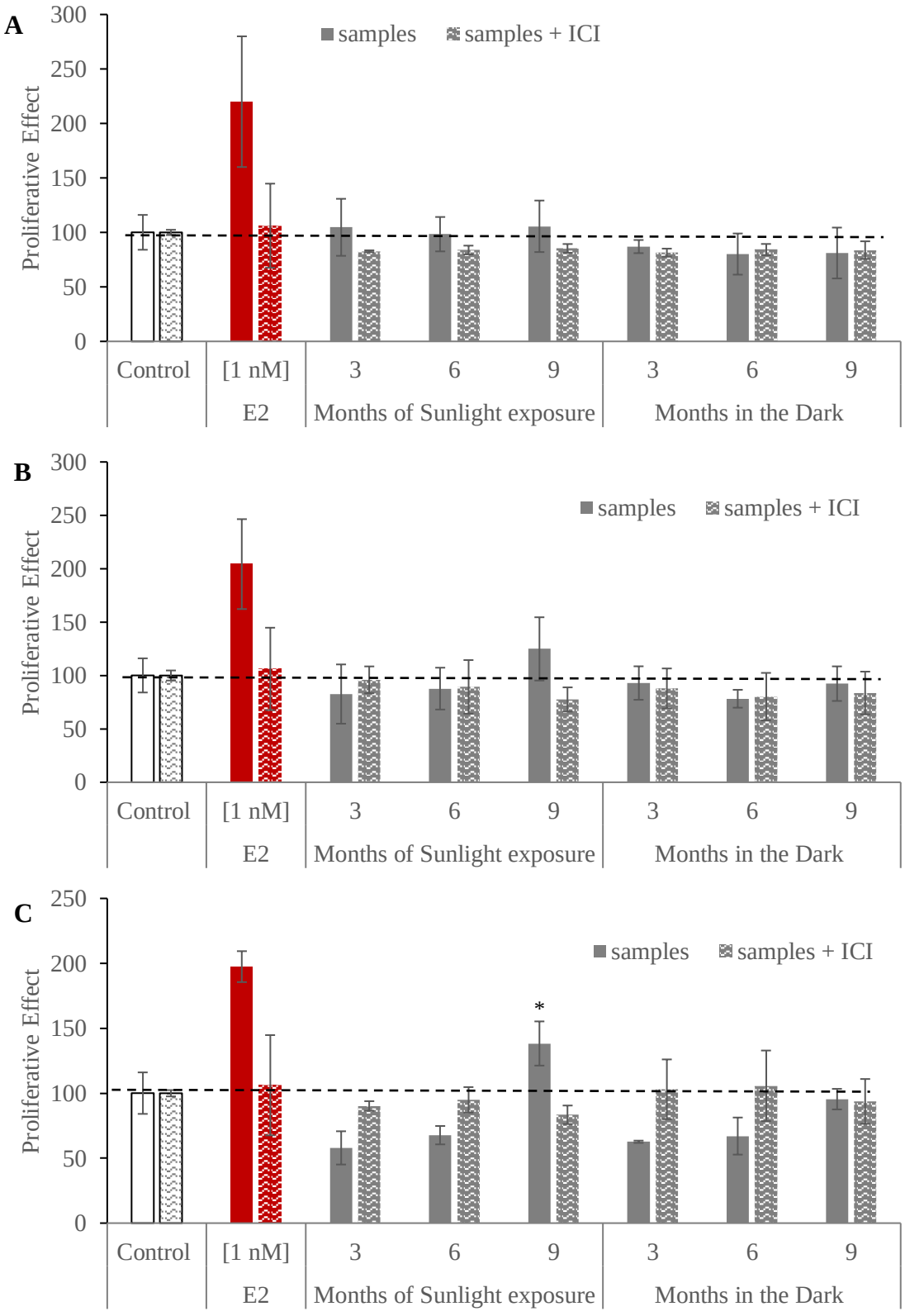


Figure 5.6 Proliferative effects of 2 L PET bottle reactor leachates in well water at Almería, Spain. *Leachates released into well water after 3, 6 and 9 months of exposure of 2 L PET bottle reactors to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

Table 5.5 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of leachates released from PET SODIS reactors into well water after 3-,6- and 9-months exposure to sunlight and darkness at Almeria, Spain. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

			concentration factor					
			10X		50X		100X	
			RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
Jerrycan SODIS reactor	Months of exposure to Sunlight	3	7.4±25.8	6.2E-05	3.2±29.7	1.1E-06	-69.5±21.4	nd
		6	8.9±25.8	1.5E-04	19.2±15.5	6.0E-03	-0.3±25.0	nd
		9	-5.1±27.3	nd	39.6±19.5	3.8E-02	69.7±30.3	0.3
	Months in the Dark	3	-3.7±13.7	nd	9.4±23.0	2.0E-04	-66.4±17.2	nd
		6	1.5±12.3	3.5E-08	7.9±26.8	8.4E-05	-24.9±31.1	nd
		9	-8.9±23.0	nd	11.5±16.3	5.1E-04	-32.5±29.8	nd
2 L PET bottle SODIS reactor	Months of exposure to Sunlight	3	3.8±10.1	2.6E-05	-28.1±26.5	nd	-53.2±14.2	nd
		6	-5.8±10.7	nd	-22.4±15.7	nd	-33.1±24.5	nd
		9	0.5±17.3	1.7E-09	29.0±18.0	8.6E-03	48.7±18.5	5.1E-02
	Months in the Dark	3	-10.3±16.8	nd	-16.8±16.2	nd	-47.6±8.2	nd
		6	-19.7±2	nd	-40.3±28.8	nd	-43.6±21.8	nd
		9	-16.7±28.6	nd	-17.7±17.6	nd	-6.0±10.1	nd

5.2.3. Testing the water using the Ames test

Leachates are the substances that migrate from a plastic container to its contents during regular use. Since the SODIS process involves the continuous re-exposure of the reactor to sunlight and other weather conditions, this study assessed if weather conditions promote the release of leachates that migrate from the plastic components of the reactor to the water that is going to be disinfected by exposing reactors with water to weather conditions for various periods of time.

Sampling was done as described in the previous section and as described in the methodology, all the water samples were solid phase extracted and were assessed for mutagenicity at a concentration factor of 1000X with the Ames test performed with and without the metabolic activator S9 by following the procedure described in the methodology section.

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 5.6**)

Table 5.6 Ames test results of leachates released from PET jerrycan SODIS reactor and 2 L PET bottle SODIS reactor after 3, 6 and 9 months of exposure to sunlight or dark at PSA, Almería, Spain.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as fold induction over the baseline which is the mean of revertant relative to the baseline ± standard deviation. A fold induction over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Location	Exposure conditions		TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	3.00±1.00	1.82±1.15	2.00±1.00	1.24±0.58
		Positive control	22.83±1.53	25.62±1.53	22.50±4.36	36.71±0.58
PET jerrycan exposed at PSA, Almeria, Spain	Months of Sunlight exposure	3	0.40±2.65	1.44±1.00	0.00±0.00	0.27±0.58
		6	0.87±1.53	1.58±3.79	0.10±0.58	0.27±0.58
		9	0.73±1.53	1.99±0.58	0.48±0.58	0.80±1.00
	Months in the Dark	3	0.33±1.15	1.37±4.51	0.29±1.00	0.54±0.58
		6	0.73±1.15	1.10±2.08	0.38±1.15	0.80±0.00
		9	0.27±0.58	1.86±1.00	0.00±0.00	0.27±0.58
PET bottle exposed at PSA, Almeria, Spain	Months of Sunlight exposure	3	0.68±1.53	1.08±4.00	0.38±1.15	0.37±1.15
		6	0.43±1.15	1.08±1.00	0.19±1.15	0.55±1.00
		9	0.94±1.53	1.44±0.58	0.38±1.53	0.18±0.58
	Months in the Dark	3	0.34±0.58	1.51±1.00	0.29±1.00	1.10±1.00
		6	0.94±0.58	1.22±1.53	0.48±0.58	0.18±0.58
		9	0.85±3.21	1.51±2.65	0.57±1.73	0.55±1.00

5.3. Summary of the toxicological studies on the PET SODIS reactors.

Testing PET plastic polymer for extractables:

- There was a direct correlation between the increase in temperature and time of incubation with the release of estrogenic substances (**Table 5.7**). No estrogenic activity was detected when samples were extracted at room temperature. Estrogenic activity was detected following extended incubation at 37 °C. At this temperature released estrogenic substances from raw and aged PET were detected after 15 and 72 hours respectively. Estrogenic activity was detected following extraction at 50 °C for all periods of incubation.
- Detection of estrogenic activity in the extracts was affected by the age of the plastic and the solvent used. When ethanol was used as a solvent estrogenicity was only detected for raw PET and not for the aged PET. Estrogenic activity was detected when DMEM was used as a solvent for raw PET and for PET aged for 3, 6 and 9 months and was concentration dependant.
- No mutagenicity was detected in any sample.

The findings of the toxicological assessments of estrogenic activity for extractables from the novel PET extractables are summarised in **Table 5.7**.

Table 5. 7 Summary of the statistically significant extractables from the plastics PET samples for assessments of estrogenic activity.

Plastic stage		solvent	Temperature extraction conditions							
			20 °C 24 h	20 °C 72 h	37 °C 5 h	37 °C 15 h	37 °C 24 h	37 °C 72 h	50 °C 5 h	50 °C 15 h
PET	raw	Ethanol				+	+	+	+	+
		DMEM (100-fold)								
		DMEM (10-fold)								+
	3-months-aged	Ethanol								
		DMEM (100-fold)								
		DMEM (10-fold)							+	+
	6-months-aged	Ethanol								
		DMEM (100-fold)								
		DMEM (10-fold)						+	+	+
	9-months-aged	Ethanol								
		DMEM (100-fold)								
		DMEM (10-fold)						+	+	+

Testing the water from the PET reactor for leachates:

- Water tested from PET reactors, both 2 L and 25 L, exposed to sunshine for 3.6 and 9 months showed estrogenic activity after 9 months but not after 3 and 6 months. The levels of estrogenic activity detected in the 9 months samples were not considered a threat to human health.
- No mutagenicity was detected in any sample

Chapter VI

6. Toxicological studies on the novel PP SODIS reactors.

Novel large-volume transparent PP buckets were proposed for use in treating water in Malawi using SODIS. During SODIS the reactors are exposed to a variety of weather and environmental conditions (temperature, UV, reuse, etc) which may induce the release of plastic additives and monomers into water, therefore, toxicity was investigated using the E-screen assay to investigate estrogenic activity and the Ames test to investigate mutagenicity. The raw and aged PP used in the construction of the reactors was tested for extractables (Section 6.1) and the water produced by the reactors was tested for leachates (Section 6.2).

6.1. Testing the PP plastic polymer for extractables

In carrying out toxicity studies for the PP bucket SODIS reactors, raw and weathered/aged pieces of polypropylene (PP) used in the construction of the reactors were tested for extractables. The plastic was subjected to a range of stress conditions as described in Section 2.2.1 and samples of both raw plastic (6.1.1) and aged plastic (6.1.2) were tested using the E-screen assay for estrogenic activity and the Ames test for mutagenicity (6.1.3).

6.1.1. Testing raw PP plastic polymer extractables with the E-screen bioassay

The E-screen was carried out as described in Section 2.2.7 Chapter 2 and with the optimised conditions summarised in Section 3.3 Chapter 3. PP extractables into cell culture medium and into ethanol were screened for estrogenic activity. Media extracts from PP pieces were diluted and presented to cells at two different concentrations (10- and 100-fold dilutions). Ethanolic extracts were diluted and tested at one concentration (100-fold dilution) so the final concentration of EtOH was 1% v/v (which is the maximum concentration of solvent at which no cytotoxic effects were observed). The positive control was 1 nM estradiol (E2) and the negative control was DMEM7/F12 hormone-free medium supplemented with dilution vehicle 1% (v/v). Half of the plate was reserved for cells that were co-treated with ICI at 10^{-7} M. The proliferative effect of the samples was assessed using the DNA quantification assay as described in Section 2.2.7.4 Chapter 2. All extracts and experimental controls were run in triplicates per plate and each plate was repeated 3 times.

Extraction of raw PP into DMEM/F12 culture medium yielded extracts that were not estrogenic (**Fig 6.1**). However, ethanolic extracts of raw PP exerted significant effects on cell proliferation relative to the negative control. Estrogenic activity was related to time and temperature of extraction. Raw PP subjected to 20 °C and 37 °C at any period of incubation yielded extracts in ethanol solvent that had negligible effects on proliferation Higher extraction temperature 50 °C in ethanol yielded extracts showing significantly ($p < 0.05$) increased proliferative activity in the range 123 to 139 % relative to the negative control (**Fig 6.1**). Co-incubation of raw PP extracts with ICI [0.1 μM], an ER antagonist significantly reduced proliferation of all extracts. The data suggest that ethanol is a more potent and effective solvent than DMEM/F12 culture medium for extracting substances with estrogenic activity from PP plastic.

Interestingly, analysis of more concentrated extracts of raw PP in DMEM/F12 medium (10-fold diluted) showed a trend towards proliferation (ranging between 141 % and 163 %) after prolonged exposure at 37 °C and 50 °C (**Fig 6.2**). Co-incubation of raw PP extracts with ICI [0.1 μM], an ER antagonist significantly reduced proliferation of all extracts to control levels confirming estrogenic activity.

Relative to E2, the estrogenic effects of the 10-fold diluted raw PP extracts in DMEM/F12 amounted to 69.6-98.0 % RPE. Thus, extracts of raw PP obtained after 24 or 72 h at 37 °C or after 5 or 15 h at 50 °C contain estrogenic components showing partial and total agonistic activity (**Table 6.1**). Estradiol equivalents of raw PP DMEM/F12 medium 10-fold diluted extracts amounted to between 281-1451 ng/L, approximately. The estrogenic effects of the 100-fold diluted raw PP extracts in ethanol amounted to 33.4-73.2 % RPE. Thus, extracts of raw PP obtained after 5 or 15 h at 50 °C contain estrogenic components showing partial agonistic activity (**Table 6.1**). Estradiol equivalents of raw PP extracts in ethanol ranged between 84- 3594 ng/L, approximately. The relatively higher EEQs of ethanolic PP extracts suggest that EtOH solvent is more efficient at extracting substances with estrogenic activity from PP plastic. (**Table 6.1**).

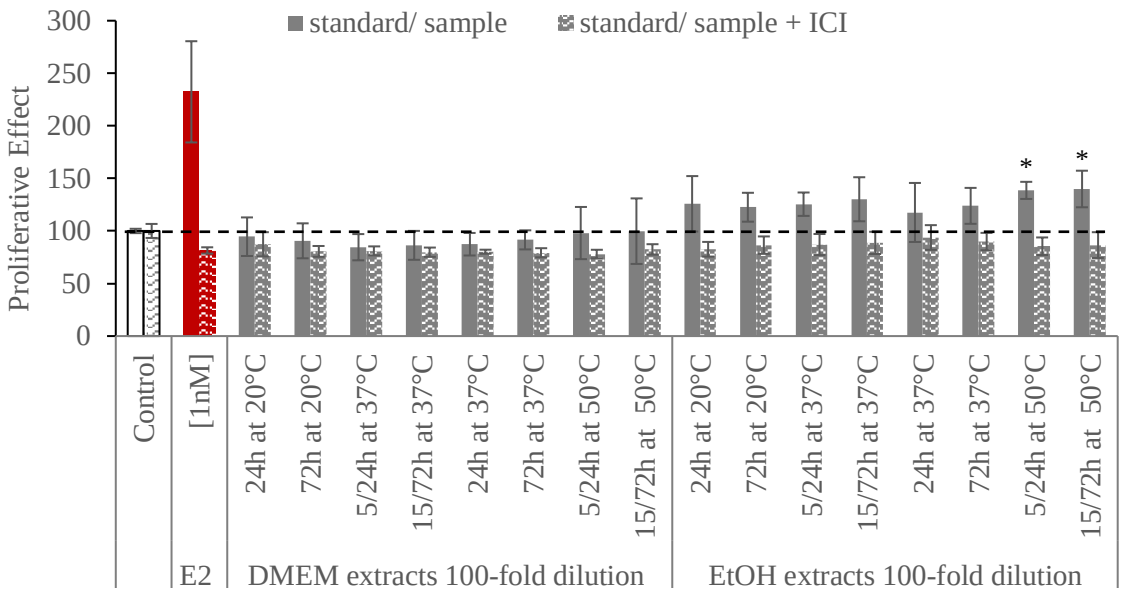


Figure 6.1 Proliferative effects of raw PP extracts (100-fold dilution). *Raw PP was submerged in DMEM /F12 media solvent or in EtOH solvent and exposed to different temperature conditions. Extractables were diluted 100-fold and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

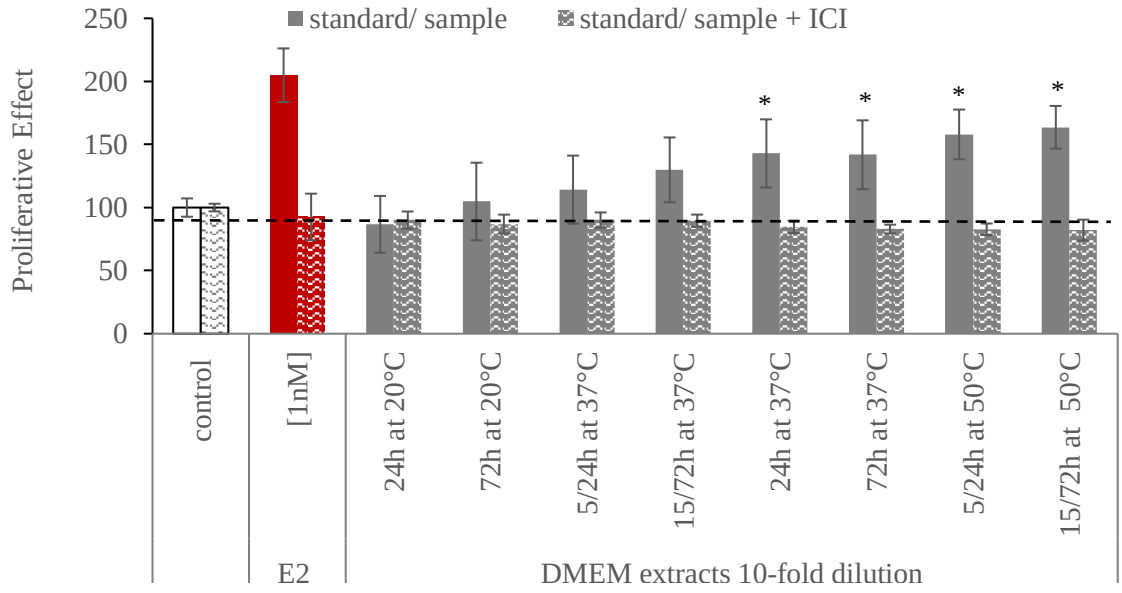


Figure 6.2 Proliferative effects of raw PP extracts (10-fold dilution). *Raw PP was submerged in DMEM /F12 media solvent or in EtOH solvent and exposed to different temperature conditions. Extractables were diluted 10-fold and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

Table 6.1 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of exudates leached from raw PP polymer into two solvents (DMEM/F12 and ethanol) during thermal stress. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

	DMEM media solvent				EtOH solvent	
Dilution →	1:10		1:100		1:100	
Temperature condition ↓	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
24h at 20 °C	-16.9±42.5	nd	-1.1±11.7	nd	48.8±45.0	514.7
72h at 20 °C	12.9±54.8	0.1	-4.9±11.0	nd	57.5±18.1	1133.3
5/24h at 37 °C	33.4±59.7	8.4	-10.5±6.5	nd	47.2±33.1	441.7
15/72h at 37 °C	58.4±50.8	122.2	-9.0±8.8	nd	65.2±29.2	2065.0
24h at 37 °C	75.4±37.9	414.6	-9.1±8.2	nd	33.4±51.5	84.2
72h at 37 °C	69.6±31.0	281.8	-8.3±8.9	nd	33.7±16.7	87.9
5/24h at 50 °C	95.9±15.6	1309.5	-2.4±19.7	nd	63.2±12.3	1771.1
15/72h at 50 °C	98.0±14.0	1451.3	-2.9±23.9	nd	73.2±30.0	3594.2

6.1.2. Testing aged PP plastic polymer extractables with the E-screen bioassay

The constant use of SODIS reactors and their constant exposure to sunlight could have an effect on the chemical stability and robustness of the plastic components of the reactor. This implies that the aging of the plastic could have a critical effect on the rate of release of substances with estrogenic activity into water.

In order to mimic the aging process of the plastic, plastic pieces of PP bucket SODIS reactors were exposed to weather conditions in Plataforma Solar of Almeria, Spain for 3, 6 and 9 months. reactor; plastic Extractables of the plastic pieces were obtained by submerging in DMEM/F12 medium or ethanol for varying time periods and temperatures ranging between 20 °C and 50 °C as indicated in Chapter 2. Aged PP plastics were diluted 10-fold and/or 100-fold as previously stated prior to E-screen analysis. The proliferative effect of each extract was assessed by measuring proliferation of MCF-7 BUS cells using the DNA quantification procedure. The % RPE of each extract and EEQ values were calculated. All extracts and experimental controls were run in triplicates per plate and each plate was repeated 3 times.

Composition of extractant solvent also had a profound effect on estrogenic activity of aged PP plastic extracts. Extracts 100-fold diluted in DMEM/F12 medium solvent from 3, 6 and 9 month-aged PP yielded proliferative effects similar to the negative control. By contrast, PP plastic aged for 3 months only and subjected to extraction in ethanol under varying time and temperature conditions yielded extracts that were proliferative.

PP aged for 3 months and subjected to 20 °C for 24 h and 72 h or to 37 °C for 5 h within a 24 h period yielded ethanolic extracts that had negligible effects on proliferation. Longer exposure of aged PP in ethanolic solvent to 37 °C or to 50 °C increased proliferation by 40-50 % relative to the negative control. Co-incubation of raw PP extracts with ICI [0.1 μM], an ER antagonist mitigated against proliferation confirming estrogenic activity of ethanolic aged PP plastic extracts obtained under thermal stress.

Extraction of 6- and 9-month aged PP pieces into ethanol for varying times and temperatures yielded extracts showing negligent proliferation relative to the negative control (**Fig 6.3**). The absence of estrogenic activity from thermally stressed 6- and 9-month PP may be a reflection of possible photodegradation.

Analysis of more concentrated PP extracts in DMEM F12 (10-fold diluted extracts) showed that proliferation was dependent on thermal stress (**Fig 6.4**). PP extracts obtained at temperatures ranging between 37 °C and 50 °C for varying periods of time increased proliferation of 3-, 6- and 9-month extracts between 50 % and 75 %, 40 and 94 % and 37-91 % respectively.

These results suggested that some of the substances with estrogenic activity extracted from the different aged PP plastic might be susceptible to photocatalysis since the most aged PP and therefore more time exposed to the sun release substances with estrogenic activity less easily than less aged PP. In other words, if substances with estrogenic activity are degraded by the sunlight action it is expected that the PP plastic pieces exposed for longer periods to the sunlight have lower or less proliferative effects since there are no substances with estrogenic activity left in the plastic to extract. This might be corroborated by an observation during the collection of the plastics and other samples at PSA, Spain, where it was noted that the older and more frequently used PP bucket became more fragile and breakable compared to the new or less used buckets.

The corresponding RPE values and EEQ values of each of the extracts are displayed in **Table 6.2** These values imply a partial and total agonistic effect of E2 exerted by the extracts that were released from the PP aged plastic under the most extreme extractions conditions used.

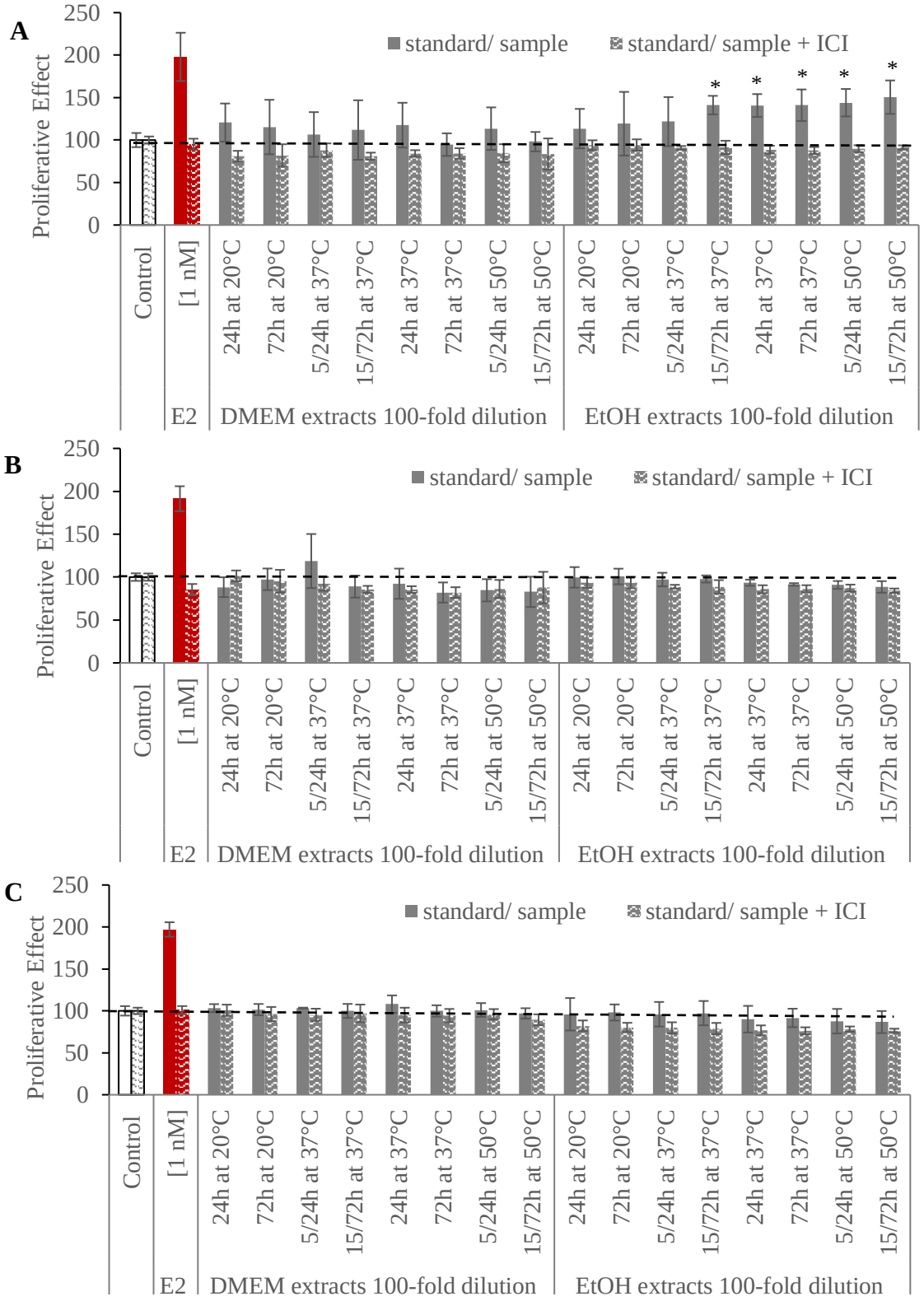


Figure 6.3 ***Proliferative effects of aged PP extracts (100-fold diluted)*** *3-months aged (A), 6-months aged (B) and 9-months aged (C) PP was submerged in DMEM/F12 media or EtOH solvent and exposed to different temperature conditions. Extractables were diluted 100 times and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

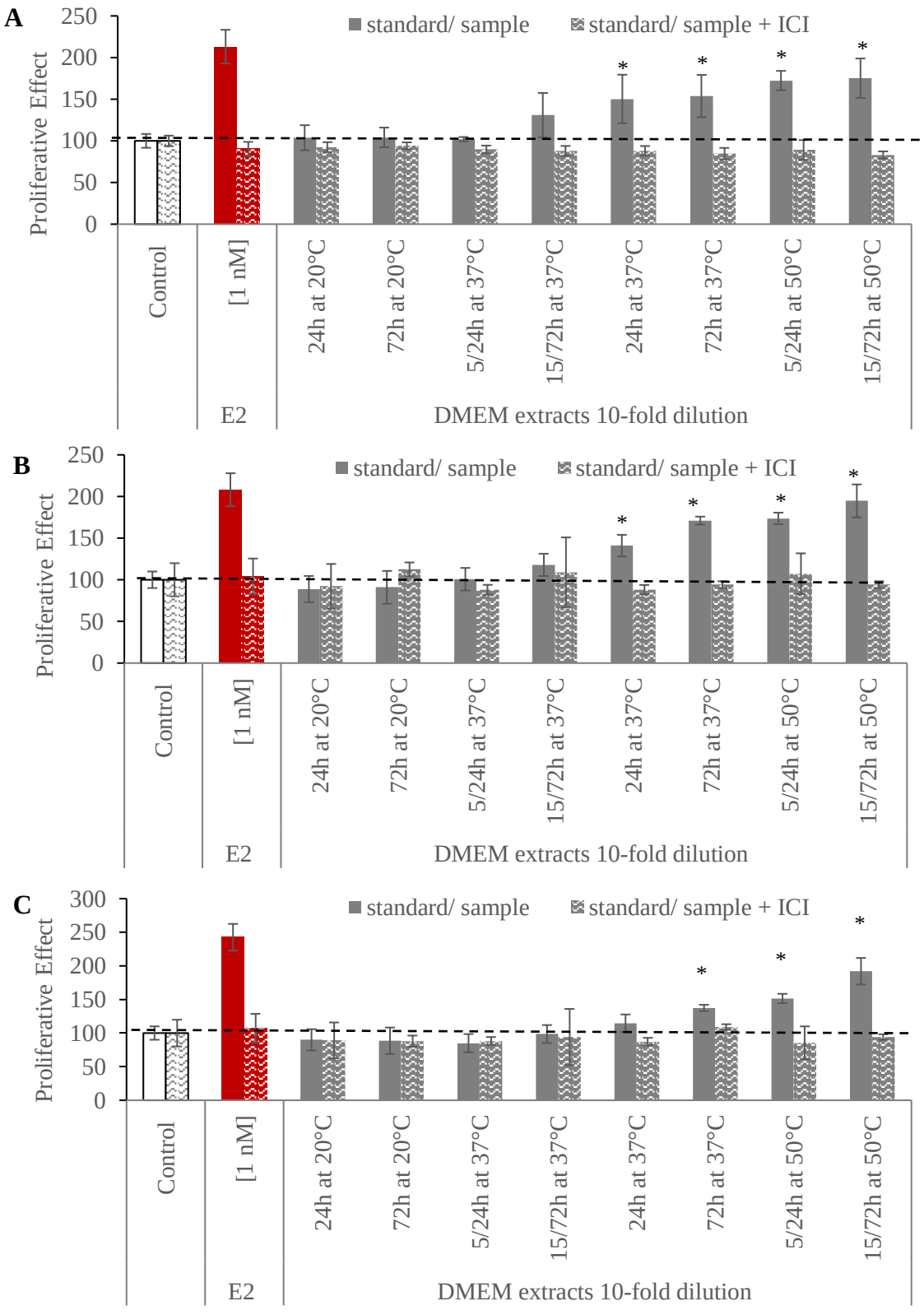


Figure 6.4 Proliferative effects of aged PP extracts (10-fold diluted) *3-months aged (A), 6-months aged (B) and 9-months aged (C) PP was submerged in DMEM/F12 media or EtOH solvent and exposed to different temperature conditions. Extractables were diluted 10 times and screened for estrogenic activity in the presence and absence of ICI [0.1 µM]. Single factor Anova indicated significantly increased proliferation (*) $p<0.05$ of the water extracts compared to the control.*

Table 6.2 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of exudates leached from 3-month, 6-month and 9-month-aged PP plastic polymer into two solvents (DMEM/F12 and ethanol) during thermal stress. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

		DMEM media solvent				EtOH solvent	
	Dilution →	1:10		1:100		1:100	
	Temperature condition ↓	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
3-months-age	24h at 20 °C	-9.7±15.8	nd	20.8±20.7	8.7	-4.7±14.7	nd
	72h at 20 °C	-8.3±20.8	nd	8.1±23.2	0.1	7.7±15.1	0.1
	5/24h at 37 °C	2.2±13.0	1.9E-05	10.8±24.5	0.4	24.6±9.7	19.3
	15/72h at 37 °C	18.0±13.7	0.4	4.6±31.5	6.4E-03	26.1±8.9	25.8
	24h at 37 °C	38.5±12.7	16.7	15.6±17.5	2.2	28.9±12.3	42.3
	72h at 37 °C	64.5±19.2	196.0	-3.6±15.1	nd	28.9±9.9	41.7
	5/24h at 50 °C	73.9±12.1	375.1	10.3±25.3	0.3	30.7±8.3	55.8
	15/72h at 50 °C	87.2±8.0	831.2	-10.6±11.5	nd	35.6±10.3	114.7
6-months-age	24h at 20 °C	-15.1±11.9	nd	-6.6±25.1	nd	1.6±8.0	4.3E-04
	72h at 20 °C	-4.8±9.2	nd	3.8±24.3	2.6E-03	-0.7±11.8	nd
	5/24h at 37 °C	-10.8±4.2	nd	27.4±41.1	32.4	-0.7±6.1	nd
	15/72h at 37 °C	-2.4±14.6	nd	-14.2±17.1	nd	-2.9±5.1	nd
	24h at 37 °C	15.0±11.7	0.1	-12.2±22.6	nd	-6.5±3.6	nd
	72h at 37 °C	24.6±15.3	1.9	-22.9±17.4	nd	-1-±4.9	nd
	5/24h at 50 °C	36.9±9.9	13.5	-20.8±18.7	nd	-9.9±7.3	nd
	15/72h at 50 °C	62.9±13.3	174.0	-21.5±23.6	nd	-10.3±3.7	nd
9-months-age	24h at 20 °C	-0.7±7.5	nd	3.4±5.0	1.5E-03	-6.0±13.2	nd
	72h at 20 °C	4.4±10.6	5.1E-04	1.5±7.1	2.8E-05	-4.5±19.2	nd
	5/24h at 37 °C	3.9±5.9	2.9E-04	2.2±5.6	2.0E-04	-6.9±19.3	nd
	15/72h at 37 °C	7.9±16.3	8.4E-03	0.0±8.8	nd	-5.7±2.0	nd
	24h at 37 °C	45.1±25.3	35.4	11.7±14.1	5.5E-01	-13.5±15.7	nd
	72h at 37 °C	47.3±21.4	44.6	3.0±11.3	7.7E-04	-12.4±19.8	nd
	5/24h at 50 °C	64.5±10.8	196.1	1.0±8.8	3.5E-06	-16.7±18.1	nd
	15/72h at 50 °C	66.8±21.0	232.0	-3.2±6.5	nd	-17.8±16.0	nd

6.1.3. Testing raw and aged PP with the Ames test

All extractables were assessed for mutagenicity with the Ames test performed with and without metabolic activator S9 by following the procedure described in Section 2.2.8. The extractables were sampled from both raw and aged PP plastic as described in Section 2.2.2. In order to simulate the aging process of the PP plastic, raw PP plastic pieces were exposed to weather conditions for 3, 6 and 9 months in the Plataforma Solar of Almeria. Spain.

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 6.3**).

Table 6.3 Ames test results for extractables released from PP plastic polymer at different aged states after different temperature extraction treatments.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as fold induction over the baseline which is the mean of revertants relative to the baseline ± standard deviation. A fold induction over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic

Age	Extraction solvent	Treatment	TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	2.00±0.00	1.82±1.15	2.00±1.00	1.91±0.58
		Positive control	22.83±1.53	25.80±1.73	23.83±0.58	24.77±0.58
Raw	PBS	24h at 20 °C	0.50±0.00	0.67±0.58	0.00±0.00	0.17±0.58
		72h at 20 °C	1.00±1.00	1.00±1.00	0.00±0.00	0.17±0.58
		5/24h at 37 °C	0.67±1.15	0.83±0.58	0.17±0.58	0.00±0.00
		15/72h at 37 °C	1.67±0.58	1.33±0.58	0.33±0.58	0.33±0.58
		24h at 37 °C	1.67±2.08	1.67±2.08	0.17±0.58	0.33±0.58
		24h at 37 °C	1.17±2.52	1.33±2.08	0.33±0.58	0.33±0.58
	EtOH	24h at 20 °C	0.94±1.15	0.80±1.00	0.17±0.58	0.00±0.00
		72h at 20 °C	0.67±0.58	0.94±0.58	0.00±0.00	0.17±0.58
		5/24h at 37 °C	1.07±1.53	0.94±1.53	0.00±0.00	0.33±0.58
		15/72h at 37 °C	1.21±3.61	1.47±1.53	0.17±0.58	0.17±0.58
		24h at 37 °C	0.54±1.15	0.67±0.58	0.33±0.58	0.17±0.58
		24h at 37 °C	1.07±1.15	1.21±1.00	0.17±0.58	0.50±0.00
3 months	PBS	24h at 20 °C	0.33±0.58	0.21±1.53	0.33±0.58	0.67±1.15
		72h at 20 °C	0.50±1.00	0.17±1.15	0.00±0.00	0.67±1.15
	EtOH	24h at 20 °C	0.32±0.58	0.28±1.00	0.23±0.58	0.67±0.00
		72h at 20 °C	0.40±1.53	0.23±0.58	0.00±0.00	0.56±0.58
6 months	PBS	24h at 20 °C	0.21±0.58	0.39±0.58	0.17±0.58	0.50±1.00
		72h at 20 °C	0.52±1.53	0.28±1.15	0.00±0.00	0.00±0.00
	EtOH	24h at 20 °C	0.44±0.58	0.29±1.00	0.12±0.58	0.44±0.58
		72h at 20 °C	0.79±2.00	0.33±1.15	0.23±0.58	0.11±0.58
9 months	PBS	24h at 20 °C	0.41±1.53	0.00±0.00	0.17±0.58	0.36±0.58
		72h at 20 °C	0.30±0.58	0.25±0.00	0.17±0.58	0.18±0.58
	EtOH	24h at 20 °C	0.55±1.53	0.04±0.58	0.17±0.58	0.50±1.00
		72h at 20 °C	0.41±0.00	0.21±0.58	0.50±1.00	0.67±0.58

6.2. Testing water from the PP buckets for leachates

Unlike extractables, which are all the chemical substances that have been released from plastic under extreme conditions, leachates are substances that migrate from the plastic to its surroundings during regular use.

Since the SODIS process involves the continuous re-exposure of the reactor to sunlight and other weather conditions, this study assessed if weather conditions promote the release of leachates that migrate from the plastic components of the reactor to the water that is going to be disinfected.

To test the PP buckets reactors for leachates the reactors were filled with well water and exposed to the weather conditions of PSA in Spain for 3, 6 and 9 months. Reactors filled with well water and stored in the dark for the same period of time served as controls. The water was tested using the E-screen assay (6.2.1) and the Ames test (6.2.2).

6.2.1. Salinity and conductivity of the water in the PP bucket reactors

Well water collected at 3, 6 and 9 months from the PP bucket reactor was analyzed with respect to mineral composition and conductivity as described in Chapter 2. The composition of well water collected from PP bucket SODIS reactors was not significantly altered over the course of the 9 months. (**Table 6.4**). There were no significant differences between the conductivity of the water from different reactors after different periods of exposure.

Table 6.4 Salinity and conductivity measurements of the well water exposed to sunshine in PP buckets at PSA, Almería, Spain

		PP Bucket SODIS reactor		
		3 months	6 months	9 months
Salinity (mg/L)	Chloride	33.34	40.94	29.72
	Nitrite	0.13	0.12	0.00
	Nitrate	0.58	1.65	0.44
	Phosphate	0.00	0.00	0.00
	Sulphate	25.51	35.00	21.59
	Sodium	48.72	44.64	46.13
	Ammonium	0.03	0.00	0.00
	Calcium	0.75	0.67	0.73
	Potassium	0.48	0.67	0.25
	Magnesium	7.08	8.62	6.90
Conductivity mS/cm		2.62	2.52	2.41

6.2.2. Testing the water using the E-screen bioassay

After the exposure period, 1L of water was solid phase extracted as described in Chapter 2. The DMSO extract (100 μl) was diluted in DMEM/F12 hormone-free medium to yield final concentration factors of 100X, 50X and 10X when compared to the original water sample.

The concentrated extracted samples of leachates were then screened for estrogenic activity in the presence and absence of ICI [0.1 μM] as described in Chapter 2. The proliferative effects and the RPE of each sample were calculated by measuring cell proliferation with the DNA quantification method. EEQs of each concentrated extracted sample of leachates were assessed in order to determine if the samples were in compliance with the ADI recommended by the WHO.

As **Fig 6.5** shows none of the PP leachates significantly increased proliferation when compared to the control.

The corresponding RPE values of these samples are presented in **Table 6.5.** However, since most of the PP leachates samples have lower proliferative effects than the control the corresponding RPE values are negative. Therefore, it was not possible to do the calculations of those EEQ values and since RPE values lower than 10% are considered non-estrogenic the EEQ values of those samples were not determined.

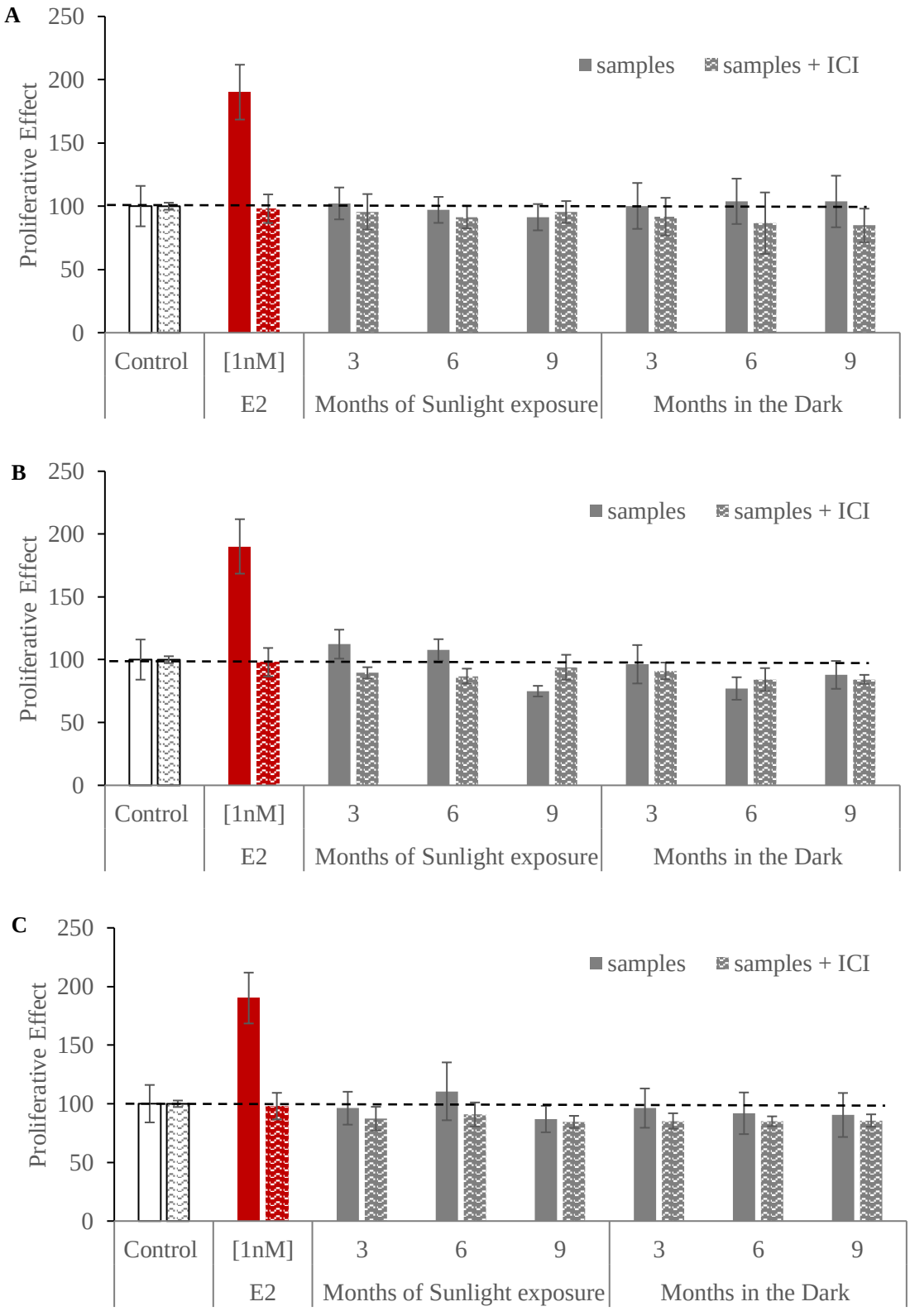


Figure 6.5 Proliferative effects of PP buckets reactor leachates in well water at Almería, Spain. *Leachates released into well water after 3, 6 and 9 months of exposure of PP bucket reactors to sunlight and darkness were concentrated 10 times (A), 50 times (B) and 100 times (C) and assessed for estrogenic activity in the presence and absence of ICI [0.1 µM].*

Table 6.5 *Relative Proliferative Effects (% RPE) and Estradiol Equivalents (EEQs in ng / L) of leachates released from PP buckets reactors into well water after 3-,6- and 9-months exposure to sunlight and darkness at Almeria, Spain. Data refer to mean ± SD of triplicate experiments. Nd refers to not determined.*

			concentration factor					
			10X		50X		100X	
			RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)	RPE (%)	EEQ (ng/L)
PP bucket SODIS reactor	Months of exposure to Sunlight	3	4.9±7.2	8.4E-05	6.8±7.2	8.4E-05	5.0±36.5	9.8E-06
		6	4.8±8.1	7.8E-05	10.1±11.5	5.5E-04	5.6±7.8	1.7E-05
		9	-2.6±13.0	nd	-14.2±16.7	nd	-16.1±11.2	nd
	Months in the Dark	3	7.5±15.3	6.7E-04	-0.3±16.9	nd	1.6±28.4	3.8E-08
		6	-1.5±18.2	nd	-14.7±16.6	nd	-10.2±20.2	nd
		9	-5.4±18.1	nd	-5.0±16.5	nd	-12.0±21.9	nd

6.2.3. Testing the water using the Ames test

Leachates are the substances that migrate from the plastic to its contents during regular use. Since the SODIS process involves the continuous re-exposure of the reactor to sunlight and other weather conditions, this study assessed if weather conditions promote the release of leachates that migrates from the plastic components of the reactor to the water that is going to be disinfected by exposing reactors with water to weather conditions for various periods of time.

Sampling was done as described in the previous section and as described in the methodology, all the water samples were solid phase extracted and were assessed for mutagenicity at a concentration factor of 1000X with the Ames test performed with and without metabolic activator S9 by following the procedure described in the methodology section.

No mutagenicity was detected for any sample when tested using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (**Table 6.5**)

Table 6.6 Ames test results for leachates released from PP bucket reactors after 3, 6 and 9 months of exposure to sunlight or darkness at PSA, Almería, Spain.

The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as fold induction over the baseline which is the mean of revertants relative to the baseline ± standard deviation. A fold induction over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Location	Exposure conditions		TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	2.00±0.00	2.24±0.58	2.24±0.58	1.91±0.58
		Positive control	22.83±1.53	20.50±1.00	21.39±2.00	24.42±1.15
PP bucket exposed at PSA, Almeria, Spain	Months of Sunlight exposure	3	0.67±0.58	0.45±1.00	1.34±2.00	0.52±1.00
		6	2.67±2.52	0.74±1.15	0.74±1.53	0.52±1.73
		9	1.33±2.89	0.74±0.58	1.34±0.00	0.52±1.00
	Months in the Dark	3	2.17±1.15	0.89±1.00	1.34±1.00	0.35±0.58
		6	1.33±0.58	0.74±2.08	0.45±1.00	0.52±1.00
		9	0.67±0.58	0.89±1.00	0.45±1.00	0.70±1.15

6.3. Summary of the toxicological studies on the PP SODIS reactors.

Testing the PP plastic polymers for extractables:

- There was a direct correlation between the increase in temperature and time of incubation with the release of estrogenic substances (**Table 6.7**). No estrogenic activity was detected when samples were extracted at room temperature. Estrogenic activity was detected when samples were extracted at 37 °C for extended periods of time and at 50 °C.
- Estrogenic activity was detected in raw and PET aged for 3,6 and 9 months but was dependant on the solvent used. When ethanol was used estrogenic activity was detected in the raw PET and the PET aged for 3 months. When DMEM was used estrogenic activity was detected in raw PET and PET aged for 3,6 and 9 months but was dependant on concentration of DMEM used.
- No mutagenicity was detected in any sample.

The findings of the toxicological assessments of estrogenic activity for extractables from the novel PET extractables are summarised in **Table 6.7**.

Table 6. 7 Summary of the statistically significant extractables from the plastics PET samples for assessments of estrogenic activity.

Plastic stage		solvent	Temperature extraction conditions							
			20 °C 24 h	20 °C 72 h	37 °C 5 h	37 °C 15 h	37 °C 24 h	37 °C 72 h	50 °C 5 h	50 °C 15 h
PP	raw	Ethanol							+	+
		DMEM (100-fold)					+	+	+	+
		DMEM (10-fold)								
	3-months-aged	Ethanol				+	+	+	+	+
		DMEM (100-fold)								
		DMEM (10-fold)					+	+	+	+
	6-months-aged	Ethanol								
		DMEM (100-fold)								
		DMEM (10-fold)					+	+	+	+
	9-months-aged	Ethanol								
		DMEM (100-fold)								
		DMEM (10-fold)						+	+	+

Testing the water from the PP reactors for leachates:

- Water exposed to sunshine in PP reactors for 3, 6 and 9 months showed no estrogenic activity.
- No mutagenicity was detected in any sample.

Overall summary of the toxicological findings for the novel SODIS reactors- Chapters IV-VI

The main toxicological findings for estrogenic activity for the extractables from PMMA, PET and PP were:

- There was a direct correlation between the increase in temperature and time of incubation with the release of estrogenic substances from all three plastics.

Considering all solvents used.

- No estrogenic activity was detected when samples were extracted at room temperature.
- Estrogenic activity was detected following extended incubation at 37 °C. At this temperature released estrogenic substances from raw and aged PMMA were detected following 15 hours and 24 hours incubation respectively, from raw and aged PET after 15 and 72 hours respectively and from raw and aged PP after 24 and 15 hours respectively.
- Estrogenic activity was detected following extraction at 50 °C for all periods of incubation.
- The estrogenic effect of PMMA extractables in cell culture medium was concentration independent while the estrogenic effects of PET and PP extractables in cell culture medium were concentration-dependent.
- When the plastics were aged for 3, 6 and 9 months, depending on the solvent, extractables were detected for up to 9 months for PET and PP but only up to 6 months for PMMA

The findings of the toxicological assessments of estrogenic activity for extractables from the novel SODIS reactors are summarised in **Table 6.8**.

Table 6.8 Summary of the findings for the toxicological assessments of estrogenic activity of extractables from the plastics PMMA, PET and PP used in the novel SODIS reactors.

NA=not applicable ND=none detected

Plastic	Solvent	Minimum time and temperature for release	Dose-effect relationship	Age of plastic and detection of estrogenic activity
PMMA	Ethanol	37 °C for 72 h	NA	3- months aged
	Cell culture medium	37 °C for 15 h	non-monotonic	3- and 6- months aged
PET	Ethanol	37 °C for 15 h	NA	ND
	Cell culture medium	37 °C for 72 h	monotonic	Up to 9 months aged
PP	Ethanol	37 °C for 15 h	NA	3- months aged
	Cell culture medium	37 °C for 24 h	monotonic	Up to 9 months aged

Toxicological findings for estrogenic activity of the leachates;

- PMMA plastic pieces tested under environmental conditions at four distinct geographic locations in Africa and Europe did not release any substances with estrogenic activity into water.
- Water sampled from the PMMA tubular SODIS reactors showed no estrogenic activity when tested under field conditions in Africa over a calendar year.
- PET reactors tested at PSA released substances with estrogenic activity into well water after 9 months of exposure to sunshine but not after 3 months or 6 months exposure to sunshine. However, levels of estrogenic activity detected were not considered a threat to human health. PP bucket reactors also tested at PSA did not release any substances with estrogenic activity into well water following exposure to sunshine for 3,6 and 9 months.

Toxicological assessments for mutagenicity

- None of the water samples analysed, extractables or leachates, from the three novel SODIS reactors and their plastic materials showed any mutagenicity.

Chapter VII

7. Advanced Oxidative Processes

The previous toxicological studies described in chapters 4-6 tested plastics used in solar disinfection of water (SODIS). Another objective of this work was to test plastics used in advanced oxidative processes for toxicity. *In-vitro* experiments were set up to test the toxicity of water from two advanced oxidative processes, photo-Fenton and persulphate activation using three plastics - polyethylene terephthalate (PET) from a transparent jerrycan (PETjc) as had been tested in chapter 5, PET from a coca-cola bottle (PETcc) and blue polycarbonate (PC) from a container used in a water dispenser. A SODIS process was also used for comparison. The treatments were applied as described in Section 2.2.5. Briefly, plastic pieces, PETjc, PETcc and PC, were submerged in 1 L of milliQ water in a glass borosilicate vessel. The irradiation experiments were performed at room temperature (25 °C) and the temperature of the solution increased up to approximately 30 °C during irradiation. Three different treatments were used. Solar disinfection (SODIS) with no additives, photo-Fenton using 10 ppm (290 µM) H_2O_2 and 1 ppm (18 µM) Fe^{2+} and a persulfate activation process using 24.3 ppm (90 µM) peroxydisulfate (PDS) and 1 ppm (18 µM) Fe^{2+}. Two time periods of irradiation were investigated: 6 consecutive hours in one day and for 6 consecutive hours for 7 successive days. Toxicity was evaluated using the E-screen assay to test for estrogenic activity (7.1) and the Ames test to test for mutagenicity (7.2).

7.1. Testing of the advanced oxidative process samples with the E-screen bioassay

The E-screen assay was carried out in the presence and absence of ICI. When the co-incubation of the samples with ICI mitigates the proliferative effects of migrating chemicals released under SODIS, photo-Fenton and persulfate activation, it confirms the presence of estrogenic activity. Proliferative effects were measured using MCF-7 BUS cells and the relative proliferative effects (RPE) and estradiol equivalent values (EEQs) were calculated.

7.1.1. Proliferative effects of migrating substances released from plastic after 6 hours of irradiation

The proliferative effects of migrating substances from PETjc, PETcc and blue polycarbonate plastic polymers following 6 h of irradiation with SODIS, photo-Fenton and persulfate activation are described in **Fig 7.1**. The calculated relative proliferative effects (RPE) are described in **Table**

7.1. Following 6 h irradiation, no significant estrogenic activity was detected for PETjc for any of the treatments (**Fig 7.1**). The RPE values (**Table 7.1**) of migrating substances from PETjc were around 5% released under photo-Fenton treatment, 7% released under persulfate activation and 18% released under SODIS. According to Kuch *et al.* (2010) classification of the agonistic activity of E2 these RPE values indicated that the samples had a very weak or non-existent agonistic effect of estradiol and since the values were not statistically significantly different from each other, all of the samples from the experiments using PETjc and 6 h irradiation were considered not to be estrogenic.

On the other hand, evidence of estrogenic activity was apparent from migrating substances from PETcc following 6 h irradiation. Proliferative effects relative to the negative control were 153 ± 20% following SODIS, 239 ± 32% following photo-Fenton conditions and 184 ± 23% following persulfate activation (**Fig 7.1**). The data show a significant ($p<0.05$) difference in proliferation of 86% between photo-Fenton and SODIS irradiation treatments, while the difference of 31% in proliferation between persulfate activation and SODIS irradiation treatment is not significant. These proliferative effects translate to RPE values of approximately 76% for photo-Fenton treatment, 67% for persulfate activation and around 43% for SODIS (**Table 7.1**). These results suggested that substances released from PETcc following irradiation act as partial agonists of the estradiol receptor, according to the classification of the agonistic activity of E2 described by Kuch *et al.* (2010). However, since there were no statistical differences between the proliferative effects exerted by the substances released after photo-Fenton and persulfate activation and there was a significant ($p<0.05$) difference between the proliferative effects of the substances released after SODIS and photo-Fenton irradiation treatment, the substances released from PETcc after 6 h of SODIS irradiation treatment were classified in this study as mild agonists of E2, the substances released from PETcc after 6 h of persulfate activation were classified as partially-strong agonists of E2, and the substances released from PETcc after 6 h of photo-Fenton treatment were classified as strong agonists of E2.

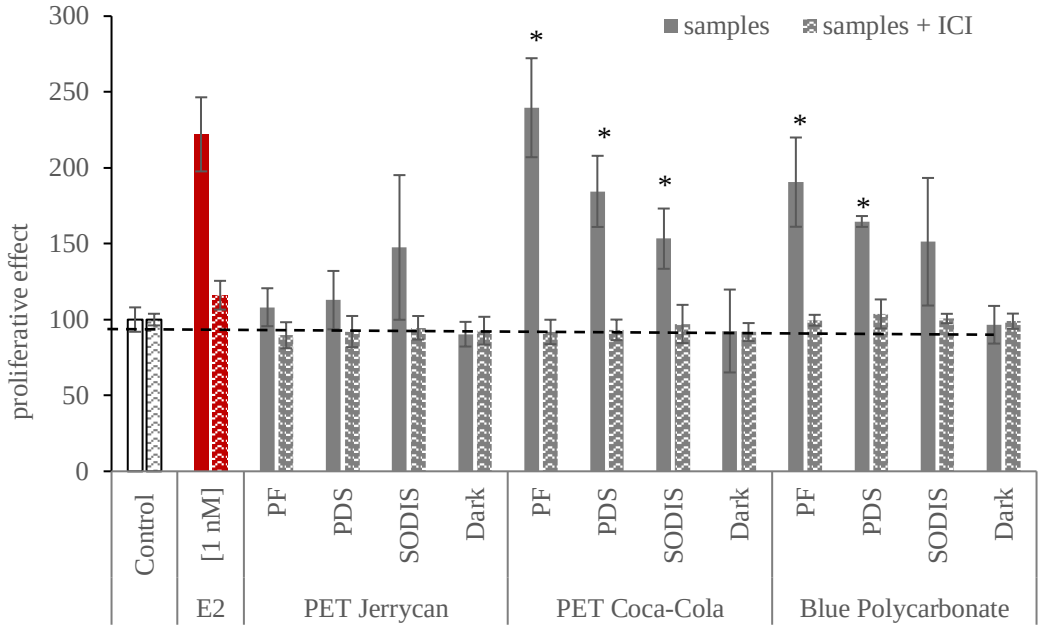


Figure 7.1 Proliferative effects on MCF-7 BUS cells of substances released from different plastic sources after 6 hours of irradiation in SODIS, photo-Fenton (PF) and persulfate activation (PDS) processes*. Single factor Anova indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

Migrating substances from blue polycarbonate stimulated growth by 151 ± 42% (relative to negative control) following SODIS for 6 h (**Fig 7.1**). Proliferative effects of substances released from blue polycarbonate after irradiation under photo-Fenton reaction for 6 hours stimulated proliferation by 190 ± 29% and by 164 ± 4% after irradiation under persulfate activation. There was a significant ($p<0.05$) difference of 39% in the proliferation exerted by the samples obtained after using photo-Fenton treatment when compared to the samples obtained after SODIS treatment but interestingly, there were no significant differences between the proliferation of persulfate activation and SDIS treatments. The substances migrating from blue polycarbonate plastic polymer after 6h of photo-Fenton irradiation treatment had a relative proliferative effect of 81% which implies that these released substances act as a total agonist of the estradiol receptor (**Table 7.1**). Both PDS and SODIS processes promoted the release of substances that triggered a relative proliferative effect of around 43-49%. In this study, the substances released after 6 h of SODIS irradiation treatment and persulfate activation treatment were considered mild agonists of E2 (**Table 7.1**).

Table 7.1 *Relative Proliferative Effects (RPE in %) of substances released into water from different plastics after 6 hours under different irradiation treatments. Values are expressed as the mean ± standard deviation.*

RPE (%)				
6 hours	Irradiation treatment	Plastics polymer		
		PETjc	PETcc	PC
	Photo-Fenton	4.87±7.10	75.58±9.72	80.64±36.27
	Persulfate	6.94±7.72	67.13±20.78	49.23±14.91
	SODIS	17.72±22.06	42.67±16.79	43.52±27.08

7.1.2. Proliferative effects of migrating substances released from plastic following 7 days of irradiation

The effect of prolonged irradiation on the release of substances from the plastic polymers is described in **Fig 7.2** PETjc, PETcc and blue polycarbonate plastic polymers were exposed to SODIS, photo-Fenton and persulfate activation for 6 h per day for 7 consecutive days. The proliferative effects results are described in **Fig 7.2**. The calculated relative proliferative effects (RPE) are described in **Table 7.2**.

Evidence of time-dependant estrogenic activity of chemicals released from PETjc after 1 week of irradiation was shown (**Figs 7.1-7.2**). Relative to the negative control (100%), these samples stimulated growth by 210 ± 21% for the samples that underwent SODIS for 1 week. Interestingly, no significant increase in proliferation above this value was observed when SODIS was enhanced. The substances released after 1 week under photo-Fenton irradiation and persulfate activation stimulated proliferation by 216 ± 18% and by 244 ± 36%, respectively (**Fig 7.2**). The RPE values of the leachates coming from PETjc polymer after 1 week under irradiation treatment were $\geq$ 80% which indicated a strong estradiol agonistic effect of the samples (**Table 7.2**). Interestingly, prolonged irradiation of PETcc for a week did not significantly enhance estrogenic activity when compared to the levels of estrogenicity detected following 6 hours irradiation of the samples. Estrogenic activity of migrating chemicals from PETcc released after 1 week under SODIS, photo-Fenton and persulfate activation were 139 ± 7 %, 180 ± 26% and 182 ± 23% respectively relative to the control (**Fig 7.2**). These values translated to RPE values between 33-67% indicating that the leachates released from the PETcc after 1 week of irradiation treatment act as mild and partial agonists of estradiol receptor.

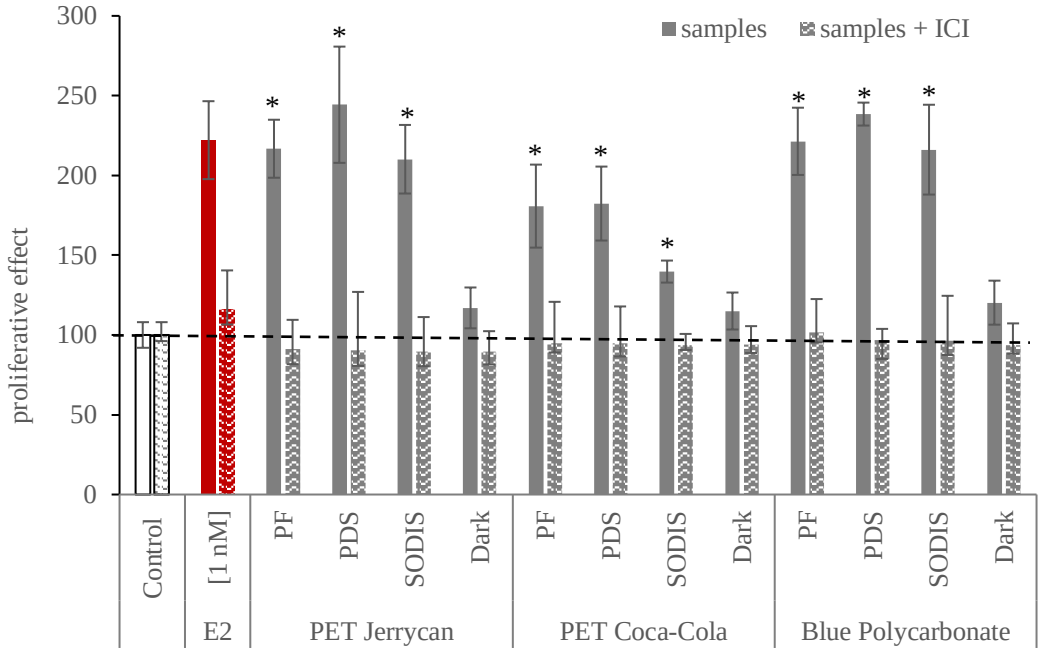


Figure 7.2 Proliferative effects on MCF-7 BUS cells of substances released from different plastic sources after 6 hours of irradiation for 7 consecutive days in SODIS, photo-Fenton (PF) and persulfate activation (PDS) processes*. Single factor Anova indicated significantly increased proliferation (*) p<0.05 of the water extracts compared to the control.*

Evidence of time-dependent estrogenic activity was also apparent for blue polycarbonate samples when comparing 1-week irradiation treatments and 6 h irradiation treatments. Prolonged irradiation over the course of 1 week of blue polycarbonate polymer enhanced estrogenic activity when compared to the estrogenic activity of the samples irradiated for 6 h. Estrogenic activity of released substances from blue polycarbonate under SODIS, photo Fenton and persulfate activation conditions were 216 ± 21%, 221 ± 21% and 238 ± 7% respectively relative to the control (**Fig 7.2**).

The RPE values of the polycarbonate samples (PC) irradiated for 1 week were ≥ 80% (**Table 7.2**), suggesting that they are strong estradiol agonists. Moreover, values for photo-Fenton and persulfate leachates were above 100% indicating that some of these leachates have an estrogenic effect more potent than that exerted by the positive control estradiol

Co-incubation of the samples released from the plastic polymers following 1 week of irradiation with ICI had a negligible effect on growth relative to the negative control (**Fig 7.2**), confirming the estrogenic effect of the samples.

Table 7.2 *Relative Proliferative Effects (RPE in %) of substances released into water from different plastics after 6 hours for 7 consecutive days under different irradiation treatments. Values are expressed as the mean ± standard deviation.*

RPE (%)				
	Irradiation treatment	Plastics polymer		
		PETjc	PETcc	PC
7 days	Photo-Fenton	82.43±37.87	62.73±15.71	115.65±18.85
	Persulfate	99.34±53.85	66.61±12.54	131.33±20.39
	SODIS	87.25±48.84	33.25±7.35	95.95±25.54

7.1.3. Estradiol Equivalents (EEQs)

Estradiol equivalent values (EEQs) were calculated for all the samples that showed an increased proliferative effect to determine the toxicity of the water in terms of average daily consumption by adults (**Table 7.3**). The values calculated from the dose-response curve of 17β-estradiol were corrected for the concentration factor used. Since the water samples were concentrated by a factor 10,000 via SPE and diluted in the E-screen 100-fold the concentration factors tested for every sample was 100X. Derived EEQs were reported as ng/L. The statistically significant EEQ values calculated ranged from 0.008 – 5.9 ng/L.

Following 6 h irradiation, the EEQ values for the samples from the blue polycarbonate polymer were in the order of 0.03-0.6 ng/L and were the highest values obtained for all three plastics. EEQ values for the PETjc samples were 10^3 to 10^6 times smaller than 1 ng/L while the EEQs values for PETcc samples were greater than the values for the PETjc and were in the order of 0.03-0.4 ng/L.

Following 1 week of irradiation, the EEQ values for the samples from the blue polycarbonate polymer were in the order of 1.3-5.9 ng/L and were again the highest values obtained for all three plastics for that period of irradiation. EEQ values of the PETjc samples were in the order of 0.6-1.5 ng/L while the EEQs levels for PETcc samples were in the order of 0.008-0.2 ng/L and unlike following 6 hours of irradiation were lower than the values obtained for the PETjc.

Assuming an average consumption of water through breast milk of a 5 kg baby is 0.95 L (EFSA, 2012), the daily consumption of estradiol of the irradiated treated sample that gave the higher EEQ value would be 1.1 ng/kg of body weight. However, these average values of body weight and liquid intake were established by a EFSA report for European populations, while the target communities of this study were African populations with reduced resources that could lead to lower body weights. Thus, for a baby of 3.5 kg that has a liquid intake of 0.52 L the daily

consumption of estradiol of the irradiated treated sample that gave the higher EEQ value would be 0.9 ng/kg of body weight. The corresponding valued for an average adult of 65 kg who drinks 2 L of water per day would be 0.2 ng/kg of body weight. All values calculated were well within the acceptable daily intake (ADI) for 17β-estradiol of up to 50 ng/kg bw/day established by the WHO (JEFCA, 2000).

Table 7.3 *Estradiol equivalents (EEQs) in ng/L of substances released into water from different plastics after 6 hours and 7 days under different irradiation processes*

EEQs (ng/L)				
Irradiation treatment		Plastics polymer		
		PETjc	PETcc	PC
6 hours	Photo-Fenton	8.4E-07	0.4	0.6
	Peroxydisulfate	4.6E-06	0.2	0.1
	SODIS	4.1E-04	2.7E-02	3.0E-02
7 days	Photo-Fenton	0.6	0.2	3.2
	Peroxydisulfate	1.5	0.2	5.9
	SODIS	0.8	8.2E-03	1.3

7.2. Testing of the advanced oxidative process samples with the Ames test

The water samples were tested for mutagenicity using the Ames Test as described in Section 2.2.8. No mutagenicity was detected for any sample as no value obtained showed a clear fold induction ≥2 above the baseline value (**Tables 7.4-7.5**).

Table 7.8 Ames test results for 6 hours of advanced oxidative processes

The results of the Ames Test for water samples from various photo-disinfection processes following 6 hours of irradiation. The plastics tested were: PET jerrycan (PETjc), PET Coca-Cola (PETcc) and blue polycarbonate (PC). The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as the mean ± standard deviation of the fold increase over the baseline. A fold increase over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Duration	Plastic Polymer	Irradiation Treatment	TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	3.34±1.17	3.42±1.03	1.9±0.7	2.78±1.3
		Positive control	41.53±1.93	45.87±2.59	23.06±1.16	40±2.87
6 hours	PET jc	Photo-Fenton	0.33±1.53	0.62±3.21	0.55±1.00	1.00±1.00
		Persulfate	1.00±1.00	0.80±0.58	0.70±1.53	0.32±1.53
		SODIS	0.82±1.53	1.00±2.00	1.00±0.00	0.44±0.58
		Dark	0.20±0.00	0.49±2.08	0.67±0.58	1.33±0.58
	PET cc	Photo-Fenton	1.00±1.00	0.44±0.58	0.55±1.00	0.67±0.58
		Persulfate	1.67±1.15	0.69±1.73	0.52±0.00	0.56±1.53
		SODIS	0.23±0.58	0.17±0.58	0.12±0.58	0.56±1.15
		Dark	0.60±1.00	0.14±1.15	0.33±0.58	0.67±2.31
	PC	Photo-Fenton	0.83±1.53	0.36±1.53	1.00±0.00	2.67±1.53
		Persulfate	1.33±1.15	1.15±0.58	0.35±0.58	0.32±1.53
		SODIS	0.58±1.53	1.00±2.00	0.12±0.58	0.56±1.53
		Dark	0.47±1.53	0.28±0.58	0.67±0.58	0.50±0.00

Table 7.9 Ames test results for 7 days of advanced oxidative processes

The results of the Ames Test for water samples from various photo-disinfection processes following 6 hours of irradiation on 7 consecutive days. The plastics tested were: PET jerrycan (JC), PET Coca-Cola (CC) and blue polycarbonate (PC). The test used the strains S. typhimurium TA98 and TAMix with (+) and without (-) S9. Results were reported as the mean ± standard deviation of the fold increase over the baseline. A fold increase over the baseline (≥2.0) upon exposure to samples relative to the control indicated that the sample was mutagenic.

Duration	Plastic Polymer	Irradiation Treatment	TA 98 (+S9)	TA 98 (-S9)	TA mix (+S9)	TA mix (-S9)
		Baseline	3.34±1.17	3.42±1.03	1.9±0.7	2.78±1.3
		Positive control	41.53±1.93	45.87±2.59	23.06±1.16	40±2.87
7 days	PET jc	Photo-Fenton	0.50±2.00	0.80±3.00	0.55±1.00	1.33±0.58
		Persulfate	1.50±1.00	0.46±0.58	0.35±0.58	0.24±1.00
		SODIS	0.82±1.53	0.17±0.58	1.00±0.00	0.67±1.00
		Dark	0.53±0.58	0.42±1.73	0.17±0.58	0.83±0.58
	PET cc	Photo-Fenton	0.17±0.58	0.27±1.00	0.18±0.58	1.67±2.08
		Persulfate	1.17±0.58	0.46±1.53	0.17±0.58	0.48±1.00
		SODIS	0.23±0.58	1.17±2.31	0.35±1.00	0.44±1.53
		Dark	0.20±1.00	0.28±0.58	0.33±0.58	1.50±1.00
	PC	Photo-Fenton	0.33±0.58	0.27±1.00	0.37±0.58	2.33±1.15
		Persulfate	1.33±2.08	0.69±2.00	0.17±0.58	0.40±0.58
		SODIS	0.35±1.00	1.33±0.58	0.47±0.58	0.33±1.00
		Dark	0.40±0.00	0.35±0.58	0.33±1.15	1.50±0.00

7.3. Summary of findings for the advanced oxidative processes

- When PET from the transparent jerrycan was tested for SODIS and the advanced oxidative processes for 6 hours irradiation no estrogenic activity was detected.
- The application of advanced oxidative processes for 6 hours did promote the release of estrogenic substances from PET from a Coca-Cola bottle and from blue PC from a water dispenser.
- Extended irradiation for seven days promoted the release of estrogenic substances from all three plastics used in all three solar disinfection processes.
- A direct correlation between the increase of the application time of the solar disinfection processes and the release of estrogenic substances from PET from the jerrycan reactor and PC from the water dispenser was observed. However, an inverse correlation between the increase of the application time of the solar disinfection processes and the release of estrogenic substances from PET from a Coca-Cola bottle was observed.
- Any estrogenic activity detected was not considered a risk to human health when the acceptable daily intake (ADI) for 17β-estradiol of up to 50 ng/kg bw/day established by JEFCA, (2000) was considered
- No mutagenicity was detected for any of the three plastics in any SODIS or advanced oxidative process.

Chapter VIII

8. Discussion

Validation and optimization of the E-screen

The E-screen bioassay was developed by Soto et al. (1995) as a tool to screen estrogenic activity of environmental pollutants. It measures estrogen receptor-dependent cell proliferation of human breast cancer cells (MCF-7). The role of the estrogen receptor in the induction of cell proliferation was confirmed when a potent specific pure estrogen receptor antagonist (ICI 182780) was identified in 1991. An analogue of estradiol, ICI 182,780 showed high affinity binding to the estrogen receptor and inhibited MCF-7 cell growth but was without effect on the growth of estrogen receptor negative human breast cancer cells (Wakeling *et al*., 1991). It is a simple assay in which the negative control is provided by cells cultured in medium supplemented with estrogen-free serum, the positive control is provided by cells exposed to E2 and test samples are cells treated with a range of concentrations of chemicals to be tested. A false positive result can be caused by mitogens that promote cell proliferation through pathways other than those involving estrogen receptor. To avoid false positive results and validate a positive answer the use of an estrogen receptor antagonist such as ICI is required. Some of the first studies to use the E-screen to assess estrogenic activity of substances released from plastic containers used as food contact materials (FCM) were conducted by Hirano et al. (2001) and Ohno *et al.* (2001, 2003). Both groups tested the estrogenic activity of substances released from polystyrene food containers and both groups reported negative results on estrogenic activity. Later, Wagner and Oehlmann (2011) used the E-screen to evaluate the estrogenic activity of water from eighteen brands of commercial PET bottles and found that eleven of the samples showed estrogenic activity. Yang *et al*. (2011) also used the E-screen assay and concluded that most plastics release estrogenic compounds when submitted to daily use stressors. Real *et al*. (2015) used the E-screen to analyse water samples from 29 brands of commercial PET bottles from the south of Spain, concluding that 79% of the samples contained estrogenic substances. However, there are no reports of the use of the E-screen assay to assess estrogenic activity of substances released during the SODIS process from plastic polymers of SODIS reactors. The only toxicological assessments in relation to estrogenic activity of SODIS samples were performed using chemical analysis (Wegelin *et al*., 2000; Schmid *et al.,* 2008). Both studies concluded that the level of leachates released from PET plastic polymer of the SODIS reactors was within the range established by different health authorities.

Though the E screen bioassay has not been officially recognised as a standard test method, our criteria for acceptance of the E-screen were: (i) that the negative vehicle control must not stimulate cell growth (ii) E2 at physiological levels (10^{-9} M) or other known xenestrogen used as positive

control must stimulate cell growth by at least 2-fold and (iii) proliferation should be inhibited by the anti-estrogenic substance, ICI. The vehicle control (medium containing DMSO 1% and 0.5%) had no effect on the proliferation of the cells when compared to blank controls of DMEM/F12 media alone. It has previously been reported in the literature that different stocks of the MCF-7 cell line have different sensitivities to estrogen (Villalobos *et al.*, 1995). Therefore, a critical parameter to meet the optimal conditions of 2-fold growth stimulation when MCF-7 cells are exposed to estrogenic controls was the selection of the MCF-7 stock. An MCF-7 stock from the European Collection of Authenticated Cell Cultures (ECACC) was selected initially for convenience and commercial availability, and its sensitivity was measured by exposing the cells to E2 and to BPA. This study showed that although MCF-7 (ECACC) cells showed a general increase in proliferation when treated with E2 or BPA neither of these substances promoted a 2-fold increase in proliferation relative to the negative control. Having not met the requisite criteria, validation of the E-screen assay with this stock of cell line cannot be claimed.

Observation of biological differences between MCF-7 stocks is not an unusual phenomenon. MCF-7 cells have been shown to differ in their responsiveness to E2 and their sensitivity to antiestrogens. Variable patterns of growth (Page *et al.*, 1983), tumour associated antigens (Hand *et al.*, 1983), amplification of *N ras* oncogene (Graham *et al.*, 1985) and tumorigenicity in athymic nude mice (Osborne *et al*., 1985) have also been documented. Such differences have been attributed to genetic instability during long term culture and or to selection of cells due to different culture conditions (Osborne *et al.*, 1987). A study carried out in 1987 showed that of 4 MCF7 cell lines obtained from different laboratories at Michigan Cancer Foundation and from a commercial source (ATCC), one MCF (ATCC) cell line was found to exhibit a distinctly different karyotype and distinctly different biological properties to the other MCF7 cells, suggesting MCF(ATCC) was not derived from the patient from whom the original pleural effusion was derived (Osborne *et al*., 1987). Like ATCC, the ECACC webpage (*ECACC General Cell Collection: 86012803 MCF7*, no date) also ascribes the MCF-7 cell stock as a cell line named after the Michigan Cancer Foundation (MCF) where it was derived from the pleural effusion from a 69-year-old female suffering from a breast adenocarcinoma. It cites the above studies that showed the susceptibility of these cells to genetic instability. This description and its associated bibliography are very similar as those provided for the MCF-7 stock from the American Tissue Culture Centre (ATCC) (*MCF7 ATCC® HTB-22™*, no date).

Villalobos *et al.* (1995) conducted a study that also showed biological differences between MCF-7 cell lines. They characterized the proliferative responses of a total of four stocks of MCF-7 cells to determine which of them should be chosen for screening industrial chemicals for estrogenic activity. Of four stocks examined by Villalobos *et al.* (1995), the MCF-7 cell stock from the American Type Culture Collection (ATCC) at passage 147 was found to be least proliferative in

response to an estrogenic environment. Based on the findings of Osborne *et al.*, (1987), Villalobos *et al.* (1995) attributed their finding to the fact the MCF-7 cells of ATCC stock came from a different patient than the other MCF-7 stocks. As shown in Chapter 3, MCF-7 ECACC stock exhibited proliferative responses to an estrogenic environment of a magnitude similar to responses exhibited by the MCF-7 ATCC stock in the Villalobos *et al.* (1995) study. The other three MCF-7 stocks compared in the study of Villalobos *et al.* (1995) were MCF-7 BUS also known as MCF-7 BOS at passage 173 of the original MCF-7 cells, donated from Tufts University, Boston, MCF-7 BB at passage 580 from Institut Jules Bordet, Brussels, Belgium who had received them from the Michigan Cancer Foundation and MCF-7 BB104 derived from MCF-7 cells in their laboratory. MCF-7 BUS stock showed a proliferative response to estrogen (E2) with cells yielding a fold over the proliferation over the negative solvent control higher than two. Villalobos *et al.* (1995) suggested that the different proliferative profiles and sensitivities to (xeno)estrogens of the MCF-7 stocks could not be attributed to culture conditions or passage number and attributed these differences to heterogeneity of cloned subpopulations of the MCF-7 cell line. This most estrogen-responsive MCF-7 BUS stock continues to be widely cited in the literature for screening estrogenic activity in bottled mineral water (Wagner and Oehlmann, 2009) and industrial wastewater (Schilirò *et al.*, 2012), evaluating estrogenic potential of emerging environmental contaminants (Rasmussen and Nielsen, 2002; Henry and Fair, 2013) and phytomedicines (de Ávila *et al.*, 2018; Lee *et al.*, 2018) and for assessing antiestrogenic potential of novel anticancer compounds (Jones *et al.*, 1997) (Ahmed and Wober, 2020; Bazioli *et al.*, 2020) and selective estrogen receptor modulators (Park *et al.*, 2019). It was of interest to use the MCF-7 BUS stock, given its use in the literature, however, it was not commercially available. As previously described in other studies that used MCF-7 BUS, the MCF-7 BUS cells used in this study were generously gifted by the group C. Sonnenschein and A. Soto (Tufts University, Boston). The data presented in Chapter 3 provided clear evidence that MCF-7 BUS stock was suitable for determining estrogenic activity. Overall, MCF-7 BUS displayed a 2-3-fold higher proliferative effect than the MCF-7 ECACC stock rendering the latter not sensitive enough to be used for the E-screen.

Another important parameter of the acceptance criteria of the E-screen was to determine the best way to measure cell proliferation during the E-screen assay. To measure proliferation there are two types of assays that can be used, direct assays and indirect assays. Direct assays determine cell proliferation and growth by measuring the number of stained or dyed cells, nuclei, DNA or protein. Indirect assays determine cell proliferation, growth and viability by quantifying the sub-product generated in a metabolic reaction. Therefore, special attention has to be paid when using indirect assays to measure proliferation in order to avoid bias results. If the effects of the screened chemicals interfere with the cellular pathway/metabolic reaction used by the indirect assay to

measure proliferation the results could be biased. Different studies have measured proliferation of MCF-7 cells during the E-screen bioassay with different methods. In the original E-screen assay developed by Soto *et al* (1995) cell proliferation was determined with a direct method by staining and counting cell nuclei. Other direct methods that were used later to determine cell proliferation include the sulforhodamine-B assay (Villalobos *et al.*, 1995; Rasmussen and Nielsen, 2002) that fixes cells and stains their protein content. Later, Yang *et al.* (2011) quantified DNA as a direct way to determine cell proliferation of MCF-7 cells during the E-screen. Indirect assays such as MTT (Körner *et al*. 1999; Wang *et al*. 2010), MTS (Henry and Fair, 2013) and Alamar Blue or Resazurin (Wagner and Oehlmann 2011) have also been used to determine proliferation of MCF-7 cells.

The acid phosphatase (AP) assay measures the amount of chromophore produced by the AP enzyme of cells after hydrolysis of the substrate para nitrophenyldiphosphate. The assay has been previously used to evaluate anti-tumorigenic properties of lead compounds in cancer cells (Martin and Clynes, 1993). For example, a recent publication by Irene and Claudio (2020) used the AP assay to measure the antiproliferative effects of doxorubicin and α-mangostin, two anticancer drugs being trialled to reduce cell stemness in MCF-7 cell model of luminal breast cancer. However, it has not been previously reported in the literature for screening compounds for estrogenic activity. Damiani et al. (2019) reported that a reliable and uniform cytotoxic profile of chemical agents with varying mechanisms of cytotoxicity in glioblastoma cells could not be achieved when using conventional assays (MTT, Alamar Blue, Acid Phosphatase and Trypan Blue). Damiani et al. (2019) concluded that due to the varying endpoints of different assays and varying mechanisms of cytotoxicity exerted by different substances accurate IC_{50} values could not be defined and highlighted the importance of understanding the advantages and limitations of each assay for better predictive responses. This study showed that there was no statistically significant difference between the proliferative effects exerted by E2, BPA and the negative control when cell proliferation was determined using the AP assay indicating that this assay is not suitable for estrogenic screening, hence it was not further used for future experiments.

In many *in vitro* investigations, tetrazolium-based assays such as MTT, MTS and WST-1 assays, are frequently used as a measure of cell growth and viability. All these assays use NAD(P)H-dependent cellular oxidoreductase enzymes in viable cells to reduce the different forms of yellow tetrazolium MTT, MTS or WST into purple formazan. The MTT assay was the first homogeneous cell viability assay for 96-well high throughput screening that used a tetrazolium oxidative reaction (Stockert *et al.*, 2018). MTS is a new kind of tetrazolium salt that is directly soluble in cell culture medium, which eliminates a liquid handling step during the assay, saving time and eliminating errors. Kröner *et al.* (1999) showed that the MTT assay that measures mitochondrial activity was comparable and as suitable as the SRB assay that stained cell protein to measure

proliferation during E-screen. Henry and Fair (2013) successfully used the MTS assay in an E-screen to assess anti-estrogenic properties of several emerging environmental contaminants on MCF-7 cells. However, it has been previously reported in the literature that metabolism linked to NADPH shows environmental plasticity on MCF-7 cells (Otto *et al.*, 2015). The endpoint of the MTS assay is based on NADPH metabolism which has been shown to be affected by the culture environment of MCF-7 cells. Wang *et al.* (2010) compared DNA quantification as a direct assay and reduction of MTS and MTT as indirect assays to measure antiproliferative effects of botanical chemicals on MCF-7 cells. Unlike the MTS assay that could be affected by number of passages, divisions and treatments, DNA measurement is not as easily affected by any of these factors and thus gives more reliable direct measurement of cell proliferation. Therefore, Wang *et al*. (2010) concluded that the MTS and MTT assays could underestimate results and that the selection of the proliferative assay should be evaluated carefully depending on the chemical to be evaluated and its effect on the cells selected. It is also noted that several structurally diverse estrogens including BPA and E2 can activate or inactivate MCF-7 cell kinases through non-genomic pathways, which could lead to unexpected effects on metabolism causing false positive or negative results if proliferation is measured through metabolic activity (Zhang and Safe, 2006).

It was expected that in accordance with previous literature MTS and DNA quantification would be suitable for measuring proliferation during E-screen. This study showed that the most sensitive assay to measure proliferation of MCF-7 BUS stock cell line is DNA quantification. Moreover, the EC_{50} 2.1 pM calculated in this study from the standard curve of Relative Proliferative Effects of E2 on MCF-7 BUS cells was similar to the EC_{50} value of 1.7 pM reported by Yang et al. (2011). However, during this study, the maximum proliferative effect reached by E2 [1 nM] was a 3-fold increase over the negative control, far from what has been reported in other studies of 4-fold to 6-fold increase (Rasmussen and Nielsen 2002; Villalobos et al. 1995; Yang et al. 2011).

The lack of standardization of the E-screen may explain the variabilities in the sensitivities to E2 reported in different studies that used the MCF-7 BUS cell stock. The difference in proliferation exerted by E2 [1 nM] in MCF-7 BUS and the proliferative effect reported in other studies could be due to variations in concentration of hormone-free serum used in the experimental medium. Medium in this study was supplemented with equal proportions of charcoal-stripped foetal calf serum (2.5%) and newborn calf serum (2.5%). Lykkesfeldt and Briand (1986) showed that both charcoal-stripped calf serum and newborn calf serum were suitable to be used as hormone-free serum to supplement the experimental media used with MCF-7 cells. Soto *et al.*, (1995) used DME medium supplemented with 5% charcoal dextran foetal bovine serum during the development of the E-screen. Later, Villalobos et al. (1995) tested different concentrations of charcoal-stripped serum in the experimental medium used with the different stocks of MCF-7 cells to determine how that affected their sensitivity and responsiveness to E2, and reported that

MCF-7 BUS cells cultured in media supplemented with 10% of charcoal striped serum yielded higher proliferative effects than other stocks and other serum concentrations. In a more recent paper by Yang et al. (2011) medium supplemented with a combination of 1% charcoal-stripped foetal bovine serum and 4% charcoal-stripped bovine calf serum was used as an estrogenic free medium in the E-screen bioassay. It yielded a 4-fold increased proliferation of MCF-7 cells with E2 over the negative control. The differences between the proliferative effects of E2 in MCF-7 BUS cells obtained in this study and previous studies could also be due to the lack of estrogen deprivation prior to experimental work. Rasmussen and Nielsen (2002) reported that maintaining MCF-7 for 2-3 days in an estrogen-free medium (estrogen deprivation) reduced the variability of proliferative effects observed during the E-screen and promoted sensitive enhancement of MCF-7 BUS to estrogenic substances. Yang et al. (2011) also deprived MCF-7 cells of estrogenic medium 2 days prior to their experimental work.

Finally, the third point of the acceptance criteria established that proliferation must be inhibited by co-incubation with an anti-estrogenic substance. This criterion was established to avoid false positives caused by other substances that promote proliferation of cells through cellular pathways other than the estrogenic receptor pathway. Tamoxifen was initially used on breast cancer cell lines such as MCF-7 as an antagonist of estrogen (Gradishar and Jordan 1997; Hissom *et al*. 1989; Jordan 1995; Wakeling and Bowler 1988), however MCF-7 cell line gained resistance to tamoxifen (Hu *et al.*, 1993; Wiebe *et al.*, 1993) and new antiestrogenic substances like ICI, were studied and used (Hu et al. 1993; Katzenellenbogen *et al*. 1997; Walton *et al.* 1994; Nawaz and Hyder 1995). Rasmussen and Nielsen (2002) tested the anti-estrogenic properties of ICI by co-incubating MCF-7 BUS cells with E2 (1nM) and different concentrations of ICI. A similar experiment was performed during this study in order to determine the most suitable concentration of ICI to be used during E-screen in order to validate and corroborate positive results. The anti-estrogenic properties and potency of ICI are in agreement with a previous study in which the maximum inhibition obtained was at 10^{-6} M and an IC_{50} of approximately 2×10^{-7} M (Zafar Nawaz and Hyder, 1995). The dose-response curves of ICI demonstrated that ICI at 0.1 μM was suitable to be used as an antagonist of the proliferative effects on MCF-7 exerted by estrogenic substances and that is similar to the dose-response curves to ICI in the presence of E2 reported by Rasmussen and Nielsen (2002). This study showed that all the acceptance criteria established to ensure optimal performance of the E-screen assay were met.

Other important parameters like the cell seeding density, the period of incubation and the antagonistic effect of ICI were studied in order to improve and optimise the performance of the E-screen bioassay. This study performed the E-screen assay in 96-well plates to maximize the number of samples and replicas that could be measured. The original E-screen assay developed by Soto *et al.* (1995) was performed in a 12-well plate. Villalobos *et al.*, (1995) optimized the E-

screen assay in a 24-well plate, and Rasmussen and Nielsen (2002) optimised and standardized the E-screen assay by performing it in a 96-well plate. In agreement with what was described by Rasmussen and Nielsen (2002), initial observations of this study led to the conclusion that it was better to avoid the use of the wells at the edge of the 96-wells plates since the results of those wells were too variable. The initial seeding density of cells to use is intrinsically related to the plate used, the number of wells in the plate and the size of the wells. Soto *et al.* (1995) used a seeding density of 20,000 cells/well. Since Villalobos *et al.*, (1995) doubled the amount of wells in the plates that they used the seeding density was reduced to a half (10,000 cells/well) of the seeding density previously used by Soto *et al.* (1995). To determine which was the more suitable seeding concentration to be used in a 96-well plate Rasmussen and Nielsen (2002) tested a range of cell seeding densities from 1500-6000 cells/well during the E-screen. Rasmussen and Nielsen (2002) determined that the best results were obtained between 4500-6000 cells/well. Similarly, this study tested the performance during the E-screen with a range of seeding densities from 500 to 4000 cells/well to determine which was the best initial cell concentration to use within the E-screen performed in a 96-well plate. The selected range was close enough to the range tested by Rasmussen and Nielsen (2002) and close to the seeding concentration of 5000 cells/well in a 96-well plate used by Yang *et al.* (2011). It was determined that the best initial cell concentration was 4000 cells/well which was in agreement with the range established by Rasmussen and Nielsen (2002). Yang *et al.* (2011) similarly used 5000 cells/well in a 96-well plate. On the other hand, Wagner and Oehlmann (2011) used far less concentrated seeding, 1,500 cells/well in a 96-well plate. A cell density of 1500 cells/well was found to be suboptimal in this study and by Rasmussen and Nielsen (2002). Other studies used far higher cell densities ranging between 8000 and 10,000 cells/well (Henry and Fair 2013; Wang *et al.* 2010); however, according to Rasmussen and Nielsen (2002) there were no statistical differences between any seeding density above 4500 cells/well.

Another important parameter to optimize the E-screen assay is the period of incubation. It was established by Soto *et al.* (1995) during the development of the E-screen that the optimum period of incubation for the E-screen was 6 days which corresponded to the late exponential phase of the proliferation of MCF-7 cells. This period of exposure has been used by many others that have used the E-screen assay (Henry and Fair 2013; Hirano et al. 2001; Jones et al. 1997; Ohno et al. 2003; Rasmussen and Nielsen 2002; Soto et al. 1995; Villalobos et al. 1995; Wagner and Oehlmann 2009, 2011; Yang et al. 2011). To ensure that this was the optimal period for MCF-7 BUS to respond to E2, cells were exposed to E2 [1 nM] and BPA [0.1 μM] for 1, 2, 3, 4, 5 and 6 days. This study showing that 6 days of exposure to estrogens was the optimal period to get a proliferative response is in agreement with the literature for E-screen assay.

Validation and optimization of solid phase extraction

As described in Chapter 2 water samples were prepared for *in vitro* bioassay by solid phase extraction (SPE). This is a sample preparation technique commonly used prior to the analysis of endocrine disrupting activity in samples. Extraction of samples is an essential step to properly prepare the water samples for analysis in order to purify the sample. By minimising matrix interferences caused by natural organic matter and ions, SPE improves the likelihood of concentrating possible xenestrogens and mutagens. Due to the simplicity and automatization of the solid phase extraction technique (SPE) compared to the time consuming, complex liquid-liquid extraction (LLE) technique SPE has been widely used as an analytical tool for the preparation, purification and concentration of water samples to be assessed in endocrine disruption assays (Pacáková *et al.*, 2009). SPE cartridges may be loaded with environmentally aqueous samples ranging in volume from 250 ml -1500 ml (Caban et al. 2015; Wagner & Oehlmann 2011). While solid phase extraction and other sample preparation techniques have been optimised and standardized for chemical analysis, standardization and optimization have rarely been conducted for bioassay studies. SPE procedures used in bioassay analysis have often been adapted from procedures used for chemical analysis. However, preparation procedures of the samples for chemical analysis could be ineffective in extracting unknown active compounds from water samples to be analysed with bioassays. Thus, optimising the SPE procedure is imperative for bioassay studies in order to avoid underestimations or false negative results. Typically, during the Solid Phase Extraction procedure a liquid sample is loaded through a sorbent (also known as the stationary phase) contained in a syringe cartridge. The choice of sorbent is determined by the nature of the chemical analytes to be separated. Typically, the desired analytes of interest are retained on the stationary phase and are then removed from the stationary phase for collection in an additional elution step. The concentration step carried out through Solid Phase Extraction was critical in this study not only to avoid false negative results, but also because it facilitated the transport of samples between the different partners in different countries of the *Waterspoutt* project.

Water samples are frequently acidified prior to analysis in order to stabilize samples and prevent biodegradation of potential toxicants. It has been proposed that acidification deactivates microorganisms that would otherwise target analytes. Of note is a recent study that systematically examined the effects of acidification of water samples prior to testing for endocrine activity, genotoxicity and mutagenicity (Abbas *et al.*, 2019). Abbas *et al.*, (2019) reported that estrogenic activity as determined by YES assay and mutagenicity as determined by Ames assay were not significantly affected by the acidification of water samples (untreated hospital effluent, conventionally treated wastewater, surface water). They suggested that aqueous samples maintained at neutral pH may better represent actual estrogenic activity of water than acidified

samples. With one exception, mutagenicity was also not significantly affected by acidification of water samples. Only untreated hospital effluent showed significantly higher mutagenicity (Ames TA98 and Ames TA100) at neutral pH than at pH 2. For those reasons, *Waterspoutt* samples were not acidified prior to SPE.

Several SPE sorbents have been used for recovering endocrine, genotoxic and mutagenic activities. Wagner and Oehlmann compared 6 sorbents (C18 silica, Oasis HLB, two SDB sorbents, IsoElute ENV+, an hydroxylated copolymer of SDB and Envi-Carb Plus, an amorphous carbon molecular sorbent) ranging in the ability to extract polar and non-polar analytes and reported that C18 silica sorbent was the most effective in recovering estrogenic activity from bottled mineral water. C18 silica sorbent uses non-polar or Van der Vaals interactions to retain non-polar analytes. They attributed estrogenic activity to non-polar chemicals in bottled mineral water. Their study showed Oasis HLB was second best and as good as Envi Carb Plus, a sorbent with affinity for both polar and non-polar analytes. Oasis HLB is made from a specific ratio of 2 monomers: hydrophilic N-vinyl pyrrolidone and lipophilic divinylbenzene which allow the sorbent to retain both polar compounds as well as non-polar hydrophobic compounds in a water sample. Considering that most plastic products subjected to common-use stresses released estrogenic activity into polar solvents (Yang et al 2011), Oasis HLB was selected for use in this study. Recently, Oasis HLB has been identified as being as good as a silica-based Telos C18/Env sorbent for recovering estrogenic activity in conventionally treated wastewater and better than carbon-based Supelco Envi-Carb sorbent. In particular, Abbas et al (2019) reported that the percentage of estrogenic activity of conventionally treated wastewater extracted using Oasis HLB, Telos C18/Env and Supelco Envi Carb was 3.05%, 2.07% and 0.66% respectively. During this study, MCF-7 BUS cells were exposed to solid phase extracted estrogens BPA and E2 spiked into water. The water samples were extracted with two different cartridges Perkin Elmer sorbent specially designed to selectively retain BPA coupled to a plastic cartridge, and N-vinylpyrrolidone-divinylbenzene sorbent coupled to glass Oasis HLB cartridges. In accordance with what was established by Abbas *et al.* (2019) the results of this study showed a better performance of Oasis HLB cartridges in extracting estrogenic compounds. It is apparent from Chapter 3 that Oasis HLB was effective at recovering estrogenic activity in water. To our knowledge, other cartridges used for solid phase extraction of estrogenic and mutagenic compounds are made of polypropylene plastic. In order to avoid possible cross-contamination or bias due to the plastic of the cartridge's columns, this study selected Oasis HLB columns that were available in glass material. There are many examples in the literature showing the use of solid phase extraction to selectively enrich water extracts in endocrine disruptive and mutagenic activities. Oasis HLB extracts of bottled mineral and flavoured waters demonstrated estrogenic, androgenic, progestogenic and glucocorticoid-like activities in a range of reporter gene assays for

endocrine disruptive chemicals (Plotan et al. 2013). However estrogenic, androgenic and progestogenic activities were at levels that were not of human concern. Oasis HLB extracts of water from sun-exposed PET bottles exhibited endocrine disruptive and mutagenic activities but activities were not at levels of human concern (Bach et al. 2013, 2014).

E-screen assay for toxicity of the extractables released from the plastics

Plastic routinely exposed to sunlight, as was the case with the plastics used in the construction of the novel SODIS reactors, undergoes light-induced degradation, also known as photo-degradation. This loss of chemical robustness induced by stress factors is known as the aging of the plastic (Andrady, 2015). Sunlight exposure of the plastic could also affect the chemical structure of the plastic by inducing the breakage of chemical bonds between the plastic polymer and the additives (Andra et al. 2011).

Since there was concern that prolonged use of plastics for SODIS treatment might affect the release of potentially toxic substances, the plastics were aged for testing by exposing them to weather conditions at Plataforma Solar de Almería (PSA), Almería, Spain, for different periods of time up to nine months. Plataforma Solar de Almería (PSA) was also selected as the exposure location for the novel SODIS reactors. PSA belongs to the Center for Energy, Environmental and Technological Research (CIEMAT) and is the largest research, development and testing center in Europe dedicated to concentrating solar technologies. The PSA carries out its activities as an R&D Division within the structure of the CIEMAT Department of Energy. The solar water treatment unit (TSA) is one of the research units at the center and was established in 2012. The experimental work in this study was carried out at this unit in collaboration with our partners at PSA. PSA is located in the south of Spain in Tabernas. Within the European partners of the *Waterspoutt* project the geographic location of PSA in the south of Spain had the most similar climatic conditions to the target communities in Africa.

Extractables from plastics are defined as substances that are forcefully released from the plastic materials with the use of solvents and exaggerated conditions of time and temperature (Dorival-García *et al.*, 2018). Ball *et al.*, (2012) categorized the different extraction processes, depending on the conditions used to perform the extraction. A simulated-use extraction is an extraction process conducted using a method that mimics the expected operational conditions of the analysed device. An exaggerated extraction uses harsher extraction conditions to release a greater amount of substances than during operational conditions. Finally, an exhaustive extraction, is an extraction that is repeated until the amount of extractables released is less than 10% of the amount of extractables obtained during the initial extraction. The most common practice is to perform at least two types of extractions, a simulated-use extraction and an exaggerated or exhaustive extraction. This is a common practice because while the simulated-use extraction reveals

information of the released substances that can be practically expected, the exaggerated or exhaustive extraction estimates the highest amount of extractables that can be extracted under the worst-case conditions.

Toxicological assessments of the extractables from the raw and aged plastics used in the construction of the novel SODIS reactors of the *Waterspoutt* project were carried out using *in-vitro* assays by performing an exaggerated extraction in an effort to mimic the worst-case-scenario. This would address possible health effects of all of the substances that might potentially migrate from the plastics of the novel SODIS reactors. The study assessed the toxicity of extractables using the Ames test to determine the mutagenicity of the extractables and the E-screen to determine the estrogenic activity of the extractables.

One of the first critical steps to take into account during the extraction process is the surface area to volume ratio. Whenever it is impractical to analyze and do the toxicological assessments of extractables from the whole plastic container (or SODIS reactor), then representative parts of the device can be utilized. The use of representative plastic parts usually involves cutting the materials into portions with dimensions of 10 mm x 50 mm or smaller in order to enhance extraction efficiency (*Biological evaluation of medical devices-Part 12: Sample preparation and reference materials (ISO 10993-12:2012)*, 2012; Ball *et al.*, 2012). The ratio of surface area/volume of solvent used during the extraction of 1g of plastic in 4 ml of solvent, was approximately 50 times higher (50X) than the contact surface to volume ratio in the reactors. To choose other optimum extraction conditions, the guidance principle is that the extraction should provide a suitable exaggeration of the predicted conditions of product use. This gives a margin of safety when evaluating the leachates, a subset of extractables that could potentially migrate from the container under operating conditions (simulated-used extraction). Hence to prevent adverse effects due to leached chemicals from the plastics an extensive knowledge of the plastic material, its contents and the potential interactions that could lead to the release of harmful chemicals is required in order to properly design the toxicological assessments of the plastic materials. Generally, the extraction solvents should be chosen to maximize the amount of extractables without dissolving the plastic polymer itself. It is advised to use both polar and non-polar extraction solvents to extract a wider range of organic substances. As a rule of thumb, molecules with low polarity will be soluble in non-polar solvents and polar molecules will be soluble in polar solvents. Also, solvent volatility should be considered in order to increase method sensitivity (Ball *et al.*, 2012; Suman *et al.*, 2019). In this study, ethanol and DMEM/F12 medium were used as solvents in the E-screen assay and ethanol and PBS were used to extract samples for the Ames test. The solvents used for the extraction were selected based on previously reported work by Yang et al. (2011) who used a saline solvent and ethanolic solvent and common daily stressors (microwave, heat and humidity of dishwasher and UV exposure) to obtain extractables from different plastic products.

Partially based on what was previously reported by Fang *et al.*, (2003), Yang *et al*., (2011) described in the supplementary material of their work critical physical-chemical structures and properties that affect the binding interaction of (xeno)estrogens to the estrogen receptor (ER). One of those critical parameters was the hydrophobicity of the compound, moreover, it was proposed by Yang *et al.,* (2011) that hydrophobicity of the entire molecule can trump all other properties and permit tighter binding than substances showing equivalence in terms of essential chemical groups. Since hydrophobic substance tends to have higher solubility in non-polar solvents, the use of non-polar solvents as contraposition of polar solvents was advised.

Another important consideration when selecting the extraction solvents is their compatibility with the analytical techniques used during the toxicological assessments. Typical non-polar solvents that have been used for extraction include hexane, chloroform and methanol. However, none of these solvents is compatible with bioassays, due to their toxicity. Ethanol is a solvent with relatively low polarity suitable to be used during the performance of bioassays, however it could not be used in the bioassay without prior dilution due to its cytotoxic effects. Therefore, when carrying out the E-screen ethanol was diluted to a 1% v/v in the media. On the other hand, water and saline solutions are typically used as polar extraction solvents. In this study, the samples to be tested with the E-screen were extracted in DMEM/F12 medium as a polar solvent to avoid dilution or changes of the media parameters/nutrients that could have an impact on cell growth. For consistency and comparison of the effects of the polar and non-polar solvents, the DMEM/F12 media samples were diluted 100 times (1% v/v) for the E-screen assay performance. Samples of extractables in DMEM/F12 were also diluted 10 times in order to test a contact surface/volume ratio closer to the contact surface/volume ratio of plastic/water in the novel SODIS reactors.

The temperatures selected for the extraction process were 50 ℃, 37 ℃ and room temperature which was around 20 °C. These temperatures represent the range of temperatures that would be experienced in the SODIS process. At the upper limit, 50 ℃ was selected as an exaggerated extraction condition. Although a synergistic effect between UV radiation and a temperature of 45 ℃ and above has been shown in the SODIS process (McGuigan *et al.*, 1998), the temperature in practice rarely reaches this temperature and more usually ranges from 25 ℃ – 40 ℃ (McGuigan *et al.*, 2012). The time periods for extraction at the various temperatures ranged from 5 hours to 72 hours. In the SODIS process, the time of exposure of the reactor to the sun is recommended to be up to 6 hours in strong sunlight and 2 days under cloudy conditions. Prolonged periods of time greater than 24 hours, therefore, represented exaggerated conditions of incubation. One of the outcomes of these experiments was that they allowed the identification of the minimum period of incubation and temperature necessary to promote the possible release of estrogenic and mutagenic substances and the effects of aging of the plastic on the release of extractables.

Polymethyl methacrylate (PMMA) was used in the construction of the novel large-volume batch solar reactors used for the treatment of rainwater in field trials in sub-Saharan Africa. PMMA, also known as acrylic or acrylic glass, is a transparent and rigid thermoplastic material. The material was particularly suited to the manufacture of novel SODIS reactors as it has a high resistance to UV light and weathering and shows excellent light transmission. The polymer has a refractive index of 1.49. It allows 92% of light to pass through it, which is more than glass or other plastics. PMMA is a useful shatterproof replacement for glass being half the weight of glass with up to 10 times the impact resistance. It also has excellent scratch resistance although less than glass. PMMA is highly biocompatible and it has been used for dentures and bone replacement (*PMMA Plastics Poly(methyl methacrylate): Properties, Uses & Application*, no date).

Only one previous study could be found that researched the leachability of monomers and plasticizer from polymethyl methacrylate (PMMA) resins. Kawahara *et al.* (2004) submerged different commercial denture restorative PMMA resins in ethanol for 1, 3, 6, 12 and 24 h and 3, 7, and 14 days, all of them at room temperature. They analysed the leachability of the residual methylmethacrylate (MMA) monomer and plasticizer additives with instrumental analytical techniques (HPLC and GCMS). Although Kawahara *et al.* (2004) did not apply exaggerated temperatures to obtain extractables, a solvent, ethanol, was used for prolonged periods of time. Therefore the findings of Kawahara *et al.* (2004) were used for comparison with the extraction results of this study. Furthermore, although the PMMA polymers used by Kawahara *et al*. (2004) may not be the same as the PMMA used in this study in terms of additives, their results could serve as preliminary guidance to what plasticizers may be extracted or leached from PMMA.

This study assessed the toxicity of extractables from PMMA (raw or unused, 3-months-aged, 6-months-aged and 9-months-aged) in the E-screen, using different conditions of time and temperature and two different solvents (ethanol and DMEM/F12 cell medium) as extraction conditions. Kawahara et al. (2004) reported that ethanol was able to release di-n-butyl phthalate (DBP) a substance that has been well described in the literature as an estrogenic endocrine disruptor. However, in this study, PMMA extractables in ethanol following extraction at room temperature were not estrogenic. This could be due to several factors - the additive composition of the PMMA polymer used in the novel SODIS reactors may not be the same as the PMMA polymer tested by Kawahara et al. (2004) or if the additive composition was similar and DBP could be extracted from the PMMA polymer tested in this study, the amount that could potentially be extracted in 72 h in ethanol may not cause a proliferative effect in MCF-7 cells. This study showed that only exaggerated extraction conditions (higher temperatures and long periods of exposure) with ethanol were able to extract a concentration of estrogenic substances potent enough to have an effect on proliferation of MCF-7 cells. According to the results of this study,

only, raw or unused PMMA plastic polymer and the 3-month-aged plastic released substances with estrogenic activity. The release of estrogenic substances from raw PMMA plastic polymer was achieved after long periods of exposure (15 h) to extreme temperature conditions equal to or above 37 °C and at any period of exposure for 50 °C in polar cell culture medium solvent. In the case of raw PMMA, only samples extracted with the polar solvent DMEM showed estrogenic activity, which suggested the extraction of polar compounds. When, however, the plastic was aged in sunlight, estrogenic activity was detected using both solvents, polar cell culture medium and the relatively less polar ethanol, but mainly after 3 months exposure, some following six months exposure and none following 9 months exposure. This result suggested that both polar and non-polar compounds were extracted following aging. The release of estrogenic substances from 3-months-age and 6-months-aged PMMA plastic polymer was achieved after 24 h and longer periods of exposure at 37 °C and at any temperature at any period of exposure at 50 °C. All together, these results suggested that either substances with estrogenic activity may be gradually released from PMMA into the environment (or if used as a container into its contents) due to the action of weather conditions, until the complete depletion of estrogenic active substances from the plastic, or alternatively, substances or additives with estrogenic activity in PMMA may be susceptible to photo-degradation. As previously pointed out in Chapter I, non-monotonic dose-effect relationships are something typical of endocrine disrupting chemicals (Khetan, 2014). The findings in this study obtained using two concentrations of the DMEM extracts suggest an effect independent of concentration which could imply a non-monotonic (not linear) relationship of the dose-effect, however more concentrations in media would have to be tested in order to confirm this dose-effect relationship.

Polyethylene terephthalate (PET) was used in the construction of the novel large-volume (25 L) transparent jerrycan solar reactors used for the treatment of water in field trials in sub-Saharan Africa. PET is a mechanical, thermal, chemically resistant thermoplastic material that belongs to the polyester family of polymers. PET is an excellent water and moisture barrier material which together with other physical properties, has made it particularly suitable for food packaging applications, such as PET bottles used for mineral water. In SODIS studies to date, the most commonly used reactors have been 2 L PET bottles due to their excellent temperature resistance and light transmission. PET is a very strong and lightweight material which makes it easy and efficient to transport. Also, due to its relatively high chemical inertness PET packages have been approved as safe for contact with food and beverages by the FDA, Health Canada, EFSA and other health agencies (*PET Plastic (Polyethylene Terephthalate): Uses, Properties & Structure*, no date).

In contrast to PMMA, the toxicity of extractables from PET bottles and containers has been widely studied using both instrumental and non-instrumental analytical techniques (Bach et al.

2013; Yang et al. 2011). Bach et al. (2013) used the transcriptional activation assay of the HepG2 cell line to assess estrogenic activity and the Ames test and the micronucleus assay to assess mutagenic and genotoxic activity of water contained in PET bottles which were submitted to different temperatures (40, 50, 60 °C) for 10 days. They found that none of the extractables released from the PET triggered estrogenic or mutagenic responses. Similar to the work done by Bach et al. (2013), in this study extractables from raw and aged PET plastic polymer were obtained following incubation for different periods of time (5, 15, 24 and 72 h) at temperatures of 20, 37 and 50 °C. However, unlike Bach et al. (2013) who did not use solvents, two solvents, ethanol and DMEM/F12 medium, were used in this study similar to the study described by Yang et al. (2011). In contrast with the findings of Bach et al. (2013) who reported no estrogenic activity in extractables from PET bottles, this study showed that extracts with estrogenic activity were released from raw PET using the solvent ethanol. As was the case for raw PMMA, estrogenic activity was only detected when the plastic was extracted at elevated temperatures and for prolonged time. However, unlike the findings for PMMA, estrogenic activity was detected when the more non-polar solvent was used – suggesting the extraction of non-polar compounds. Estrogenic activity was detected also when the polar solvent DMEM/F12 medium was used but only under extreme extraction conditions of time and temperature (50 °C for 15 h). Aged PET on the other hand only showed estrogenic activity when extracted using DMEM at elevated temperatures and prolonged time of incubation. In contrast to the findings with PMMA, estrogenic activity was detected when the PET was aged for 3, 6 and 9 months demonstrating an ability of the active compounds to survive with time as the plastic ages. Yang *et al.* (2011) used the E-screen to assess the estrogen activity of extractables released from different plastics under common daily stressors (microwave, dishwasher and UV radiation). They showed that 75% of the PET extracts were estrogenic. Similar to the results of this study obtained with the raw PET polymer Yang *et al.* (2011) reported that solvents with different polarities were able to extract different subsets of estrogenic substances. They recommended that both polar and non-polar solvents be used to extract from plastic to reduce the probability of not detecting estrogenic activity. The differences in the findings of this study and that of Yang *et al.* (2011) with the findings of Bach *et al.* (2013) study could be attributed to several factors. One factor to explain these differences is the use of an SPE procedure by Bach *et al.* (2013) before assessing the estrogenic activity of the extractables while this study and Yang *et al.* (2011) did not use SPE with the extraction process. Another factor that could explain the different results between the studies is the use of a different bioassay with different cell lines to assess estrogenic activity of the samples of extractables. While this study, used the E-screen with MCF7-BUS cells, Yang *et al.* (2011) used the E-screen with a stock of non-commercially available MCF-7 cells obtained from Dr. V. Craig Jordan, Northwestern University Medical School. It has already been discussed how different MCF-7 stocks show different sensitivities and different proliferative

responses to an estrogenic environment. On the other hand, Bach *et al.* (2013) used HepG2 cells transiently transfected with hERα and with an ER-responsive luminescent reporter gene. As reviewed by Mueller, (2004) the reporter gene assays are high-throughput methods that give information about the interaction between the substances and the ERs, however their main limitation is that these kind of bioassays do not give any information about the biological response. Finally, the PET extractables results of this study also suggested a concentration dependent response of the PET extractables in DMEM/F12 medium. This result is in contrast to the response observed with the PMMA extractables. However as previously discussed, a wider range of concentrations of the test sample would need to be tested in order to clarify if this implies a monotonic or non-monotonic dose-effect response.

Polypropylene (PP) is a tough, rigid and crystalline thermoplastic produced from propene (or propylene) monomer. Polypropylene is widely used in various applications due to its good chemical resistance and weldability. For example, its good barrier properties, high strength, good surface finish and low cost make polypropylene ideal for several packaging applications. However, due to its poor resistance to UV and low heat-ageing resistance, additives are added during the PP polymerization to provide light stabilization and enhance the lifespan of the PP device (*Polypropylene (PP) Plastic: Types, Properties, Uses & Structure Info*, no date). According to the manufacturer's specifications of the PP buckets, the PP was polymerised with 8.86 g of UV stabilisers per 0.101 m^2 of illuminated surface.

This study showed that raw PP plastic, similar to raw PMMA, released extractables with estrogenic activity in DMEM/F12 medium under extreme temperature conditions (24 h at 37 °C or at any period at 50 °C). The raw PP also released compounds with estrogenic activity when extracted with ethanol at 50 °C. Extractables from aged plastic also showed estrogenic activity when extracted using DMEM and ethanol at elevated temperatures and extended incubation times. When DMEM was used as a solvent, estrogenic activity was detected in the extracts following 3, 6 and 9 months. However, when ethanol was used, estrogenic activity was detected in the extracts following 3 months only. It had been observed that the PP buckets, unlike the other plastic reactors became fragile and breakable in the sun. This observation along with the results for the PP plastic extractables suggested that stress factors may have a weathering effect on the stability of chemical bonds linking additives in the plastic resulting in the early release of stabilisers from the plastic when exposed to local sunlight for extended periods of time. The age-related differences observed in the extractables could also be attributed to photodegradation of the plastic. Other studies of PP have shown variable results. Kirchnawy *et al.* (2014) used the YES assay to test the estrogenic activity of extractables released from different plastics including four sources of PP. They incubated the plastics for 10 days at 60 °C in ethanol (10, 20, 50 and 95%) and reported that one PP sample released substances with estrogenic activity. However, the

levels detected were not considered harmful to human health. In contrast to the findings of this study, Mertl *et al.* (2014) and Yang *et al.* (2011) did not find substances with estrogenic activity released from PP into ethanol following thermal stress. Riquet *et al.* (2016) assessed the estrogenic activity of polypropylene with a selection of stabilizer additives (Irgafos 168, Irganox 1076 and Tinuvin 326). They used electron beam or microwave radiation with isopropanol/cyclohexane mixture (92.5 / 7.5 v/v) as solvent. They reported that substances with estrogenic activity (2,6-DTBP), were released from only one PP polymer with the highest concentration of one specific additive (Irganox 1076). This finding suggested that different sources of PP may have different susceptibilities to substance release with estrogenic activity during the extraction process. In contrast with Yang *et al*. (2011) who used the E-screen assay to assess the estrogenic activity of different plastics including PP under different extraction conditions and reported that 81% of the PP extractables in saline solvent were estrogenic, results from this study showed that both raw and aged PP plastic released extractables with estrogenic activity in DMEM/F12 media only under extreme temperature conditions (24 h at 37 °C or at any period at 50 °C).

Having shown that plastics used for SODIS reactors released estrogenic activity under exaggerated conditions, the question of whether estrogenic chemicals are generated on the inner surface of reactors and therefore migrate into water needed to be addressed.

E-screen assay for toxicity of water leachates

Leachates represent a subset of extractables released from the plastic-contact material under operating conditions (Dorival-García *et al.*, 2018). Sunlight exposure is the operating condition to which the plastic SODIS reactors will be exposed on a daily basis, while the water disinfected by the reactors would be considered the solvent (polar extraction solvent) into which the potential leachates of the device (the SODIS reactors) will be released. Therefore, the novel SODIS reactors were filled with water, or pieces of the reactors submerged in water and exposed to sunlight and weather conditions for several months. Leachates are extracted from a sample or device of analysis with a simulated-use extraction (Ball *et al.*, 2012). The rationale was to perform a simulated-use extraction condition to determine how combined effects of sunlight exposure and heat from the sun would affect the leachability of potentially toxic substances from the SODIS reactors after different periods of usage. The exposure period of the samples was selected in order to determine how the life-span and continuous use of the reactors could have an effect on the leachability of potentially toxic substances. The simulated-use extraction of all the reactors was first carried out in PSA, in the south of Spain because of its excellent research facility, the team of competent professionals in the field of solar disinfection and its geographic location (Spain; latitude 37.0947° N, longitude 2.3584° W, altitude 500 m). The average maximum temperature

historically (1985 – 2020) reached in Tabernas south Spain during spring/summer months has been 19-32°C, while the average minimum temperature historically reached during spring/summer months has been 9-20 °C. The average maximum temperature historically (1985 – 2020) reached in Tabernas south Spain during autumn/winter months has been 15-28°C, while the average minimum temperature historically reached during autumn/winter months has been 4-17°C (*Clima Tabernas - meteoblue*, no date). The annual average value for precipitation in Tabernas (1982-2012) was 26.5 mm, however during the dry summer months, the average level of precipitation is only 8 mm and 30 mm on average during the rest of the year (*Clima Tabernas: Temperatura, Climograma y Tabla climática para Tabernas - Climate-Data.org*, no date). UVA irradiance values of up to 50 W/m^2 have been reported at PSA (Polo-López *et al.*, 2019).

Both South Africa and Uganda have distinct rainy seasons when water is plentiful and distinct dry seasons when the water is scarce. Harvesting rainwater during the rainy season is widely practised. However the quality of the rainwater is often not of drinking water standard and the use of SODIS to treat the water using 2 litre PET bottles was shown to be an effective treatment technology (Nalwanga *et al.*, 2018). Disadvantages associated with the use of 2 L vessels have been described by Nalwanga *et al.*, (2018) including the disadvantage of the small treatment volume and the labour intensive nature of filling several bottles in order to treat an adequate volume. While limitations of solar disinfection when it is scaled-up through the use of large batch volumes or continuous flow recirculation reactors have been described (Ubomba-Jaswa *et al.*, 2010), use of a 25 L borosilicate glass tube reactor was shown to be suitable for treating drinking water both at household level and institutional level in Sub-Saharan Africa (Nalwanga *et al.*, 2014). However, while glass has been shown to be effective for disinfecting water using SODIS under sub-Saharan weather conditions (Asiimwe *et al.*, 2013), glass is heavier and more susceptible to breakage than plastic. So, one of the objectives of the *Waterspoutt* project was to develop large scale batch solar reactors made using the plastic PMMA for the treatment of rainwater in sub-Saharan Africa.

While waiting for the PMMA reactors to be constructed, it was decided to test pieces of PMMA for leachates. Pieces of the plastic were immersed in local water at four distinct geographic locations and exposed to sunlight in glass vessels. The four locations included the test facility at PSA, Stellenbosch in South Africa and rural Uganda where it was planned to site the PMMA reactors and at Dublin City University, Dublin where the toxicological studies were carried out. It was of interest to see if the very different weather conditions at the four locations would influence leaching from the PMMA when exposed to the local environment. The plastic pieces were exposed for up to nine months.

Ireland is said to have a temperate climate – the weather is rarely very hot or very cold. The maximum average temperature in Dublin reported during the warm season (June to September) was 19°C, while the minimum average temperature during the warm season was 13°C. The maximum average temperature in Dublin during the cold season (November to March) was reported as 7°C, while the minimum average temperature in Dublin during the cold season was 5°C. In Dublin, the average percentage of the sky covered by clouds varies over the course of the year. Rain falls throughout the year with the heaviest rainfall centered around October. The average total accumulation in October was 79 mm and the lowest levels of rainfall centered around February with an average total accumulation of 49 mm (*Monthly Data - Met Éireann - The Irish Meteorological Service*, no date). UV radiation in Ireland is not as intense as in Spain. While a UV index value of 10 or above is a frequent occurrence during the summer months in Spain, in Ireland the maximum UV index is closer to 7 or 8.

Stellenbosch is a town in the Western Cape province of South Africa, situated about 50 kilometres (31 miles) east of Cape Town, at the southern extremity of the African continent. Located in the southern hemisphere, the summer season runs from December to March and the winter season from June to September. In Stellenbosch, the summers are warm, dry, and mostly clear; the winters are long, cold, wet, and partly cloudy; and it is windy year-round. A total rainfall of 431.4 mm was recorded from July 2018 to September 2018 (high rainfall period), while 183.8 mm was recorded from October 2018 to January 2019 (medium rainfall period). The rainfall then decreased to 146.2 mm during February to April 2019 (low rainfall period). The mean ambient UVA radiation at both sampling sites ranged from 7.16W/m2 (12/09/2018) to 31.29 W/m2 (14/01/2019), while the mean ambient UV-B radiation ranged from 1.33 W/m2 (12/09/2018) to 4.63 W/m2 (14/01/2019) (Reyneke *et al.*, 2020). In South Africa, the average maximum temperature measured on the solar lab roof in Stellenbosch for the past 20 years was 41°C approximately, while the average minimum temperature measured was 2°C approximately (*Stellenbosch Weather Services temperature of the last 20 years* , no date).

Uganda is a country in Africa located on the equator. The climate is tropical, with two wet seasons and two dry seasons however, the timing of these is no longer reliable due to the effects of climate change (Nalwanga *et al.*, 2018). In general, April - May and August – December are considered wet months while January – March and June – July dry months. The study area lies just south of the equator where total monthly rainfall values vary and range from 2.9 mm to 135.9 mm. There is little difference in the temperatures between the wet and dry seasons which range on average between 22.5°C to 27.5 C from a minimum value of 19.5°C and a maximum temperature of 33.6°C. Values for UVA+B radiation of up to 60 W/m^2 have been reported by Nalwanga *et al.*, (2018).

Facilitating the transport of the water samples from the different partnership countries to Dublin for analysis was a critical aspect of this toxicological study. To improve transportability the water samples were first extracted using solid phase extraction. The solid phase extraction was carried out at the exposure location by the staff of the different partners of the *Waterspoutt* project, with the exception of PSA in which staff from DCU performed the solid phase extraction during an internship. Solid phase extraction not only improved the transportability of the leachate samples but it also allowed the use of DMSO as the bioassay compatible vehicle solvent. Although the pieces of PMMA were exposed to a range of temperatures (2 °C – 40 °C) and UV radiation values up to 60 W/m^2 over a period of nine months, no toxicity was detected in any sample of water in which the pieces had been immersed. At PSA, when the salinity and conductivity of the local well water and distilled water used to immerse the pieces was tested following the nine-month period, increases in the levels of ammonium concentration and the sulfate concentration of the water were observed however, these differences were deemed not statistically significant.

Following the installation of the PMMA reactors in Africa, a comprehensive study of water samples taken from the reactors in Stellenbosch over a nine-month period together with a number of random samples taken from the reactors in Uganda showed no toxicity associated with the reactors. These findings demonstrated the robustness of the reactors and their safety for use in SODIS for the treatment of harvested rainwater in sub-Saharan Africa.

All of the novel SODIS reactors evaluated in this project were designed to upscale the volume of water treated from 2 liters in the traditional PET bottle to 20 liters or more. In Ethiopia, jerrycans are commonly used as water containers. These jerrycans are normally yellow in color and opaque. They range in volume from 5L suitable for a child to carry to a full-size vessel capable of containing 20 – 25 liters of water. The objective in *Waterspout*t was to develop a transparent 25-liter jerrycan that could be used in the SODIS process. PET plastic was the material of choice. Transparent PET vessels capable of holding 25 liters were sourced in Spain. They had been designed for holding olive oil and the PET material was food grade quality. Testing for leachates was carried out by filling the transparent 25-liter jerrycans with water and exposing them to sunlight at PSA for up to nine months. Two-liter PET bottles were also studied for comparison. According to the information provided by PSA partners, the average minimum and maximum temperature reached inside the reactors were 25 °C – 40 °C, respectively.

In this study on PET reactors, leachates with estrogenic activity were obtained, unlike the case with the PMMA reactors. However, estrogenic activity was only detected in the most concentrated samples following exposure to sunlight for nine months. No estrogenic activity was detected following 3 or 6 months. In the SODIS process, it is recommended to replace the plastic container used every six months (*Sandec: Sanitation, Water and Solid Waste for Development SODIS*

manual; Luzi *et al.*, 2016). So, the result of this study indicated that no toxicity was associated with the PET reactors if used according to recommended practice. However, estrogenic activity was detected in the most concentrated water samples taken from both the 2-litre PET bottle and the 25-litre transparent jerrycan following 9 months of exposure to the sun.

The estrogenic activity detected in the water from the PET reactors exposed to sunlight for 9 months reached a maximum EEQ value of 0.3 ng /L. Our findings provide first evidence for migration of xenestrogens from PET SODIS reactors into water after prolonged 9-month period of sunlight exposure. Bach *et al.*, (2014) had previously reported negative results on endocrine disrupting chemicals released from PET or glass bottles after exposing the bottles to the effects of sunlight. The main difference however between this study and the Bach study is that the latter investigated the effect of sunlight exposure on PET bottled water for 2, 6 and 10 days.

The estrogenic activity of water from commercial PET bottles has been widely studied with varying EEQ levels. Typically, xenestrogens reported from PET bottled water are in the range of 0.002-40 ng EEQ / L with maximum values of 75 ng EEQ /L. Pinto and Reali (2009) were among the first to investigate estrogenic activity of mineral water stored in PET bottles in Italy. They used the YES bioassay and reported that only 10% out of 30 samples had detectable estrogenic activity (EA) reaching a maximum EEQ value of 23.1 ng/L, which is almost 100 times higher than the EEQ value obtained in this study. It is still a value that is considered harmless to human health through consumption because it is within the accepted daily intake (ADI) range (0-50 ng/kg body weight) set by JECFA. Similarly, Wagner and Oehlmann (2009) used the YES assay to test the estrogenic activity of water from 20 brands of commercial PET bottles. They found that 60% of the samples exhibited an appreciable estrogenic activity with a maximum value equivalent to 75.2 ng/L, which is more than 200 times higher than the EEQ value obtained in this study; however, this EEQ level indicated that the water is still considered safe to consume by adults because it is within the ADI limit. A follow-up study of PET bottled water from three European countries using the E-screen also showed as much as 60% waters tested were estrogenic. However, estrogenic activity was lower than reported in their earlier study and corresponded to EEQs of 1.9-12.2 pg/L. They argued that methodological differences in sample preparation can account for variations in estrogenic activity reported (Wagner and Oehlmann, 2011; Abbas *et al.*, 2019). Plotan et al. (2013) used reporter gene assays to test the estrogenic activity of solid phase extracted water from 14 brands of PET bottles. Endocrine disrupting activity (estrogenic, androgenic, progestogenic and glucocorticoid activity) was found in 78% of the samples with an average concentration of 10 ng EEQ /L for the samples that showed estrogenic activity, which was not considered a health risk. The EEQ value reported by Plotan et al. (2013) is around 30 times higher than the highest EEQ value for the PET reactors reported in this study. Maggioni et al. (2013) used HELN-ERα reporter cell line to analyse estrogenic activity of water from 5

commercial brands of PET bottled mineral water in Italy. Estrogenic activity was low across all samples with maximum estrogenic activity of 13.6 pg EEQ / L. In a more recent study, Real et al. (2015) used receptor-specific bioassays based on reporter gene expression in MCF-7 cells and found estrogenic activity averaging 0.113 pM EEQ in 79% of 29 PET-bottled water sold in the South of Spain. This last EEQ value reported by Real et al. (2015) is the lowest EEQ value reported in the literature, it does not pose a risk to human health and it is almost 10-fold lower than the highest EEQ value reported in this study for PET reactors.

Some studies, in contradiction with these previous studies, were not able to detect estrogenic activity in the water of commercial PET bottles. Two studies from the same year and the same research group, Mertl et al. (2014) and Kirchnawy et al. (2014) used similar approaches to test the estrogenic activity of several plastic polymers using a battery of assays (YES, YAS, ERα and AR CALUX on human osteoblast cells). The main differences between these studies were the number and type of plastic polymers. Kirchnawy et al. (2014) assessed the leachability of seven composite films (CF), nine PS plastic products, seven PP plastic products, eight PE plastic products, six PET plastic products and five plastic products that use a combination of PET and HDPE. On the other hand, Mertl et al. (2014) assessed the leachability of PET, PP, PE, PS, composite films (CF) and food cartons (FC). As was the case in this study, both studies used ethanol as an extraction solvent and used Oasis HLB solid phase extraction prior to bioassay. However, PET was exposed to 10-20% ethanol (a food simulant to represent worst-case scenario according to EC 10/2011) for 10 days at 60 °C. Both studies concluded that none of the PET samples analysed released estrogenic active compounds. In 2016, Chevolleau *et al*. used stable reporter gene cell lines derived from Hela cells to study different types of endocrine disrupting activity (estrogenic, androgenic, progestogenic, glucocorticoid and thyroidogenic activity) in water stored for 10 days at 40 °C and 60 °C in glass, virgin PET and recycled PET coloured bottles. No endocrine activity was detected in any of the 5 reporter cell lines.

The quantifiable estrogenic activity of commercial PET bottled water reported by Pinto and Reali (2009), Wagner and Oehlmann (2011), Plotan et al. (2013) and Maggioni et al. (2013) may be due to contamination of the spring water source, the PET bottle production process and or migration from packaging between bottle plant and local retail store. Pinto and Reali (2009) suggested that differences in the chemical quality of the raw PET polymers and /or differences in manufacturing technology may account for contaminants. For the duration of this study, the source of the water used to fill the 2 L and 25 L PET reactors was the same. Similarly, the respective PET SODIS reactors came from the same source. The highest EEQ values obtained in this study for each PET SODIS reactors (2 L and 25 L capacity) exposed to sunlight for 9 months was between 25 and 150 times higher than the EEQ values obtained by Wagner and Oehlmann (2011) for commercial PET bottles. Though water extracts were prepared by SPE and E-screen

was used to assess estrogenic activity, the main difference between this study and that of Wagner and Oehlmann (2011), that might explain the different results is the level of stress to which the PET was exposed in this study. In this study the PET reactors were exposed for a prolonged period of time, nine months, to strong sunshine at PSA where UV levels of up to 50 W/m^2 were reported and where the temperature of the water in the reactors was recorded to range from 25 °C – 40 °C. in contrast to the study by Wagner and Oehlmann (2011), where the bottled water studied was stored in local retail stores. Furthermore, exposure of the same PET to darkness for 9 months yielded negligible estrogenic activity suggesting the primacy of solar radiation over time and temperature as a contributory factor to migration. When compared with estrogen positive extractables that were detected when PET pieces were exposed to 100% ethanol at temperatures ranging from 37-50 °C for periods between 5 and 72 h, this study confirms that leachates obtained following solar radiation for 9 months represented a tiny proportion (0.04%) of extractables from PET SODIS reactors.

Pinto and Reali (2009), Wagner and Oehlmann (2009, 2011), Plotan et al. (2013) and Real et al. (2015) assessed the estrogenic activity of leachates in water from different brands of commercial PET bottles and got results that varied from one brand of PET bottle to another. Since the techniques of extraction and bioassay used in each study had not varied for water from different brands of PET bottles analysed, this suggested that the source of the PET plastic and the additives used in its polymerization may play a critical role to determine the leachability and estrogenic activity of the leachates. This hypothesis would explain the controversial and contradictory results published in different studies of estrogenic activity of PET leachates, and is in concordance with what was previously discussed by Groh and Muncke, (2017), regarding the discrepancy between toxicological studies from PET bottles and how those discrepancies could be attributed to differences in PET bottle manufacture.

To further investigate novel large volume SODIS reactors, transparent PP buckets, manufactured in Malawi, were investigated for use by the local community. The testing of the vessels for toxicity was carried out at PSA. The 20-litre buckets were filled with water and exposed to sunshine at PSA with the PET reactors for nine months. No toxicity was detected in the water when tested at 3, 6- and 9-month intervals.

Unlike with PET, few studies have investigated leaching from PP containers. As described earlier, Mertl et al. (2014) and Kirchnawy et al. (2014) assessed the estrogenic activity of several plastics including PP using a battery of assays (YES, YAS, ERa and AR CALUX on human osteoblast cells). Both studies used ethanol as the extraction solvent, creating a much more stressful environment than was used in this study. Even so, Mertl et al. (2014) did not find estrogenic activity above the limit of detection of the YES and ERs CLAUX assay. In the case of Kirchnawy

et al. (2014), only one out of seven PP samples showed estrogenic activity. The levels detected had an EEQ level of 1.9 ± 0.6 ng/L with the YES assay, which does not represent a risk for human health. While it was noted previously that Riquet et al. (2016) assessed the estrogenic activity of polypropylene with a selection of stabilizer additives (Irgafos 168, Irganox 1076 and Tinuvin 326), they reported that substances with estrogenic activity (2,6-DTBP), were released from only one PP polymer with the highest concentration of one specific additive (Irganox 1076) and when extracted using electron beam or microwave radiation with isopropanol/cyclohexane mixture (92.5/7.5 v/v) as solvent.

Overall, the results of this study showed that all three novel SODIS reactors were safe to use under the recommended conditions for SODIS.

Advanced oxidative processes

The use of AOPs in the treatment of drinking water was first proposed in the 1980s (Glaze, 1987). The application of these processes for drinking water production has been limited although UV/H_2O_2 treatment followed by granular activated carbon (GAC) filtration was shown to be effective in the removal of organic compounds (Kruithof *et al*., 2007). Concern about the production of toxic by-products in these systems prompted the need for toxicity studies. Linden *et al.*, (2004) tested the toxicity of water after UV/H_2O_2 treatment and studied estrogenic activity and acute toxicity in the water. Heringa *et al.*, (2011) reported the first study on the formation of genotoxic by-products during UV/H_2O_2 AOP and later Martijn and Kruithof, (2012) and others also studies the formation of genotoxic by-products during UV/H_2O_2 AOP. Their findings showed that if mutagenic by-products are formed, they can be effectively removed using a filtration step over activated carbon. Thus, Hofman-Caris *et al.*, (2015) pointed out that since such a filtration step is in most cases standard in UV/H_2O_2 processes to remove the excess of H_2O_2, advanced oxidation processes based on UV will not cause mutagenic activity in finished drinking water.

As previously mentioned during the discussion of estrogenic activity of leachates it has been widely reported that exposing plastics to UV radiation and sunlight releases chemicals into the water, and studies have shown that some of these released substances from the plastic have estrogenic effects. Plastics are usually polymerized with antioxidants and UV stabilisers to prevent its oxidative degradation during sunlight exposure. The amount of these additives depends on the chemical structure of the additive and of the plastic polymer, and are normally added in amounts that take into consideration their most common household use (Zweifel *et al.*, 2009; Hansen *et al.*, 2013). Thus, the more frequently used amounts of antioxidants in plastic polymers may not be sufficient to protect the plastic from continuous exposure to sunlight or to exposure to oxidative stress generated through AOPs. Some of the most common antioxidants added to plastic are phenolics, which include BHT, BHA, BPA and Irganox; and

organophosphites (used to reduce hydroperoxides) which include TNPP and Irgafos. Phenolic antioxidants are used in lower amounts than organophosphites (Hahladakis *et al.*, 2018). Yang et al. (2011) discussed with supporting points from the study of Fang et al. (2003) that one of the most effective chemical groups to ensure a high binding affinity to the estrogen receptor is the presence of a phenolic ring structure, a chemical group present in all of the phenolic antioxidants. Among the most commonly used antioxidant phenols, BPA has been widely reported as a possible leachate from plastic with estrogenic activity. Organophosphite antioxidants are commonly used in combination with phenols to provide synergistic oxidation protection. Organophosphites cannot be used in estrogenic-free plastics since these antioxidants are hydrolytically unstable and can produce phenols when exposed to water (Murphy, 2001). In their study on the effect of reactor material and its reuse on the photo-Fenton process, López *et al.*, (2017) found improved disinfection using photo-Fenton in reused PET reactors which they attributed to additional ROS produced by material degradation. They questioned the stability of PET after continuous exposure to a highly oxidative process such as photo-Fenton and suggested that toxicity testing be carried out on such systems to evaluate the safety of the water. To our knowledge, there has not been a study to date on the effect of photo-Fenton or peroxydisulfate reagents on the release of estrogenic leachates from SODIS plastics. The specific question that this research addressed was ‘ how does the generation of an enhanced oxidative stressed environment in the water matrix affect the leachability of solar reactors, and how do advanced oxidative processes impact plastic SODIS reactors?

Six hours of exposure is the commonly required time to achieve inactivation of pathogens during the SODIS process. The results from this study showed that after 6 hours of exposure to different irradiation processes (SODIS, photo-Fenton and persulfate activation) PET plastic from the transparent SODIS jerrycan did not release substances with estrogenic activity. However, after 6 hours of exposure, all three irradiation processes, SODIS, photo-Fenton and persulfate activation, promoted the release of substances with estrogenic activity from both PET plastic from a Coca-Cola bottle and from blue PC plastic from a water dispenser. These results indicated that the source of the plastic is a critical factor that determines the leachability of estrogenic substances (Groh and Muncke, 2017). PET is a very strong and lightweight material with a high chemical inertness that has allowed its approval as a safe contact material for food and beverages. Some of the common phenolic antioxidant additives used in PET polymerization are Bruggolen, GC Greenox, GC rainox, Jadewin ao, Evernox and Alvinox (*Additives for Polymers - The Universal Selection Source – SpecialChem - additives antioxidants phenolics - PET* n.d.). Sax (2010) discussed in a review that although PET is not manufactured with phthalates or precursors of phthalates several studies have detected and reported different levels of phthalates, such as DEP, DEHP and DMP, in mineral water bottled in PET bottles while the same levels of phthalates were

not found in counterpart mineral water bottled in glass bottles from the same commercial brand. Sax (2010) theorised that this could be due to the use of recycled PET in the polymerization and manufacture of "new" PET bottles. This recycled PET polymer may have absorbed contaminants during the previous use or from the washing and recycling treatment. Sax (2010) also theorised that PET from different suppliers may differ in the degree to which it is homopolymer or copolymer, the extent to which the material is "virgin" or recycled PET, and in details of the manufacturing process. It is worth mentioning therefore that one difference between the PET plastics used in this study was the age of the plastic. While the PETjc plastic was new, the PETcc plastic while of food grade was not new. *Andra et al.*, (2011) found that the greatest contributor to the release of chemicals from PET and PC was the number of times the container was used. The maximum EEQ values of substances released from PETcc and PC after 6 h of photo-Fenton process were 0.4 ng/L and 0.6 ng/L, respectively. Being within the acceptable daily intake (ADI) established by JECFA (2000), neither of these values represented a risk for water consumption. The estrogenic activity of water from commercial PET bottles has been widely studied with varying EEQ levels in the range of 0.002-40 ng EEQ / L with maximum values of 75 ng EEQ / L (Pinto and Reali, 2009; Wagner and Oehlmann, 2009, 2011; Maggioni *et al.*, 2013; Plotan *et al.*, 2013; Real *et al.*, 2015; Abbas *et al.*, 2019). The EEQ values reported in this study described leachates released from PET plastic pieces submitted to oxidative stress, while the EEQ values reported in the aforementioned literature studies described leachates released from PET plastic under simulated use. Despite the exaggerated conditions used in this study the EEQ levels were within the range of what has been previously reported in the literature and were considered safe for human consumption.

In recent times, interest has grown in using plastics other than PET in the SODIS process. Fisher *et al*., (2012), investigated the use of 1L polycarbonate bottles while Keogh *et al.*, (2015) carried out comparative studies of the bacterial inactivation efficacy of 19 L polycarbonate water dispenser containers (WDCs) similar to that used in this study. In their review Borde *et al.*, (2016) considered the challenges faced using large plastic bottles such as WDCs made of polycarbonate in SODIS studies including the fear of leaching from the plastic container into the water. Polycarbonate (PC) is a transparent and tough thermoplastic polymer with organic functional groups linked together by carbonate groups that are responsible for its unique combination of high-performance properties, that include among others high impact strength and dimensional stability. PC is an extremely clear plastic that can transmit over 90% of light as effectively as glass. Polycarbonate sheets are available in a wide range of shades that can be customized depending on an end-user application. It is also a lightweight plastic polymer, which makes it a fantastic candidate for the construction of a transportable SODIS reactor. More than 50 phenolic antioxidants and other UV stabilizers based on benzotriazole are commonly used during the

polymerisation of PC to guarantee its excellent UV radiation resistance and photo-degradation. Because of its heat resistance PC is used in applications for direct contact with foods and beverages (*Polycarbonate (PC) Plastic: Properties, Uses, & Structure - Guide*, no date). The PC container used in this study like the PETcc was also reused plastic and leaching of BPA from reusable PC drinking bottles has been reported by Kubwabo *et al.*, (2009). Another distinguishing feature of the PC plastic was its blue colour. Colorants are among the additives that can be used in plastic manufacture. In nearly all cases, additives are not chemically bound to the plastic polymer (Hahladakis *et al.*, 2018) and so could be leached under stressful conditions. There are few studies about the leachability of estrogenic substances from PC plastic. Guart *et al.*, (2013) used the YAES and YAAS bioassays to test the migrating substances of PC bottles after 10 days at 40 °C, however, all the results were negative. During their study chemical analysis was also used and leaching of 0.748 µg of BPA per kg of Titan was detected in the water after 10 days of incubation at 40 °C. Yang *et al.*, (2011) used the E-screen to assess the estrogenic activity of several plastics, which included a few plastic samples of PC polymer. 100% of unstressed PC resin showed detectable estrogenic activity in saline solvent. However, this study was not quantitative and so EEQs are not available for comparison.

Interestingly, data from this study suggested that prolonged exposure to advanced oxidative processes increased the estrogenic activity of leachates from the PET jc (EEQ values 0.6-1.2 ng/L) and blue PC water dispenser (EEQ values 1.3-5.9 ng/L), but the estrogenic activity of leachates released from PETcc bottle decreased. There are studies showing that solar photo-Fenton technology is able to partially or totally degrade chemical substances in water that have been reported in the literature to act as endocrine disrupting chemicals or estrogens. For example, in 2004 Katsumata *et al.* observed a 90% decomposition of BPA antioxidant after 36 hours of photo-Fenton treatment. The decomposition of BPA produced six different ring intermediates phenol, p-hydroquinone, 4-isopropylphenol, 4-isopropenyl-phenol, 4-hydroxyacetophenoneand and methyl benzofurans. Neamţu and Frimmel (2006) reported that photo-Fenton irradiation of BPA for 120 min was enough to drastically reduce the estrogenic activity of BPA in the YES assay. This result suggested that the phenolic intermediates of BPA previously identified must actually have a really low affinity for ER; however, the YES assay does not provide information about the possible biological effects of these intermediates. According to Bajt *et al.*, (2001), due to the lack of response to light at wavelengths greater than 300 nm and the presence of a benzene carboxylic structure, phthalates are recalcitrant to photo-degradation and biodegradation. Rocha *et al.*, (2013) showed that photo-Fenton treatment was able to reduce 77% of DBP concentration of sanitary landfield leachates, however, they failed to show similar reductions in BBP and DEHP concentrations. Zhang and Li (2014) showed that photo-Fenton treatment was able to remove six phenolic endocrine disrupting compounds (EDCs) (estrone, 17β-estradiol, 17α-ethinylestradiol,

estriol, bisphenol A, and 4-nonylphenols) from waste activated sludge (WAS). However, the identity of the manufacturers that produce the PETcc bottle and the blue PC container is not known. For an accurate representation of the possible EDC additives used in the polymerization of these plastics, that might be responsible for the observed biological effect or that might be susceptible to photo-degradation chemical analysis would be needed. Most of the studies about photo-Fenton as a technology to degrade micro-pollutants focus on the impact micro-pollutants have on the environment and how to eliminate them from wastewater. To our knowledge, the studies that research the degradation of micropollutants with photo-Fenton are focused on the use of photo-Fenton to reduce the environmental impact that some wastewaters could have, but none of them are focused on the use of photo-Fenton for the treatment of water for human consumption.

The RPE values obtained in this study indicated that while after only 6 hours of exposure the leachates from the PET jerrycan SODIS reactor were deemed not estrogenic, after 1 week of exposure to advanced oxidative processes, leachates acted as total estrogen agonists with EEQ values of 0.6, 1.5 and 0.8 ng/L for substances released after photo-Fenton, PDS activation and lamp simulated SODIS respectively. The RPE values of the blue PC water container after prolonged exposure to advanced oxidative processes also indicated a total agonistic estrogen effect of the leachates with EEQ values of 3.2, 5.9 and 1.3 ng/L for substances released after photo-Fenton, PDS activation and lamp simulated SODIS respectively. Despite these increases in the EEQ values after prolonged exposures to advanced oxidative processes, none of these values represented a risk for human health through water consumption. RPE values of leachates released from the PET Coca-Cola bottle after prolonged exposure to advanced oxidative processes suggested they act as partial agonists of estrogen. The EEQ values of the leachates released from the two PET polymers after prolonged exposure to advanced oxidative processes are within the range of 0.002-40 ng EEQ / L reported in the literature for water from PET bottles under simulated use stress (Pinto and Reali, 2009; Wagner and Oehlmann, 2009, 2011; Maggioni *et al.*, 2013; Plotan *et al.*, 2013; Real *et al.*, 2015; Abbas *et al.*, 2019).

Overall, the results of this study showed that more estrogenic activity was detected in leachates of plastic samples subjected to advanced oxidative processes than in the leachates of plastic samples subjected to UV light lamp simulated SODIS treatment. This implied that a high oxidative stress on the water matrix may have enhanced the release of estrogenic substances from the plastic polymers. Some of these estrogenic substances released from the plastic could be phenols or phenolic ring structures of antioxidants such as BPA. Other plastic polymer additives that might be responsible for the observed estrogenic effect and that have been previously found as plastic leachates in PET bottled mineral water could be phthalates, such as DEHP and DEP. Since phthalates are quite resistant to photo-degradation (Khetan, 2014) it would not be surprising that advanced oxidative processes will promote the release of some phthalates without being able

to totally degrade them and eliminate their endocrine disrupting effects. Although this study showed that there is an increased biological proliferative effect associated with the use of advanced oxidative processes in plastic SODIS reactors, the EEQ values calculated from these biological effects indicated that the levels of estrogenic substances released into the treated water from the plastic containers were safe for human consumption. Though the results of this study are preliminary, other more inert plastic polymers should be screened for estrogenic responses to AOP.

The Ames Test

In vitro testing for genotoxicity is required by EU and US legislation in applications made for food contact material authorisation and a variety of bioassays used include tests to assess DNA mutations, chromosomal aberrations or alterations in DNA processes (Groh and Muncke, 2017). The Ames test, developed in the 1970s by Bruce Ames, Professor of Biochemistry at UC-Berkeley, is widely used as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA (Ames *et al.*, 1973; Mortelmans and Zeiger, 2000). The Ames test was used in this study to investigate toxicity associated with using plastics in solar disinfection of water by testing for mutagenicity. However, no mutagenicity was detected in any sample throughout the study.

One of the earliest investigations for the presence of toxins in bottled water was carried out by De Fusco *et al.* in 1990. They studied the leaching of mutagens into mineral water from PET bottles using the Ames test following short and long-term storage in light and dark. The water samples were concentrated for testing using silica bonded-phase cartridges and the results identified leaching of mutagens after 1 month of storage but not after 3 or 6 months of storage. The mutagenic activity detected in the water stored for 1 month in PET was higher after storage in daylight compared with storage in the dark. A later study from the same group (Monarca *et al.*, 1994) focused their research on non-volatile compounds leached into distilled water contained in green PET bottles, detected several compounds including acetaldehyde, acetic acid, propanal, terephthalic acid, dimethyl terephthalate, phenol-2, 6-bis(1,1-dimethylethyl)-4,4-methyl and 1,2benzenedicarboxylic acid butyl-2-methyl-propyl ester but found no mutagenicity using the Ames test on water stored for 1, 3 and 6 months in daylight. The water samples were concentrated using silica C18 cartridges. According to Groh and Muncke (2017), the discrepancy between the two studies could be attributed to differences in the PET bottle manufacture as the bottles for the two studies were sourced from different bottle manufacturers. However, discrepancies between studies might also be attributed to the differences in the tests used and even different approaches used to any given method such as the Ames test.

Over the last few decades, a number of variations to the initial Ames test protocol have been developed. The standard Ames assay as described in the OECD Test Guideline (TG) 471 (OECD, 1997), uses the plate incorporation and pre-incubation methods in 100 mm plates. Miniaturised versions of the assay using 6-well and 24-well plates and the Ames II^{TM} assay, a second-generation bacterial reverse mutation assay developed as a screening assay, are useful for high-throughput, pre-screening purposes (Pant *et al.*, 2016). In this study, the Ames test was carried out using the Ames II kit by Xenometrix AG (Allschwil, Switzerland). Use of the kit offers many advantages including ease of handling, cost-saving on consumables and the provision of quality-controlled strains of bacteria which removes the need for genotype analysis of the cultures. The kit allows for the testing of samples in the presence and absence of S9. Many mutagenic substances, such as polycyclic hydrocarbons, do not interact with DNA unless they are activated via metabolism, mainly in the liver. Bacteria lack the metabolic enzymes of higher eukaryotic organisms, but this can be overcome via the addition of an external metabolic system consisting of a liver extract (S9) from chemically induced rats. Rainer *et al.* (2018) pointed out that S9 must be included in all Ames studies, as was the case in this study because, without metabolic activation, numerous genotoxic substances cannot be detected at all. The concern that the genotoxic/mutagenic quality of solar-disinfected drinking water might be compromised as a result of photodegradation of polyethylene terephthalate (PET) bottles used as SODIS reactors was investigated by Ubomba-Jaswa *et al.* (2010) using the Ames fluctuation test. PET bottles containing mineral water were exposed to sunlight for up to 6 months. Two approaches were used. In one instance the bottles were refilled every day to simulate the way in which PET bottles are used during SODIS and in the second instance there was continuous exposure of the bottles with no refill of the bottles. No genotoxicity was associated with un-concentrated SODIS water (daily refill) suggesting that if users apply the SODIS technique correctly, they are unlikely to experience any health hazards from genotoxins generated by SODIS if they replace their bottles every 6 months. Genotoxicity was detected after 2 months in water stored in PET bottles and exposed continuously (without refilling) to sunlight but also in PET bottles stored in the dark after 2 months making it unlikely that the genotoxicity was related to solar treatment. The study by Ubomba-Jaswa *et al.* (2010) did not include the use of S9. In this study, storage of water in PET reactors, 2 L and 25 L volume, and in other plastics including PMMA and PP for up to nine months showed no mutagenicity.

Katsonouri *et al.* (2012) reported on a project aimed to introduce a mutagenicity test in the battery of biological tests applied in the ecotoxicological testing of water sources in Cyprus. Recycled, surface, ground and drinking water samples were assessed for mutagenicity using two commercially available Ames test kits - the Ames MPF^{TM} Mutagenicity 98/100 AQUA kit (Xenometrix AG, Switzerland) and the Muta-Chromo Plate^{TM} kit (EBPI, Canada). They reported

that the kit supplied by Xenometrix, as used in this study, offered several advantages when compared to the MutaChromo™ kit. In their study, mutagenicity was associated in particular with the recycled water. 53% of recycled water samples tested positive for mutagenicity while surface water showed no mutagenicity and only one of three groundwater samples tested positive for mutagenicity but only in the absence of metabolic activation using S9 and not in its presence. Their findings highlight the importance of the source of water when testing for mutagenicity. In this study, water from a well on-site at PSA was used to test the PET and PP reactors and rainwater in South Africa and Uganda was used to test the PMMA reactors. No mutagenicity was detected in any sample. At PSA, the water was taken from a 200 m deep borehole well. The characteristics of this water were previously reported by Polo-López *et al.*, (2019). Naturally occurring organisms were below the detection limit (2 colony-forming units per mL, CFU/mL) determined by Endo agar standard plate count techniques. Turbidity was measured using a turbidity meter (Hach-2100N, Loveland, CO, USA). Total Organic Carbon was determined using a TOC analyser (Shimadzu TOC-5050, Japan). Iron concentration in the water samples was determined by UV-spectrophotometry using the ISO 6332. The main properties of the well water were pH 7.8, turbidity 1.5 NTU, TOC 5 mg/L and iron 0.05 mg/L. The quality of the collected untreated and prototype treated rainwater samples in Stellenbosch South Africa was reported by Reyneke *et al.*, (2020). All measured physico-chemical parameters (pH, turbidity, electrical conductivity, total dissolved solids and dissolved oxygen) adhered to the drinking water guideline limits of the South African Department of Water Affairs and Forestry (DWAF) (DWAF, 1996), South African National Standards (SANS) 241 [South African Bureau of Standards (SABS), 2005], Australian Drinking Water Guidelines (ADWG) (NHMRC, 2011) and WHO (2017), with no significant difference (p N 0.05) observed for the data collected for the untreated and treated water samples or between sites 1 and 2.

Treatment of drinking water using advanced oxidative processes such as UV/ H2O2 has given cause for concern about the generation of toxic by-products. The first submitted study on the formation of genotoxic by-products during UV/H2O2 AOP was reported by Heringa et al. (2011) who used the Ames 11 kit from Xenometrix as used in this study. No genotoxic activity was detected using the Ames II TAMix strain with and without S9 under all applied conditions. An increase in genotoxic activity in the Ames II TA98 strain both with and without S9 was measured in some samples however GAC post-treatment effectively reduced this genotoxic activity. They suggested that the genotoxic compound(s) were formed from components of the water such as natural organic matter (NOM) or other contaminants. Hofman-Caris et al. (2015) also investigated the formation of mutagenic byproducts in UV/ H_2O_2 processes using the Ames test. As in this study, they extracted the water samples using SPE cartridges with Oasis®HLB which they found to be most suitable for use with the Ames test. They found that potentially mutagenic byproducts

formed during drinking water treatment were dependent on nitrate and NOM concentrations. Investigations on advanced oxidative processes in this study showed no mutagenicity however all studies were carried out in Milli-Q water.

Rainer *et al.,* (2018) reviewed the suitability of the Ames test to characterize genotoxicity of food contact material migrates including the limit of detection of the method. Working with a minimum requirement for the analytical limit of detection to be 0.01 mg kg−1 for unknown substances they addressed the question of the lowest effective concentrations (LECs) obtained in the test and their use as surrogates of the limit of detection (LODs) for genotoxins. In their calculations they made a number of assumptions including - a 1000-fold concentration factor can be achieved during the sample preparation, the sample is transferred into 100% dimethyl sulphoxide (DMSO) as a solvent and the final DMSO concentration in the Ames test is 4%, resulting in a 'global concentration factor' of 40. The assumed concentration factor of 1000 was based on laboratory experience of the authors who point out that not all studies concentrate the sample before testing. In the study by De Fusco *et al.,* (1990), two independent experiments were conducted with unconcentrated and concentrated water. Mutagenicity was only detected in concentrated samples and the authors suggested that it was due to the use of a concentration factor. However, in the study by Monarca *et al.,* (1994) where no mutagenicity was observed, the samples tested had also been concentrated. Bach *et al.*, (2013) who studied the effect of temperature on the release of intentionally and non-intentionally added substances from polyethylene terephthalate (PET) bottles into water and Bach *et al.*, (2014) who investigated the effect of sunlight exposure on the release of intentionally and/or non-intentionally added substances from polyethylene terephthalate (PET) bottles into water found no mutagenicity using the Ames test. They extracted the water samples using Oasis HLB glass cartridges as were used in this study and ethyl acetate extracts were concentrated 500-fold and used in the plate incorporation method for the Ames test. They chose the concentration factor of 500 in order to reproduce realistic consumer exposure *in vitro.* The Ames II kit used in this study was recommended for all kinds of environmental samples including drinking, surface or wastewater and for testing drinking water after sample concentration with SPE columns (*Ames Test Kits - Xenometrix,* no date). Samples for testing were prepared in agreement with the assumptions made by Rainer *et al.* (2018). The water was concentrated 1000-fold in DMSO and the final DMSO concentration used was 4% suggesting that the limit of detection of 0.01 mg kg−1 would have been achieved.

A refocus on health and safety

According to the guidelines of the World Health Organisation (WHO), the acceptable daily intake (ADI) of 17-β estradiol in the diet is 0-50 ng per kg of body weight per day. This range was determined during a clinical trial performed by Moore *et al.*, (1978) in which 3 postmenopausal

women were administered oral doses (0.3, 0.6, 1.2, and 2.5 mg/day) of conjugated estrogens. When administered orally estrogens increased total cortisol levels by mediating an increase in serum corticosteroid binding globulin (CBG) capacity (Cobey *et al.*, 1956). CBG was shown to be increased in a dose-dependent manner for 14 days. However, the increase was not significant in women who were administered the 0.3 mg/day dose. Therefore, it was determined that the non-observable effect level (NOEL) was 0.3 mg/day which, for a 60 kg body weight translates into 5 μg/kg bw/day. Then a safety factor of 10 was used to account for normal variations among individuals and an additional safety factor of 10 was applied to protect sensitive populations such as children or pregnant women to yield an ADI range of 0-50 ng/kg/day.

Food and water are potential contributors of naturally occurring and anthropogenic estrogens (Fritsche and Steinhart, 1999; Connolly, 2009). Their consumption must be at levels commensurate with ADI. Caldwell *et al.*, (2010) reported that exposure to estrogens in drinking water was negligible and inconsequential when compared to exposure to estrogens from the diet, primarily milk and dairy products. The estimated adult exposure from drinking water was 82 times lower than from diet and children's exposure was 152 times lower than from diet. They proposed the level of naturally occurring estradiol in drinking water represented a safe level for all vulnerable subgroups of the population irrespective of benchmarks used (eg ADI, occupational exposure limits and threshold for toxicological concern). This is supported by evidence that estradiol is well absorbed from the gut. However, the bulk of an oral dose (~ 95%) does not reach the systemic circulation due to first pass metabolism in the liver (O'Connell, 1995). Oral bioavailability is estimated to be 5% of which only 2% is unbound in plasma (O'Connell, 1995) and available to elicit a biological response.

It is of note that the study of Caldwell *et al.*, (2010) was performed using data available from food intake-and estrogen residue databases in the US. The wide variety of foodstuffs (milk and dairy products, eggs and poultry, cereals, vegetables, pork and other meat products and a wide array of soy-derived food products) reported to contain β estradiol (Fritsche and Steinhart, 1999) may be a contributory factor to the high margin of exposure that a varied diet provides relative to US drinking water. It also took into account the amount of estrogens consumed due to pharmaceutical prescriptions. However, this was considered relatively low by comparison with dietary intake. It is unlikely that dietary consumption data for people living in a developed country such as US, could be extrapolated to people living in the poor, resourceless, local target communities in Africa considered by the project *Waterspoutt*. The wide disparity in cuisine, economic factors, animal husbandry and extent of food processing together with the concern that plastics used in SODIS of water might leach xenestrogens raises the question of whether drinking water in Africa is a significant contributor to the estradiol body burden in subgroups of the African population.

In this study the ng EEQ / L values of the extractables from raw plastic polymers exceeded ADI. However, the EEQ values of the extractables were obtained with exaggerated extraction conditions and/or using solvents such as ethanol with a different polarity to the water that would normally be contained in the reactors. On the other hand, the ng EEQ /L values of the leachates of this study were obtained after simulated use extraction conditions for both SODIS and advanced oxidative processes. In this study, the ng EEQ / L values of the different leachate samples from PMMA, PET, PP and PC were well below the ADI suggesting that the novel SODIS reactors developed by the *Waterspoutt* project are safe to use with SODIS and AOPs such as photo-Fenton and persulfate activation.

Nevertheless, in recent years concern has been raised by pediatricians and scientific organizations about the influence of exogenous estradiol on human health and development particularly of prepubertal children. Is there an acceptable level of exposure? And if so, what is it? Even low-level exposure to exogenous estradiol may impact on total activity of the endogenous β-estradiol hormone. Aksglaede *et al.*, (2006) reported on research that indicated that the rate of endogenous production of β-estradiol in prepubertal boys was 140-350-fold lower than the level (14 μg/day) currently used for assessment of ADI. This is of huge concern as sensitivity of children to exogenous low doses could be even more enhanced than previously thought. Until ADI is revised, scientists are addressing this concern by deriving trigger values to differentiate between an acceptable effect level of an exogenous source of estrogens and an unacceptable effect level and by predicting the physiological impact of exogenous exposure on hormone activity.

One approach to determining if estradiol equivalents in drinking water are acceptable or unacceptable is to derive a trigger value as described by Brand *et al.*, (2013). They formulated an equation to derive a trigger value for estrogenicity without the need to perform *in vitro* to *in vivo* extrapolations. It takes into account the ADI value, pharmacokinetic factors defining bioavailability of estradiol, estimations of the bioavailability of unknown compounds with equivalent hormonal activity, relative endocrine potencies, physiological factors and drinking water allocation factors. If trigger values are exceeded, a safety evaluation is warranted. Using this approach, a trigger value of 3.8 ng 17β-estradiol-equivalents/L in drinking water was reported for estrogenicity bioassays (Brand *et al.*, 2013). Using a different approach based on a statistical method and chemical guideline values from the literature Escher *et al*., (2015) derived an effect-based trigger value for the E-screen bioassay of 0.9 ng EEQ/L. In this study the ng EEQ / L values of the different leachate samples from PMMA, PET and PP were well below the trigger values proposed by both Brand *et al.*, (2013) and Escher *et al*., (2015). Therefore, it could be inferred that EEQ values of leachates are in agreement not only with the ADI recommended by the WHO but also with effect-based trigger values and that the novel SODIS reactors developed by the *Waterspoutt* project are safe to use with SODIS treatment. In the study of the oxidative

processes, only the PC pieces (4x4 mm^2) immersed in 1L water and exposed to solar irradiation and persulfate activation yielded leachates (5.9 ng EEQ / L) after 7 days that exceeded the trigger value of 3.8 ng 17β-estradiol-equivalents/L established by Brand *et al.*, (2013). However all 7-day irradiation treatments of PC and the PDS treatment of PET_{JC} yielded leachates that exceeded the trigger value of 0.9 ng EEQ//L established by Escher *et al*., (2015).

Having exceeded the trigger value, a safety evaluation is warranted. One approach to conducting a safety evaluation of an EEQ level that exceeds the trigger value would be to predict the influence of exogenous exposure on the daily exposure to 17β estradiol in all vulnerable subgroups of the population. This approach was first described by Plotan *et al*., (2014) who reported EEQ values in the range 400-6500 ng/kg/day in three sports supplements. They showed that estrogenic burden of the supplements may vary depending on the age, gender, body weight and hormonal fluctuation of individuals. The impact of consuming products with an average EEQ of 2.4 μg/kg /day was predicted to be greatest in postmenopausal women (2500 %) and prepubertal males (1200 %) in whom estradiol biosynthesis activity is minimal. The impact of consumption was predicted to be least in females at the preovulatory and luteal stages of the menstrual cycle (30-100%). By analogy, and taking into account the daily estrogen production levels as described by JECFA (2000), the impact of consuming PC-bottled water containing 5.9 ng EEQ /L on daily exposure to estradiol was calculated to be negligible ($\leq$0.12 %) for all vulnerable subpopulations. If however daily production rate is adjusted by a factor of 140 downwards as proposed by Aksglaede *et al*., (2006), the impact of consuming PC-bottled water treated by solar AOP containing 5.9 ng EEQ /L on daily exposure to estradiol would not be negligible and would amount to 12-17 % higher physiological impact for prepubertal males and females (JEFCA, 2000), an outcome qualitatively similar to applying the trigger value of 3.8 ng/L.

In summary, water extracts obtained after simulated-use extraction of 3 novel SODIS reactors yielded estrogenic activity with corresponding EEQ values that are in agreement with the limits of ADI established by the WHO and with effect-based trigger values of 3.8 ng/L and/or 0.9 ng/L. Even allowing for 140-350 fold lower rate of endogenous estradiol production as recommended by Aksglaede *et al.*, (2006) the most potent agonist activity (0.3 ng/L) observed in PET bottled water exposed for 9 months in the sun is predicted to induce negligible impact on all subgroups of the population.

This study focused on evaluating the mutagenicity and estrogenic activity of extractables and leachates from 3 novel SODIS reactors. However, humans drinking water from SODIS reactors are likely exposed to a mixture of chemicals and so toxic effects other than mutagenicity and estrogenicity may also be relevant. Testing the overall migrates of SODIS reactors for cytotoxicity and a broader range of genotoxic and endocrine disruptive activity should be considered in future

work. As mentioned previously, the E-screen bioassay used in this work gives information about the physiological response upon binding of xenestrogens to cells that endogenously express ERs, however it does not give information about mechanisms. When extractables and /or leachates inhibit MCF-7 proliferation relative to the negative control it is impossible to determine if the reduced proliferation is due to cytotoxic effects of the samples, or due to a mixture of estrogenic and anti-estrogenic substances in the sample without expanding the panel of toxicity assays. As mentioned in results chapters 4-6 some extractables and leachate samples induced lower proliferative responses than the negative control. For example, the apparent cytotoxic effect of the 100x concentrated 3-months PET leachate samples from both the 25 L jerrycan and 2 L bottle exposed to sun and darkness is a case in point. Potential cellular targets of such leachates may be functioning mitochondria and /or MCF-7 cellular membranes. This could be explored further using a panel of cytotoxicity tests that target mitochondrial function, energy status of cells, membrane integrity and protein synthesis as recommended by Groh and Muncke, (2017). Other explanations might be that leachates lack estrogenic substances and arrest MCF-7 cell cycle at G_1/G_0 as reported previously by Villalobos et al. (1995); or leachates are anti-estrogenic; or leachates exhibit a mixture of estrogenic and anti-estrogenic effects. It is only with expansion of *in vitro* toxicity testing of extractables and leachates that all endpoints of imminent human health relevance can be covered.

Chapter IX

9. Conclusions

- The E-screen assay was used for the first time to evaluate estrogenic activity in leachates and extractables from plastics used in solar water disinfection processes. The optimal conditions for the assay were determined to be the use of the MCF-7 (BUS) cell line, in a 96 well plate assay at a seeding density of 4000 cells/well, incubated for 6 days at 37 ^{o}C. The most suitable assay for the determination of cell proliferation was a DNA quantification assay using Hoechst 33258.
- The three plastics, PMMA, PET and PP, used in the novel SODIS reactors, showed the presence of estrogenic activity when extracted with solvents at elevated temperatures for extended periods of time. The levels of estrogenicity persisted in the plastics when aged in strong natural sunlight for up to 9 months. No mutagenicity was detected in any of the extractables.
- No estrogenicity or mutagenicity, was detected by the bioassays used in this study in samples of water taken from the novel SODIS reactors when exposed to strong natural sunlight for up to 9 months for the PMMA tubular reactor and the 20 L PP buckets and for up to 6 months for the 25 L transparent PET jerrycan thus making them safe for use in SODIS when used under recommended conditions.
- Estrogenicity was detected in samples of water taken from 2 L PET bottles and the 25 L PET jerrycan when exposed to natural sunlight for 9 months. However, the levels detected were not considered harmful to human health when the acceptable daily intake for 17β-estradiol of up to 50 ng/kg bw/day was considered.
- Preliminary toxicity studies on laboratory scale AOPs, photo-Fenton and persulfate activation, showed for the first time that oetrogenicity was detected in some water samples when the three plastics PETjc, PETcc and PC were tested.
- All three plastics tested in the AOPs showed estrogenicity after prolonged irradiation of up to 1 week. However, while PETcc and PC showed estrogenicity following 6 hours irradiation, no estrogenicity was found for PETjc showing that the source of PET plastic needs to be considered.
- The levels of estrogenicity in the AOP studies were not considered a threat to human health when the acceptable daily intake for 17β-estradiol of up to 50 ng/kg bw/day was considered.

Chapter X

10. Future Work

- In this study, the plastic material used in solar water disinfection reactors was found to influence leaching of toxic substances. It would be interesting to expand the toxicity studies to developing countries worldwide to further investigate the influence of the plastic material used and other local environmental conditions including water quality on the behaviour of solar water disinfection reactors.
- Migrates of various plastics showed apparent toxicity dependent on the polarity of the extractant solvent, concentration and duration of exposure to thermal stress or sunlight/darkness exposure. Possible cytotoxicity pathways should be examined to identify optimal concentration range for further more specific *in vitro* toxicity testing. This would require examining the most common cytotoxic endpoints (the breakdown of cellular permeability, cell proliferation) in the most appropriate cell model.
- Estrogenic activity of migrates from SODIS reactors was assessed using the E screen bioassay. Further characterisation of estrogenic activity by different in *vitro* bioassays (YES, YAS, ERa, AR CALUX) would help to extend the systematic assessment of estrogenic activity. Analysis of mixture effects on other aspects of endocrine disruption would be helpful to assess hazardous properties of overall migrates of SODIS reactors.
- When testing for genotoxicity using *in-vitro* tests, the need to detect gene mutations, chromosomal damage and aneuploidy suggest the use of both bacterial and mammalian cells. While the Ames test has been widely used in toxicity testing of drinking water, including solar disinfected water, the test only detects gene mutations. Future work should consider using the Ames test in combination with another test such as the *in-vitro* micronucleus test (MNvit), which detects both chromosomal aberrations and aneuploidy.
- The impact of AOPs on the leachability of PET and PC was examined. Further work should include a wider range of plastics, such as PMMA and PP from the *Waterspout* project, in order to perform a high-throughput analysis of the safety of SODIS enhancements.

- The identity of migrate components responsible for estrogenicity was not examined. Future work should consider chemical analysis of migrates e.g with chromatographic analysis (GC-MS, HPLC-MS), in order to identify and quantify (if the limit of detection allows) substances or mixture of substances responsible for the observed toxic effects. This would also provide data to consider the suitability of safety thresholds for the consumption of endocrine disrupting chemicals.

Chapter XI

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