H2Open Journal

IWA PUBLISHING

H2Open Journal Vol 4 No 1, 204 doi: 10.2166/h2oj.2021.108

In vitro toxicity studies of novel solar water disinfection reactors using the E-screen bioassay and the Ames test

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ABSTRACT

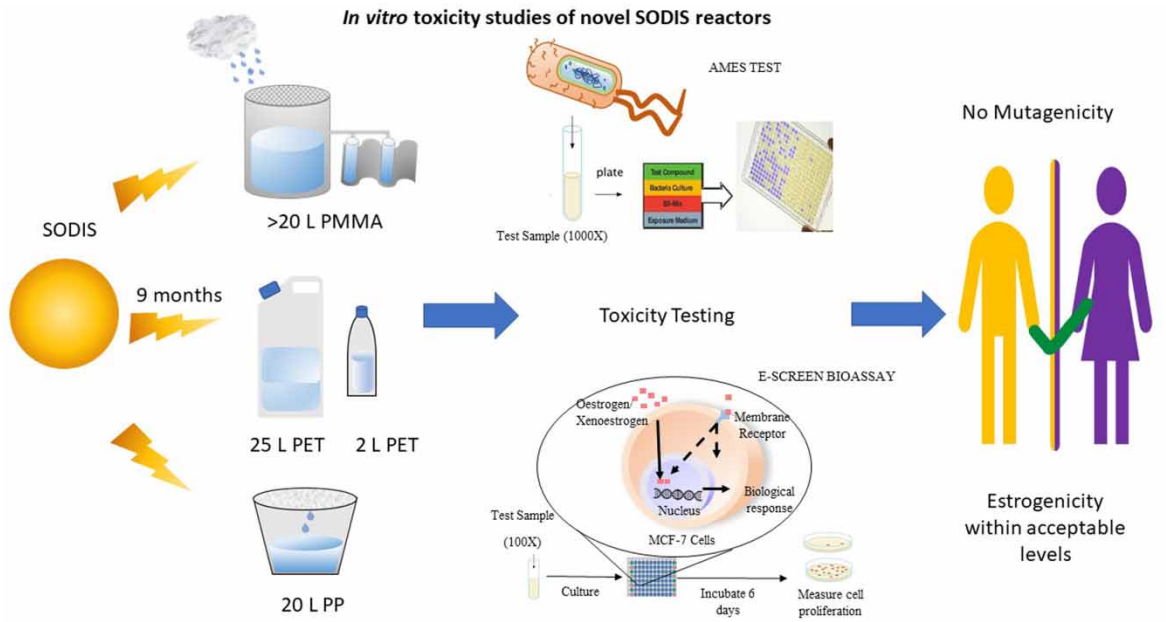
Solar water disinfection (SODIS) is a cost-effective point of use method for disinfecting water, usually in a 2 L polyethylene terephthalate (PET) plastic bottle. To increase the volume of water disinfected, three novel transparent reactors were developed using PET in 25 L transparent jerrycans, polymethyl methacrylate (PMMA) in tubular solar reactors capable of delivering >20 L of water and polypropylene (PP) in 20 L buckets. *In vitro* bioassays were used to investigate any toxic substances leached from the plastic reactors into disinfected water as a result of exposure to sunshine for up to 9 months. The Ames test was used to test for mutagenicity and the E-screen bioassay to test for estrogenicity. No mutagenicity was detected in any sample and no estrogenicity was found in the SODIS treated water produced by the PMMA reactors or the PP buckets. While water disinfected using the PET reactors showed no estrogenicity following exposure to the sun for 3 and 6 months, estrogenicity was detected following 9 months' exposure to sunlight; however levels detected were within the acceptable daily intake for 17β-estradiol (E2) of up to 50 ng/kg body weight/day.

Key words: Ames, E-screen, leachates, plastics, SODIS, toxicity

HIGHLIGHTS

- No mutagenicity or estrogenicity was detected in water from novel PMMA and PP SODIS reactors continuously exposed to sunshine for nine months.
- The E-screen bioassay was used for the first time to test for estrogenicity in the SODIS process.
- Water from 2 L and 25 L PET SODIS reactors showed no mutagenicity. Levels of estrogenicity detected were within acceptable limits.

GRAPHICAL ABSTRACT



INTRODUCTION

Where access to safely managed drinking water services is unavailable, a variety of household water treatment (HWT) technologies including solar water disinfection (SODIS), are recommended to be used at the point of use to ensure a safe drinking water supply (WHO 2017). 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. The containers are exposed to sunlight for at least 5–6 hours to achieve disinfection through the synergic effects of heat and UV radiation (Wegelin *et al.* 1994; Joyce *et al.* 1996; McGuigan *et al.* 2012). 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 (McGuigan *et al.* 2012).

However, there is concern that toxic substances might leach from plastic materials used for SODIS particularly when the plastic containers are exposed to strong sunlight for prolonged periods of time (Asiimwe *et al.* 2013; Bach *et al.* 2014; Borde *et al.* 2016). 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. However, while intentionally added substances in plastics may be safe for the consumer, non-intentionally added substances in the final plastic material can be toxic to the consumer (Bach *et al.* 2013).

Groh & Muncke (2017) suggested that *in vitro* bioassays may offer a robust and economic solution to screen the toxicity of food contact materials and described genotoxicity and endocrine disruption potential among the main categories of toxicity that can be exerted by migrating substances from food contact materials. They described genotoxicity as the ability of toxic agents to damage the genetic material in cells and pointed out that *in vitro* genotoxicity tests commonly assess the occurrence of DNA mutations, chromosomal aberrations or alterations in DNA repair processes. The Ames test, developed in the 1970s by Bruce Ames, is widely used as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA (Mortelmans & Zeiger 2000). The bacteria used in the test include strains of the bacterium *Salmonella typhimurium* that are auxotrophic for histidine and are constructed to detect either frameshift or point mutations. The test has been widely used in studies investigating mutagenicity in bottled water exposed to sunlight from the earliest

studies of De Fusco *et al.* in 1990 to the more recent studies of Bach *et al.* (2014) and Szendi *et al.* (2017). The E-screen bioassay was the first *in vitro* bioassay developed for screening estrogenic activity (Soto *et al.* 1995). The E-screen bioassay also known as the MCF-7 cell proliferation bioassay, measures cellular estrogen-dependent proliferation of the MCF-7 human breast cancer cell line.

Two litre (2 L) polyethylene terephthalate (PET) bottles are generally used for SODIS however disadvantages associated with the use of 2 L vessels have been described by Nalwanga *et al.* (2018) including the small treatment volume and the labour-intensive nature of filling several bottles in order to treat a volume sufficient for a family. To increase the volume of water produced, a range of large volume (≥20 L) sustainable SODIS technologies was developed by the European Union (EU) Horizon 2020 WATERSPOUTT project (grant agreement no. 688928). The objective was to provide affordable access to safe drinking water for remote and vulnerable communities throughout Sub-Saharan Africa and other resource-poor countries. In addition to increasing the volume of the SODIS reactors, the project also investigated the use of plastics other than PET. Three different novel reactors were designed: 25 L transparent jerrycans made of PET; polymethyl methacrylate (PMMA) tubular solar reactors capable of delivering >20 L of water and polypropylene (PP) 20 L buckets.

While studies have shown that the novel solar reactors developed in the WATERSPOUTT project are suitable for water disinfection (Polo-López *et al.* 2019; García-Gil *et al.* 2020; Reyneke *et al.* 2020), the aim of this study, which was part of a larger study (Ozores Diez 2021), was to investigate the safety of the reactors in terms of toxicity by investigating the presence of toxic leachables in the disinfected water. To date, toxicity studies of SODIS have largely focused on small scale reactors made of PET (Schmid *et al.* 2008; Ubomba-Jaswa *et al.* 2010; Bach *et al.* 2014). In this study, *in-vitro* toxicity assays were used to test large scale SODIS reactors made not only of PET but also made of polymethyl methacrylate (PMMA) and polypropylene (PP). The Ames test, previously used in SODIS studies (Ubomba-Jaswa *et al.* 2010), was used to investigate mutagenicity and the E-screen bioassay was used for the first time in SODIS studies to investigate the presence of estrogenicity. Toxicity testing was further challenged by exposing the reactors to sunlight for an extended period of time.

MATERIALS AND METHODS

The SODIS reactors

SODIS reactors made from three types of plastics were investigated – PMMA tubular solar reactors capable of delivering >20 L of water, 20 L PP buckets and two sizes of PET reactor – 2 L PET bottles and 25 L transparent PET jerrycans.

The PMMA reactors were manufactured by Ecosystem Environmental Services (EES; www.ecosystemsa.com), Barcelona, Spain using food grade quality PMMA supplied by GEHR GmbH. Two prototype reactors, of similar configuration, were constructed and consisted of several PMMA tubes placed in the centre of a V-shaped solar trough reflector (constructed from anodized aluminium). Prototype I solar reactor (140 L treatment volume) consisted of three 200 mm diameter PMMA reactor tubes and Prototype II solar reactor (88 L treatment volume) consisted of eight 100 mm diameter PMMA tubes (Supplementary Material, Figure S1). Further details of the PMMA reactors are described in Reyneke *et al.* (2020).

Two sizes of PET reactors were used in the study. 2 L PET bottles (Nestlé Aquarel) and 25 L transparent PET jerrycans supplied by Envases Solplados, Spain (www.envasessoplados.com). The transparent jerrycan was 52 cm in height, with a base of 24 by 26 cm and 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 (Supplementary Material, Figure S2A) 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 (Supplementary Material, Figure S2B).

Experimental set-up and sampling

The SODIS studies were carried out at two locations – Stellenbosch, South Africa and Plataforma Solar de Almería (PSA) in Spain. PSA which belongs to the Centre for Energy, Environmental and Technological Research (CIEMAT) and is the largest solar research, development and testing centre in Europe dedicated to concentrating solar technologies, was selected as the location for the long-term exposure studies on PMMA pieces and the novel PP and PET SODIS reactors. The PMMA reactors were tested at Stellenbosch. Water samples for toxicity testing were extracted on-site using solid phase extraction and tested for toxicity at Dublin City

University (DCU), Ireland using the E-screen bioassay to test for estrogenicity and the Ames test to test for mutagenicity.

At PSA (GPS coordinates: 37°84′N,2°34′W), long-term studies for leachates from PMMA were carried out using pieces of PMMA (2 × 5 cm) prepared from 5 cm cylinders of PMMA (each approximately. 57 g) supplied by EES. The pieces of PMMA (30 pieces of 2 × 5 cm) were immersed in 2 L volumes of distilled water and 2 L volumes of local well water contained in glass bottles (Supplementary Material, Figure S2C). 2 L PET bottles, 25 L PET jerrycans and 20 L PP buckets were filled with local well water. The well water was autoclaved at 121 °C for 15 minutes prior to use, to avoid potential growth of any microorganisms during the long experimental period. Each treatment was set up for 3-, 6- and 9-month periods of exposure to the sun and similar treatments were set up in the dark as controls. All the treatments were set up in triplicate. The vessels were sampled following 3-, 6- and 9-month intervals in sunshine and darkness and tested for toxicity, conductivity and salinity. Water samples for toxicity testing were extracted using solid phase extraction and tested for toxicity at DCU. The conductivity was measured using a conductivity meter GLP31 CRISON. The salinity or the ion concentration of the water (chloride, nitrite, nitrate, phosphate, sulphate, sodium, ammonium (NH_4), calcium, potassium and magnesium) was measured using a Metrohm ion chromatograph Model 850, that consisted of two 872 extension modules: (i) module for determination of anions and polycarboxylic acids through a gradient analysis in a column METROSEP A Supp 7-250 (250 mm × 4.0 mm ID); (ii) module for determination of cations and amines through an isocratic analysis in a column METROSEP C4-250/4.0 (250 mm × 4.0 mm ID). The mean and standard deviation (SD) were calculated using Microsoft Excel 2019 and the different samples were compared with a t-test using Microsoft Excel 2019.

Field testing of the PMMA reactors was carried out at Stellenbosch, South Africa. Prototype I solar reactor was installed at an informal settlement in Enkanini (GPS coordinates: 33°55′28.1″S 18°50′35.8″E) and the Prototype II was installed 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). Each reactor was supplied with water from a rainwater harvesting (RWH) tank. Samples were taken once a month during the period August 2018–April 2019. An untreated 10 L sample was collected directly from the RWH tank at each site on the morning of a sampling event. The respective solar reactor prototypes at each site were then immediately filled with water from the RWH tanks and exposed to direct sunlight for 6 hours (August–November 2018) or 8 hours (December 2018–April 2019). Following the completion of the solar exposure, 10 L of each treated sample was collected directly from the solar reactors. The physicochemical characteristics of the harvested rainwater and the solar disinfected water were monitored and are reported in Reyneke *et al.* (2020). Water samples for toxicity testing were extracted using solid phase extraction and tested for toxicity at DCU.

Solid phase extraction of the water

Water samples (1 L) were extracted prior to toxicity testing using a modification of the solid phase extraction method described by Wagner & Oehlmann (2011) and Abbas *et al.* (2019). Details of the method are described in Ozores Diez *et al.* (2020). Final extracts (concentration factor: ×10,000) in 100 µL dimethyl sulphoxide (DMSO) were stored in glass vials with PTFE caps at −20 °C until analysed.

E-screen bioassay

The solid phase extracted (SPE) samples (in DMSO) were diluted 100-fold with cell culture medium resulting in a final solvent negative control concentration of 1% (v/v). The cell culture conditions for the E screen, described by Soto *et al.* (1995) were used with minor modifications and the cell number was quantified based on DNA binding to the fluorescent Hoechst 33258 dye. Further details of the assay are described in Ozores Diez *et al.* (2020).

Data interpretation

Fluorescence emission of each water extract was normalized to fluorescence emission of the negative control giving the proliferative effect (PE) of the water extract (Equation (1): Calculation of Proliferative Effect).

$$PE = \frac{\text{Water extract fluorescence}}{\text{Negative Control fluorescence}} \times 100 \quad (1)$$

The estrogenic activity of the water extracts was evaluated by determining relative proliferative effect (RPE). The RPE compares the maximal fluorescence produced by the water extract with the fluorescence emitted by

the positive control (1 nM 17β-estradiol (E2)) (Equation (2): calculation of Relative proliferative effect index, % E2).

$$RPE = \frac{\text{Water extract fluorescence} - \text{Negative Control fluorescence}}{\text{E2 fluorescence} - \text{Negative Control fluorescence}} \times 100 \quad (2)$$

Total agonists of 17β-estradiol were defined by 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. Finally, non-estrogenic substances will have RPE values between 0 and 10%. Estradiol equivalents (EEQs) were calculated by interpolation from a dose response curve of 17β-estradiol in the range 0.01 pM to 1 nM 17β-estradiol (data not shown) (Equation (3): Equation for the interpolation of EEQ values from the dose response curve of 17β-estradiol).

$$y = 8500.6x^{0.2096} \quad (3)$$

EEQs were corrected for final concentration factors and reported as ng/L of the original water sample. Three independent experiments were conducted on each sample.

Graphs were plotted as the mean $\pm$ SD of the PE using the Excel Microsoft office software. 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 $p < 0.05$.

Ames test

Following extraction, using the SPE method described above, samples were concentrated 1000-fold and 10 µL volumes were tested for mutagenicity/genotoxicity using the Ames II kit by Xenometrix AG (Allschwil, Switzerland). The kit contained *S. typhimurium* TA98 used for the detection of frameshift mutations and TAMix, a mixture of equal proportions of the Ames II TA7001-TA7006 strains, to detect base-pair substitutions. Freshly prepared overnight cultures of TA98 and TAMix were 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 several cell divisions. After 90 minutes exposure, cultures were diluted in pH indicator medium lacking histidine and aliquoted into 48 wells of a 384-well plate. Within 2 days, cells that had undergone the reversion to histidine prototrophy – either spontaneously, or as a result of the exposure to a mutagen, grew 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 was counted for each sample and compared to a solvent (negative) control. Mean values for triplicate samples were determined and data analysis was performed using Excel Office 2019 software. The results were expressed as the fold induction over the baseline which was the ratio of the mean number of positive wells (revertants) for each sample divided by the baseline. The baseline was obtained by adding one standard deviation to the mean number of positive wells of the solvent control. A sample that showed a clear fold induction $\geq$2 above the baseline was classified as a mutagen. The mutagenic potential of the samples was assessed directly and in the presence of liver extract S9.

RESULTS AND DISCUSSION

In order to test the plastic reactors for toxic leachates during the SODIS process, long-term studies were carried out at PSA where the plastics in contact with water were exposed to sunlight for up to 9 months. The choice of the water matrix was important to avoid the presence of any contaminants that might contribute toxicity. Well water, sourced at PSA, and distilled water were chosen for use in the studies. Sections of PMMA tubing used in the construction of the PMMA reactors were cut into small pieces to increase the surface area. To investigate possible leaching from the plastic, the pieces were immersed in well water and in distilled water, exposed to sunlight for up to 9 months and the water was tested for toxicity at 3, 6 and 9 months. None of the samples showed proliferative activity relative to the negative control in the E-screen and co-incubation of cells with extracts and ICI had no effect consistent with an absence of estrogenic leachates (Figure 1). Estimation of corresponding RPE

values shown in Supplementary Material, Table S1 indicated that all extracts showed % RPE <10% and were not estrogenic.

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 (Supplementary Material, Table S2). Salinity and conductivity measurements of the well water and the distilled water are described in Supplementary Material, Table S3. The conductivity of the well water, 2.5 ± 0.0 mS/cm was slightly lower than the conductivity of the distilled water, 4.0 ± 1.9 μS/cm and the conductivity did not change significantly over the 9-month exposure period for either type of water. The concentration of ions in the well water indicated that the well water was of drinking water standard. Any differences in the ion concentration of the two types of water and any changes in the ion concentration over the 9-month exposure period did not affect the toxicity testing as the results of the toxicity assays were similar for both types of water and no toxicity was detected with either water type. Well water was used to study leaching in the transparent PET jerrycan and in the PP bucket.

Field studies of the PMMA reactors were carried out at Bonfoi and Enkanini in Stellenbosch. The reactors were supplied with water from harvested rainwater tanks. The physico-chemical and chemical quality of the rainwater and the solar treated rainwater was found to adhere to the respective drinking water guidelines (Reyneke *et al.* 2020). The reactors were sampled once a month between August 2018 and April 2019 which allowed for a study of seasonal variation. When tested using the E-screen bioassay, none of the samples taken from either Bonfoi or Enkanini showed proliferative activity relative to the negative control (Figure 2).

Proliferative effects of extracts collected over 9 months from August 2018 to April 2019 in Bonfoi ranged between 81 and 105% of the negative control (100%) and in Enkanini ranged between 74 and 103% of the negative control (100%). Proliferative effects of extracts from the harvested rainwater tanks feeding the tubular PMMA SODIS reactors ranged between 76 and 98% of the negative control (100%) in the Bonfoi samples and between 71 and 88% of the negative control in the Enkanini samples. Co-incubation of cells with extracts and ICI had no

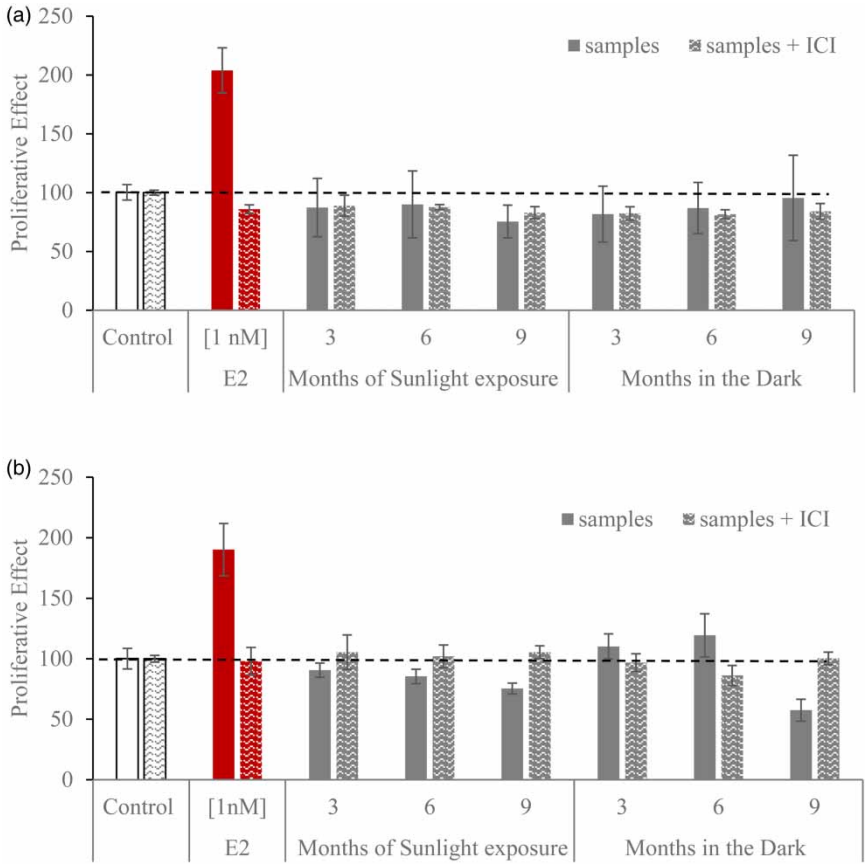


Figure 1 | Proliferative effects of water samples following immersion of pieces of PMMA in distilled water (a) and well water (b) at PSA, Almería, Spain and exposure to sunlight and darkness for 3, 6 and 9 months. Estrogenic activity was assessed in the presence and absence of ICI [0.1 μM]. E2 – 1 nM 17β-estradiol. Control – cells in hormone-free DMEM/F12 medium containing 1%(v/v) dimethyl sulphoxide.

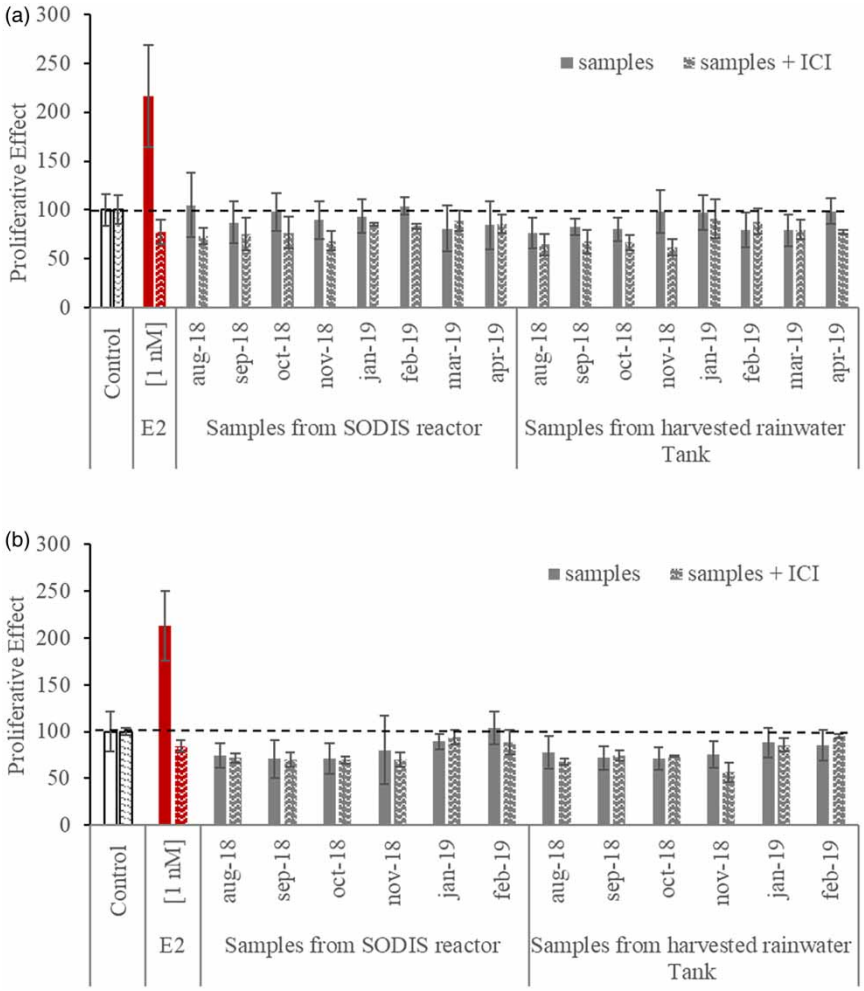


Figure 2 | Proliferative effects of water samples from the PMMA reactors and their associated harvested rainwater tanks (controls) (a) – Bonfoi and (b) – Enkanini. The samples were tested in the presence and absence of ICI [0.1 μM]. E2 – 1 nM 17β-estradiol. Control – cells in hormone-free DMEM/F12 medium containing 1%(v/v) dimethyl sulphoxide.

effect consistent with an absence of estrogenic leachates. Estimation of corresponding RPE values shown in Supplementary Material, Table S4 indicated that all extracts showed % RPE <10% and were not estrogenic. 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 (Supplementary Material, Table S5). These findings together with those of the long-term studies on PMMA pieces demonstrated the safety of PMMA for use in SODIS. 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 since it is half the weight of glass with up to 10 times the impact resistance. It also has excellent scratch resistance although less than glass. Reyneke *et al.* (2020) have shown the effectiveness of the reactors in water disinfection and the results of the toxicity studies confirmed the suitability of the PMMA reactors for SODIS.

Polypropylene (PP) is a tough, rigid and crystalline thermoplastic produced from propene (or propylene) monomer. It is widely used in various applications due to its good chemical resistance. Following exposure of the PP reactors to the sun at PSA for 3-, 6- and 9-month periods, none of the samples taken from the PP reactors showed significantly increased proliferation when compared to the control in the E-screen bioassay (Figure 3). The corresponding RPE values of these samples are presented in Supplementary Material, Table S6. Most of the PP leachate samples had lower proliferative effects than the control and so the corresponding RPE values are negative and the corresponding EEQ values were not determined. All extracts showed % RPE <10% and were therefore not estrogenic.

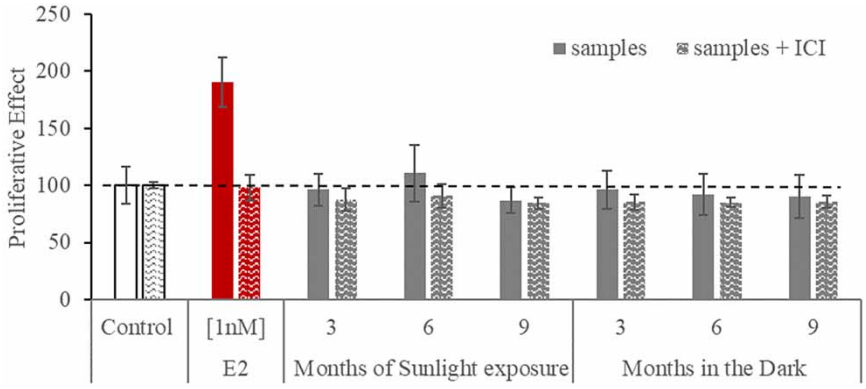


Figure 3 | Proliferative effects of water samples from the PP reactors assessed for estrogenic activity in the presence and absence of ICI [0.1 μM]. E2 – 1 nM 17β-estradiol. Control – cells in hormone-free DMEM/F12 medium containing 1%(v/v) dimethyl sulphoxide.

No mutagenicity was detected for any sample from the PP buckets or from the 2 L and 25 L PET reactors using the Ames test as no value obtained showed a clear fold induction ≥2 above the baseline value (Supplementary Material, Tables S7 and S8). As was the case with the PMMA pieces, only slight changes in ion concentrations were observed in the well water in the PP and PET reactors following incubation in the sun for 3-, 6- and 9-month intervals and there was no significant change in the conductivity of the well water over the 9-month period (Supplementary Material, Tables S9 and S10). The effectiveness of the transparent 20 L PP buckets for solar disinfection of bacterially contaminated (*E. coli*) water has been reported by Polo-López *et al.* (2019). The toxicity results confirmed the suitability of the PP reactors for SODIS.

The absence of mutagenicity in the solar disinfected water in this study is in agreement with previous findings (Bach *et al.* 2013, 2014). Ubomba-Jaswa *et al.* (2010) used the Ames fluctuation test to study PET bottles containing mineral water exposed to sunlight for up to 6 months using two approaches. 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 unconcentrated 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.

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 and Rainer *et al.* (2018) pointed out that S9 must be included in all Ames studies. The methodology used in this study was in agreement with Rainer *et al.* (2018) who 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% 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.

While no mutagenicity was detected in the water samples collected from the PET reactors (Supplementary Material, Table S8), a time-dependent estrogenic activity was recorded for these water samples (Figure 4).

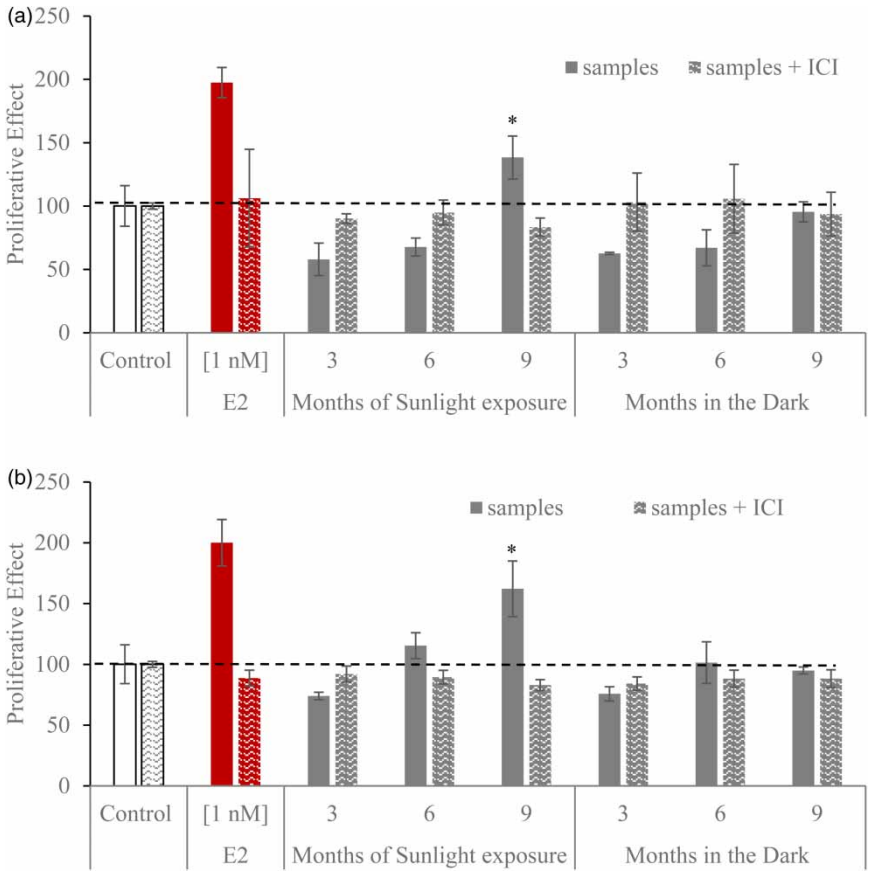


Figure 4 | Proliferative effects of water samples from the PET reactors. (a) 2 L PET bottle and (b) 25 L transparent jerrycan assessed for estrogenic activity in the presence and absence of ICI [0.1 μM]. Single factor Anova indicated significantly increased proliferation (*) $p < 0.05$ of the water extracts compared to the control. E2 – 1 nM 17β-estradiol. Control – cells in hormone-free DMEM/F12 medium containing 1%(v/v) dimethyl sulphoxide.

Extracts prepared from well water stored in 25 L PET jerrycans 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 extracts. A similar pattern was observed with 2 L PET SODIS bottles. 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%). Co-incubation of 9-month extracts with ICI [0.1 μM] mitigated proliferation confirming estrogenic activity of the 9-month extracts. Thus estrogenicity was detected in the PET vessels after 9 months' exposure to sunshine but not after 3 or 6 months in sunlight. It is recommended to replace PET bottles after 6 months to help avoid the effects of ageing such as scratches which may hinder effective absorption and transmission of UV light hence affecting SODIS efficacy (Ubomba-Jaswa *et al.* 2010). This practice would also help to minimise any threat of toxic leachables from PET.

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.

Corresponding estradiol equivalents (EEQs) of water extracts amounted to 0.3 and 0.05 ng/L respectively (Table 1). The PET reactors were exposed for a prolonged period of time, 9 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 to 40 °C. 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.

Table 1 | Relative proliferative effects (% RPE) and estradiol equivalents (EEQs in ng/L) of water samples from PET SODIS reactors following 3, 6 and 9 months' exposure to sunlight and darkness at Almeria, Spain

			RPE (%)	EEQ (ng/L)
Jerrycan SODIS reactor	Months of exposure to Sunlight	3	−69.5 ± 21.4	nd
		6	−0.3 ± 25.0	nd
		9	69.7 ± 30.3	0.3
	Months in the Dark	3	−66.4 ± 17.2	nd
		6	−24.9 ± 31.1	nd
		9	−32.5 ± 29.8	nd
2 L PET bottle SODIS reactor	Months of exposure to Sunlight	3	−53.2 ± 14.2	nd
		6	−33.1 ± 24.5	nd
		9	48.7 ± 18.5	5.1E-02
	Months in the Dark	3	−47.6 ± 8.2	nd
		6	−43.6 ± 21.8	nd
		9	−6.0 ± 10.1	nd

Data refer to mean ± SD of triplicate experiments. nd, not determined.

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 182780 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). In the assay, the negative control is provided by cells cultured in medium supplemented with estrogen-free serum, the positive control is provided by cells exposed to 17β-estradiol 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. To our knowledge, there are no previous reports of the use of the E-screen bioassay 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 temperature and sunlight-exposed PET were performed using *in vitro* gene reporter bioassays (Bach *et al.* 2013, 2014), both of which showed no release of estrogenicity into water.

According to JECFA (WHO 2006), the acceptable daily intake (ADI) of 17β-estradiol in the diet is 0–50 ng per kg of body weight per day. In this study, the ng EEQ/L values of the different leachate samples from PMMA, PET and PP were well below the ADI suggesting that the novel SODIS reactors are safe to use to deliver water for drinking, food preparation and similar purposes. Nevertheless, in recent years concern has been raised by paediatricians and scientific organizations about the influence of exogenous estradiol on human health and development particularly of pre-pubertal 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 17β-estradiol hormone. Aksglaede *et al.* (2006) reported that the rate of endogenous production of 17β-estradiol in pre-pubertal 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 greater than previously thought. Until the ADI is revised, scientists are addressing this concern by deriving trigger values to differentiate between acceptable and unacceptable effect levels of an exogenous source of estrogens and by predicting the physiological impact of exogenous exposure on hormone activity. Brand *et al.* (2013) 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 17β-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, in terms of toxicity, in the SODIS process. This study focused on evaluating the mutagenicity and estrogenic activity of extractables and leachates from three 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.

CONCLUSIONS

SODIS reactors made from PMMA, PP and PET and exposed to sunshine for nine months were found to be safe for use in the SODIS process, in terms of toxic leachables, when tested using the E-screen assay and the Ames test. No mutagenicity was detected in any reactor and the only estrogenicity detected was associated with the PET reactors following nine months', but not six months', exposure to sunshine. Estrogenicity was detected not only in the large volume PET jerrycan but also in the 2 L PET bottle more usually used in the SODIS process. While the levels of estrogenicity detected in the PET reactors at nine months were within acceptable limits and not considered harmful to human health, the findings reinforce the recommendation to replace PET bottles used in SODIS every six months. Our findings provide first evidence for migration of xenoestrogens from PET SODIS reactors into water after a prolonged 9-month period of sunlight exposure.

CONFLICTS OF INTEREST

The authors have no conflicts to declare.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from the European Union's Horizon 2020 Research and Innovation Program under grant agreement no. 688928 (WATERSPOUTT H2020-Water -5c) and the MCF-7 BUS cells generously gifted by the group C. Sonnenschein and A. Soto (Tufts University, Boston).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 30 June 2021; accepted in revised form 18 August 2021. Available online 31 August 2021