Studies on the Effects of Paraben Mixtures on MCF-7 Breast Cancer Cells in Culture

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Abstract

Parabens are the esters of p-hydroxybenzoic acid and are commonly used as preservatives in personal care products, pharmaceutical preparations and cosmetics. Recently parabens have been found to be estrogenic, bringing into question if exposure to them is adversely affecting human health. Given exposure to multiple xenoestrogens is constant; research has been carried out to determine what effect combinations of xenoestrogens might have on human and environmental health. Parabens are almost always present in combinations in formulae as this increases their antimicrobial activity, so it is important to know what the effect of this is. The main aim of this study was to determine what the effect of combining methylparaben and butylparaben together has on the proliferation of MCF-7 breast cancer cells, which proliferate in the presence of estrogen. This study was carried out by exposing MCF-7 breast cancer cells to combinations of methylparaben and butylparaben and measuring cell proliferation by counting cells using a cytometer. The results show that butylparaben caused a greater increase in cell proliferation compared to methylparaben. When methylparaben and butylparaben were combined together, the resulting cell proliferation was greater than the cell proliferation produced by either methylparaben or butylparaben alone at a concentration twice the amount of either paraben concentration contained within the mixture. These results were analysed using Analysis of Variance, which determined the combination treatments were statistically different from the single treatments according to Fishers method. This suggests that there is a synergistic effect produced when methylparaben and butylparaben are combined together, however large variation and dose dependent discrepancies means this result is uncertain and further studies need to be carried out.

Abbreviations

A list of abbreviations used:

ANOVA = Analysis of Variance

B-para = butylparaben

cAMP = cyclic Adenosine monophosphate

DPBS = Dulbecco's Phosphate Buffered Saline

E2 = 17β-Estradiol

ER = Estrogen Receptor

ERα = Estrogen Receptor alpha

ERβ = Estrogen Receptor beta

FBS = Fetal Bovine Serum

MEM = Minimum Essential Medium

M-para = methylparaben

Paraben = para-hydroxybenzoate

PRFMEM = phenol red-free Minimum Essential Medium

SHBG = sex hormone-binding globulin

TrypLE = TrypLE™ Express (1X), no phenol red

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1 Introduction

1.1 Hormones and the endocrine system

The endocrine system synthesises and secretes hormones into the circulatory system and extracellular fluids in order that the hormones can exert their effects on distant organs, tissues or cells through receptor mediated responses. There are 3 classes of hormone in mammals; steroid hormones (e.g. testosterone, 17β-estradiol), protein and peptide hormones (e.g. Insulin) and hormones derived from single amino acids (e.g. Dopamine). Hormones carry out their function in a narrow concentration range and as a result, need to be highly regulated (Leblebicioglu et al. 2013). A good example is the female hormone 17β-estradiol which is produced in tissues such as the ovaries, mammary glands and the adrenal cortex (Leblebicioglu et al. 2013). 17β-Estradiol is essential for developing primary and secondary sex characteristics in females, maintaining the female reproductive cycle (e.g. formation of the uterus lining) and pregnancy (Zhu & Conney 1998). All hormone actions are receptor-mediated; the receptors are either extracellular or intracellular. Extracellular or cell surface receptors are signal transducers; the hormone binds and activates the receptor, which changes an extracellular signal into one or more intracellular signals that causes a change in the cell. Hormones bind to intracellular receptors to activate them, which leads to the regulation of transcription of specific genes (Gupta 2009).

Steroid hormones are all derived from a common precursor, namely cholesterol. They include androgens, estrogens, corticosteroids and progestogens (Fig 1.1). A feature of the steroid hormones is their very low water solubility on account of their hydrophobic molecular structures. For this reason they are transported in the blood on a carrier protein known as sex hormone binding globulin (SHBG; see section 1.2.5). SHBG releases the steroid once the complex reaches the target tissue cells so the steroid can bind to receptors on the cell surface or passively diffuse across the cell membrane, because of their hydrophobic nature and bind

to intracellular receptors, activating them (Anderson 1974). Steroid hormones are responsible for controlling reproductive development and function as well as influencing other physiological processes. Steroid hormones play an important role in a developing fetus as they determine whether the fetus develops male or female sex organs. The ratio of steroid hormones is crucial. If there are more estrogens present than there should be in a male fetus, this will affect the formation of sex organs and can cause birth defects like hypospadias where the urethra forms abnormally, and cryptorchidism where one or both testes fail to descend (Aksglaede et al. 2006).

Steroid hormones are mostly metabolised in the liver by cytochromes P450 and in some target cells. When steroids are no longer required, functional groups (e.g. sulphate, glucuronide) are added to steroids to make them more water soluble so they can be excreted in urine, sweat and bile (Hanukoglu 1992).

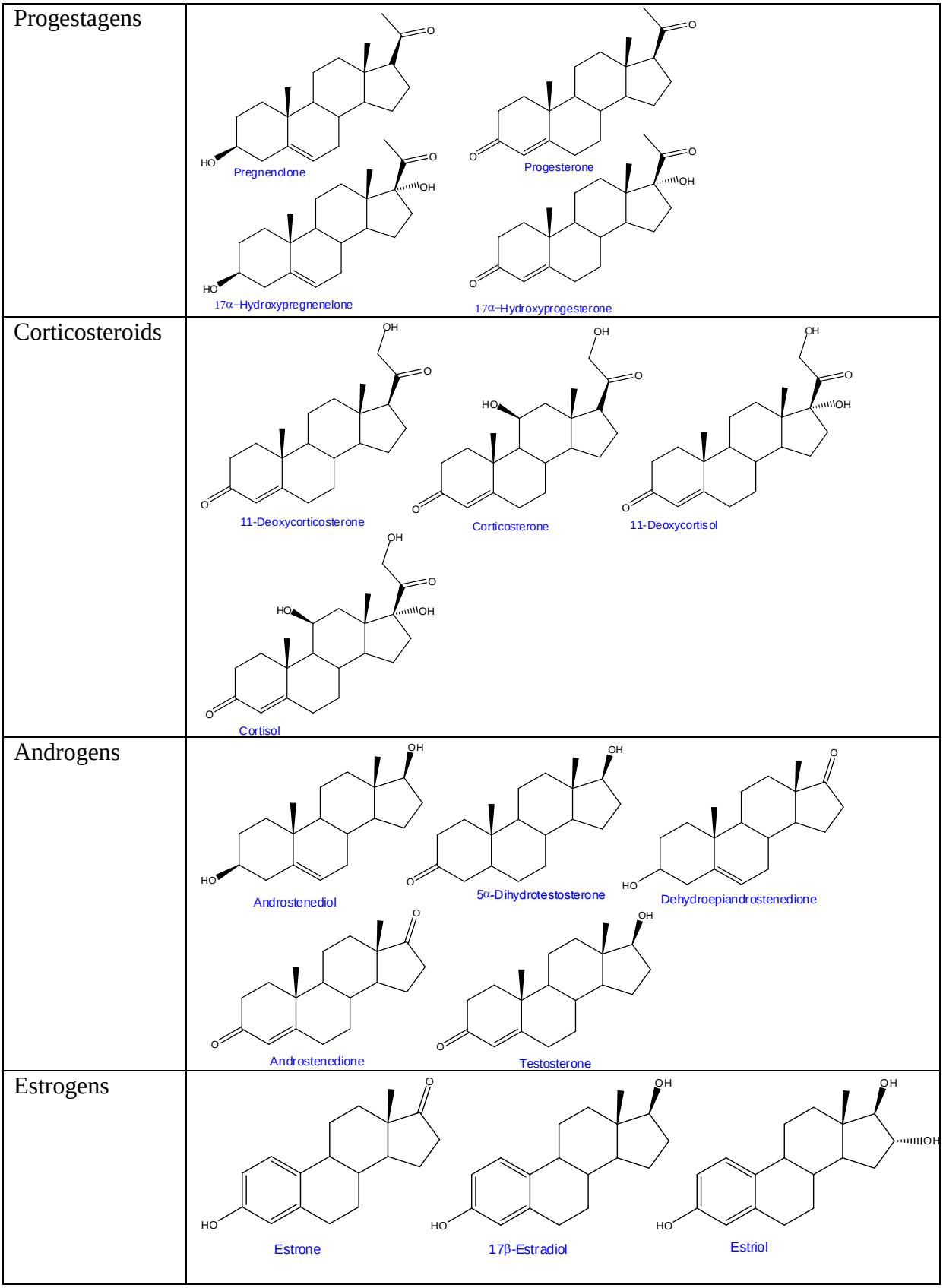

Figure 1.1: The key progestogens, corticosteroids, androgens and estrogens.

1.2 Estrogens

1.2.1 What are estrogens?

Estrogen is the name given to the 3 endogenous female hormones 17β -estradiol, estriol and estrone (Fig 1.2) that are produced by vertebrates. Estrogens were discovered in the 1920's and 1930's by Adolf Butenandt and Edward Adelbert Doisy. Butenandt extracted them from horse urine and in 1939 won The Nobel Prize in Chemistry for his work on sex hormones (Tata 2005). Estrogens are responsible for female reproductive development and maintaining secondary female characteristics and are biosynthesised from the androgens, primarily testosterone. Estrogens cannot be stored in tissues, so they are synthesised when they are required (Hanukoglu 1992). Estrogens are produced in the ovaries, testis, placenta, liver, adrenal cortex, mammary glands and fat cells. The roles and presence of 17β-estradiol, estriol and estrone in the body differ greatly depending on sex, age, stage of development or if pregnant (Green & Leake 1987). The use of estrogens has had a huge impact especially in human health and reproduction.

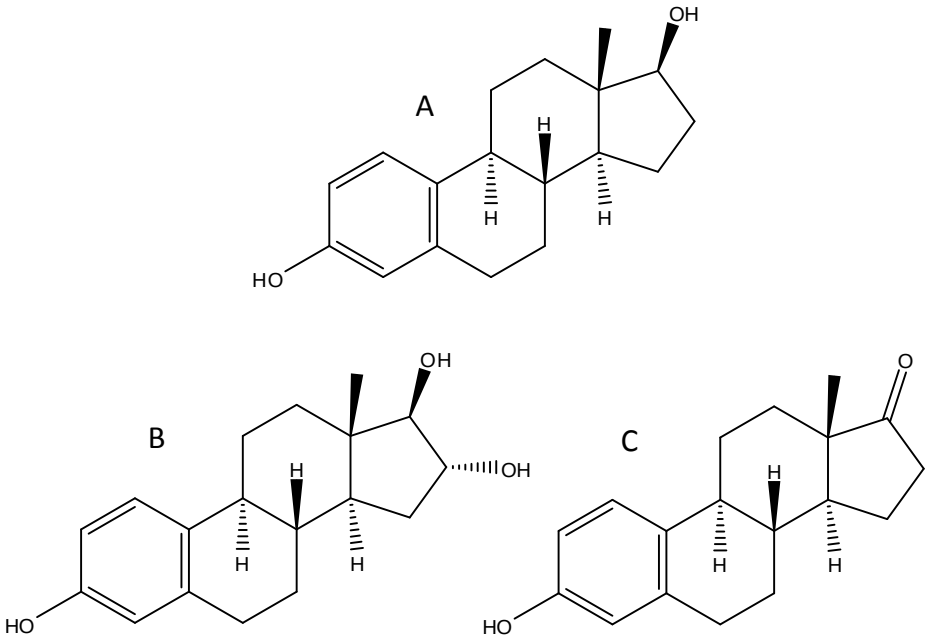

Figure 1.2: Endogenous female hormones 17β –estradiol (A), estriol (B) and estrone (C).

The contraceptive pill

The development of the oral contraceptive known as "the pill" occurred in the 1950's. Its primary mechanism is based on controlling ovulation with synthetic derivatives of estrogen and progesterone. In the 1960's and 1970's estrogen alone was used as a method of contraception, however this was shown to increase the incidence of endometrial cancer. The risk was lowered by adding a progestogen (NTP 2011). Currently the synthetic estrogen used in most oral contraceptives is 17α-ethinylestradiol (Fig 1.3) which was first synthesised in 1938 and given FDA approval in the mid 1940's (Tata 2005). It has remarkable structure activity relationships with 17β-estradiol. Ethinylestradiol is more suitable for use as a contraceptive than 17β-estradiol because it is not metabolized as quickly due to the carbon-carbon triple bond functional group on carbon 17. Ethinylestradiol is, however, metabolised by cytochromes P450 followed by glucuronide or sulphate conjugation and is excreted in the urine and bile (Johnson & Williams 2004).

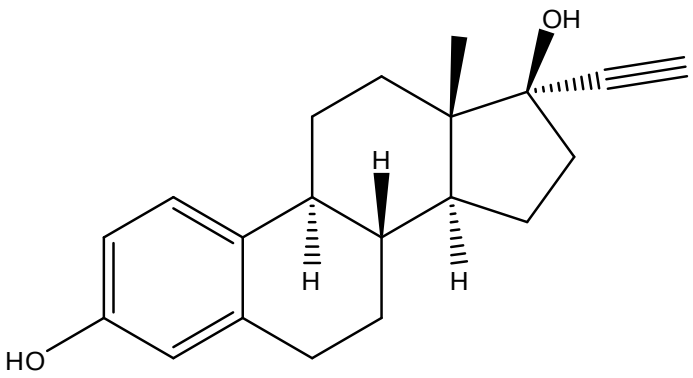

Figure 1.3: The chemical structure of 17α-ethinylestradiol used in the contraceptive pill.

Estrogens, including some synthetic derivatives are used in hormone replacement therapy (HRT) for relief of menopausal symptoms and treatment of low levels of estrogen (hypoestrogenism) (Tata 2005). Recently HRT has come under scrutiny as studies were carried out that showed increased risks of breast cancer, heart attacks or strokes (Frazzetto

2003; Dixon 2001). This has led to questioning about the risks associated with the use of estrogen in all health areas including the contraceptive pill. The full role that estrogens fulfil in the body is still up for debate; for example, there have been links to heart health (Mendelsohn & Karas 1999), increased bone reformation and reduced bone resorption (Kameda et al. 1997; Khosla et al. 2012), mental health (Hill et al. 2007) and the immune system (Nadkarni et al. 2012). Studies show that the elevated risk of cardiovascular disease in postmenopausal women compared to premenopausal women is reduced when they are given estrogen therapy. It has also been suggested that the effect estrogen has on blood vessels is protective against cardiovascular disease. Estrogen increases vasodilation and inhibits the blood vessels responding to injury and the development of atherosclerosis (Mendelsohn & Karas 1999). There is a lot of conflicting evidence as other studies show that estrogen therapy makes no noticeable difference, or even increases the risk of developing cardiovascular disease (Hulley et al. 2013; Manson et al. 2003).

1.2.2 17β-Estradiol

17β-Estradiol (E2; Fig 1.2; A) is the primary and most potent endogenous female steroid hormone. Synthesised from testosterone in many tissues, E2 is responsible for sex determination as a fetus, the onset of puberty in females and maintaining female secondary sex characteristics including the reproductive cycle (NTP 2011). E2 is also present in males but the levels are much smaller; it is involved in the sperm maturation process and may be important for a healthy libido (Heindel et al. 2012). The levels present in an individual differ depending on sex and age. Prepubescent females, post-menopausal females and males all have very similar levels. Females of a reproductive age have higher E2 levels which continuously change as the menstrual cycle progresses. During the first part of the menstrual cycle, levels of 17β-estradiol rise slowly and peak sharply, initiating ovulation (Fig. 1.4) (Klump et al. 2013).

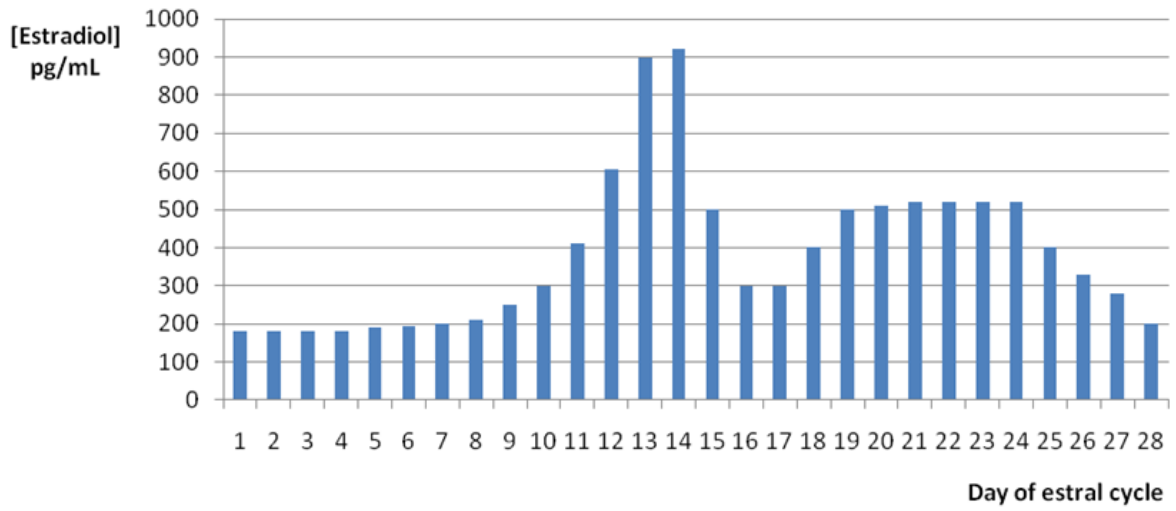

Figure 1.4: Blood levels of estradiol in women showing the large changes during the estral cycle – ovulation occurs on day 13 or 14.
(data from http://commons.wikimedia.org/wiki/File:Estradiol_during_menstrual_cycle.png).

1.2.3 Estriol

Estriol (Fig 1.2; B) was first isolated in 1930 from urine from pregnant women by Doisy and associates (Merrill 1958). Estriol is made in significant amounts during pregnancy by the placenta. When isolated from human placenta in 1931, the concentration found was much higher than estrone and E2 (Merrill 1958). Studies show that although the estriol levels present in pregnant women are high compared to non-pregnant and menopausal women, estriol is not as potent as E2 (Heller 1940). Estriol was originally used to treat undesirable symptoms of menopause. This is because the amount of estriol present in the blood of menstruating females was higher than E2 and estrone, and estriol was rarely found in the blood during menopause (Merrill 1958). More recently, studies show that treating multiple sclerosis patients with oral doses of estriol has the potential to be a treatment (Sicotte et al. 2002). Estriol is a metabolite of E2 (Fig. 1.5) and its biochemical function is not as well understood as other estrogens (Barlow & Logan 1966).

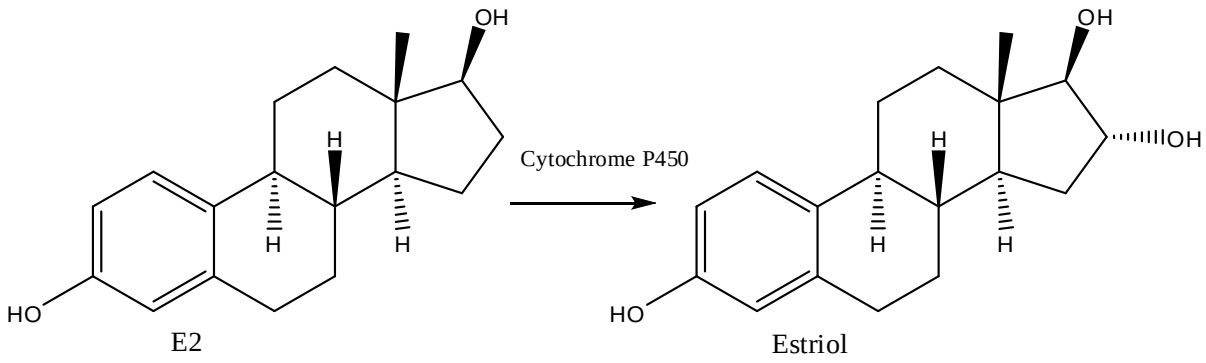

Figure 1.5: E2 is metabolised to Estriol via cytochromes P450.

1.2.4 Estrone

Estrone (Fig 1.2; C) is the least abundant of the endogenous estrogens. Estrone and its sulfonated conjugated form, estrone sulphate, are most commonly found in menopausal women as their main form of estrogen. Most of the naturally occurring estrogen in post-menopausal women is synthesised in the adrenal cortex and other peripheral tissues from androstenedione (NTP 2011). Estrone is a metabolite of E2, however is converted when needed to E2, the more active form of estrogen (Fig. 1.6) (Hanukoglu 1992). It is secreted by the ovaries along with E2 in women with normal menstrual cycles and by the placenta in pregnant women (NTP 2011).

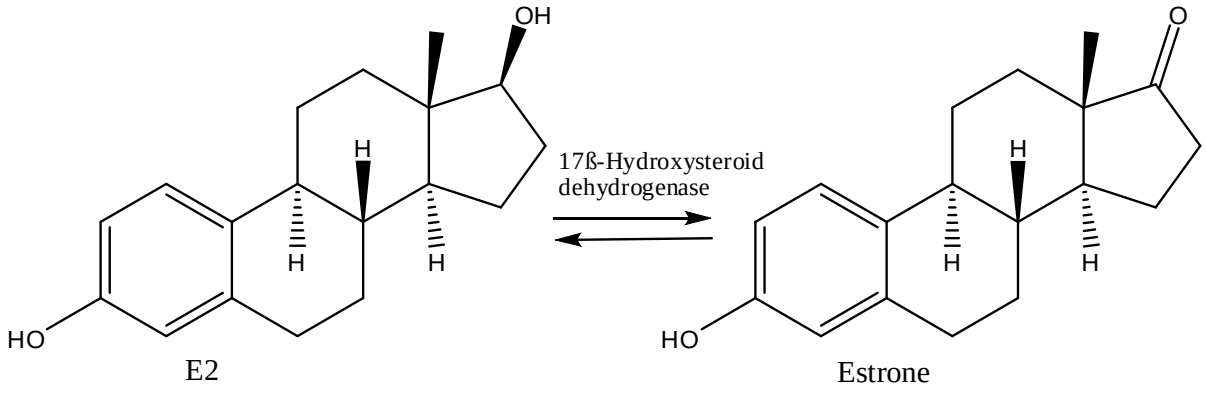

Figure 1.6: E2 is metabolised to Estrone via 17β-hydroxysteroid dehydrogenase. This reaction is reversible.

1.2.5 Transport of estrogen in the body

E2 is transported in the blood bound to sex hormone binding globulin (SHBG; Fig 1.7). The existence of SHBG was first demonstrated in 1965 by Mercier and colleages (Mercier 1965) showing the binding of testosterone. SHBG was given its name due to being able to bind particular androgens and estrogens with a high, yet reversible, affinity (Anderson 1974). The role of SHBG is complex. Originally it was thought to just regulate the access of steroid hormones into the target cells by binding them and controlling the bioavailable amount present. Since only 1-2% of E2 is unbound or "free", the level of E2 circulating in the blood is high and readily available to be delivered into target cells (Rosner 2006; Anderson 1974). Now studies show that specific cell plasma-membranes are able to bind to SHBG with a high affinity, through a SHBG-receptor, changing the model of how SHBG is thought to behave (Fissore et al. 1994; Rosner et al. 1999; Rosner et al. 2010). This binding of SHBG to the SHBG-receptor occurs in MCF-7 cells. The binding of E2 then occurs, causing a significant accumulation of intracellular cyclic adenosine monophosphate (cAMP), which is a secondary messenger in the cell (Fissore et al. 1994).

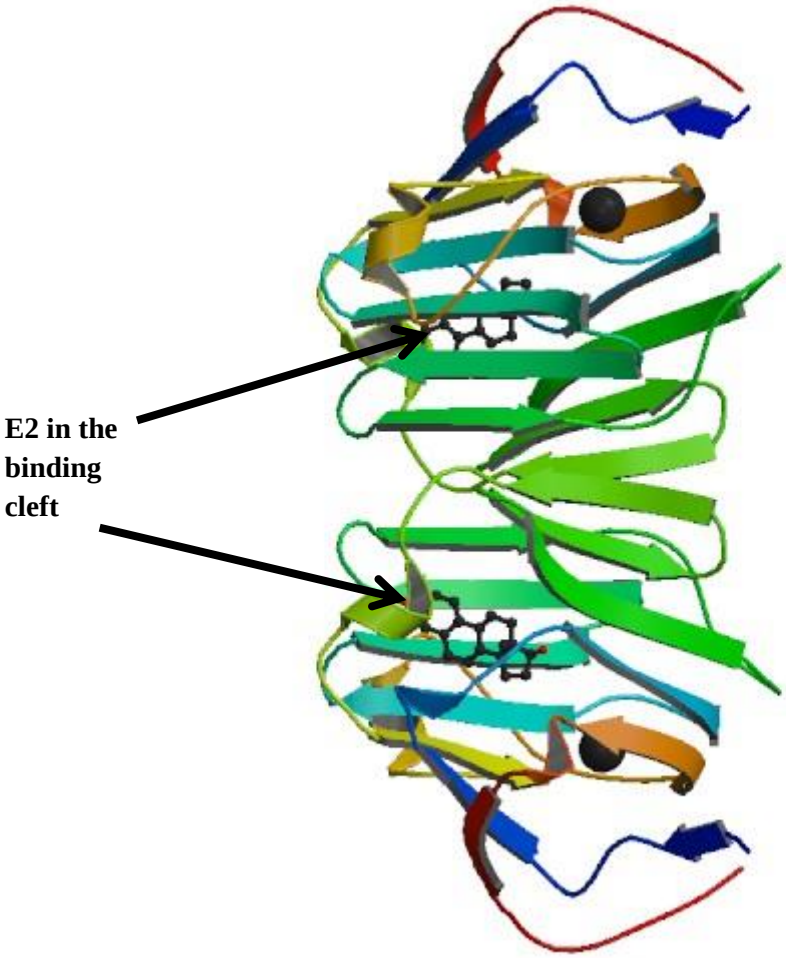

Figure 1.7: Solved crystal structure of the N-terminal domain of SHBG in complex with E2.

PDB ID: 1LHU
Grishkovskaya, I., Avvakumov, G.V., Hammond, G.L., Catalano, M., Muller, Y.A. (2002) Steroid ligands bind human sex hormone-binding globulin in specific orientations and produce distinct changes in protein conformation. J.Biol.Chem. 277: 32086-32093.

1.3 The estrogen receptor

To carry out its role, E2 binds to the estrogen receptor (ER; Fig. 1.8; A) and activates it, causing a ligand-induced conformational change in the receptor (Nilsson & Gustafsson 2000). Estrogen receptors are nuclear receptors that are ligand activated transcription factors; they regulate target gene expression (Tsai & O'Malley 1994). When the hormone binds, receptor dimerization occurs and the hormone-receptor complex moves into the nucleus

where it binds to specific estrogen responsive elements (EREs) located within promoter regions of target genes. At these specific binding sites, the hormone-receptor complex initiates transcription of estrogen-responsive genes, causing a feminising process, which is carried out by the proteins that are produced (Halachmi et al. 1994). There are two estrogen receptors, ER alpha (ERα) and ER beta (ERβ). ERα was discovered in 1962, but not isolated until 1986 (Green et al. 1986; Pettersson & Gustafsson 2001). ERα is found typically in mammary and uterine cells. ERβ was first cloned in 1996 and has a wider tissue distribution compared to ERα; it is found in many tissues such as the ovary in the granulosa cells and the prostate in the epithelial cells of the secretory alveoli (Kuiper et al. 1996). ERβ is also expressed in some tumour cells that are ERα negative, such as tumours of the colon, stomach and prostate (Younes & Honma 2011). ERβ is partially homologous with ERα; they share 95% of the DNA binding domain, yet only 55% c-terminal ligand binding domain. ERβ requires an E2 concentration 5 – 10 times greater than ERα to get the same transcriptional response (Pettersson & Gustafsson 2001). Even though ERβ requires a higher concentration of E2 and E2 binds with a lower binding affinity, some xenoestrogens have a preference for ERβ over ERα such as genistein (Kuiper et al. 1998).

When E2 binds in the ER binding site (Fig. 1.8; B), hydrogen bonding occurs between the phenolic hydroxyl and amino acid residues Glu 353 and Arg 394 and a free water molecule. Hydrogen bonding also occurs between the aliphatic hydroxyl and His 524, while the central part of E2 binds through hydrophobic interactions. The estrogen receptor binding site is non-specific and flexible. Molecules that are the right size and have hydroxyl groups positioned in approximately the right location can bind, even if they only share a few structural similarities with E2 (Elsby et al. 2000).

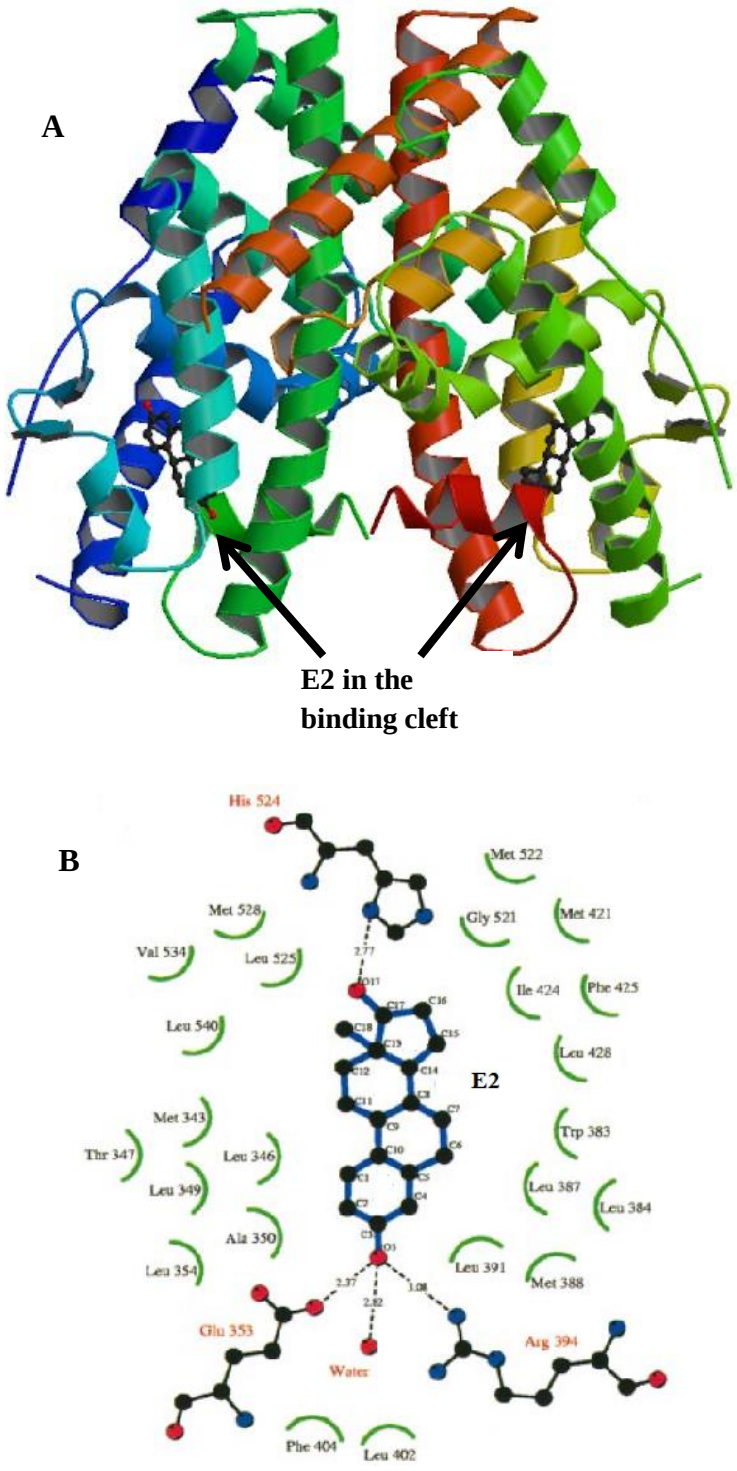

Figure 1.8: (A) Human estrogen receptor ligand-binding domain in complex with E2. (B) Human estrogen receptor ligand-binding site with E2 bound, showing key amino acid residue interactions.
PDB ID: 1ERE
Brzozowski, A.M.,Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389: 753 – 758.

1.4 Xenoestrogens

Xenoestrogens are chemicals that structurally mimic E2 and possess estrogenic activity (Heindel et al. 2012). They can be natural or man-made chemical compounds. Phytoestrogens are natural xenoestrogens and are synthesised by plants (e.g. soy produces isoflavones such as genistein (Fig. 1.9; A)). Man-made xenoestrogens are molecules made by man that end up in the environment such as nonylphenol (a surfactant; Fig. 1.9; B) (Soto et al. 1991) and bisphenol-A (a plastics monomer; Fig. 1.9; C) (Le et al. 2008). Many xenoestrogens share structural relationships with E2 and are ER agonists; they bind to the ER with a range of binding affinities and trigger a response (Kuiper et al. 1998). Phytoestrogens were first known to have a biological effect in the 1940's when sheep grazing on red clover containing formononetin (an isoflavone; Fig. 1.9; D) became temporarily infertile (Bennetts et al. 1946). Since then there have been many cases of xenoestrogens having adverse effects on wildlife. In the 1980's, it was found that male alligators living in Lake Apopka in Florida following a DDT pesticide spill, had shorter phallus's than alligators living in nearby Lake Woodruff (Guillette et al. 2000). Also in 1995 it was found that male rainbow trout living in rivers fed by sewage effluent containing estrogenic compounds were producing vitellogenin, a protein expressed in the ovaries of egg laying vertebrates (Sumpter & Jobling 1995). Studies suggest that xenoestrogens have adverse effects on human health, such as the declining human sperm count (Sharpe & Skakkebaek 1993) and the increase various cancers such as breast and testicular (Soto et al. 1997).

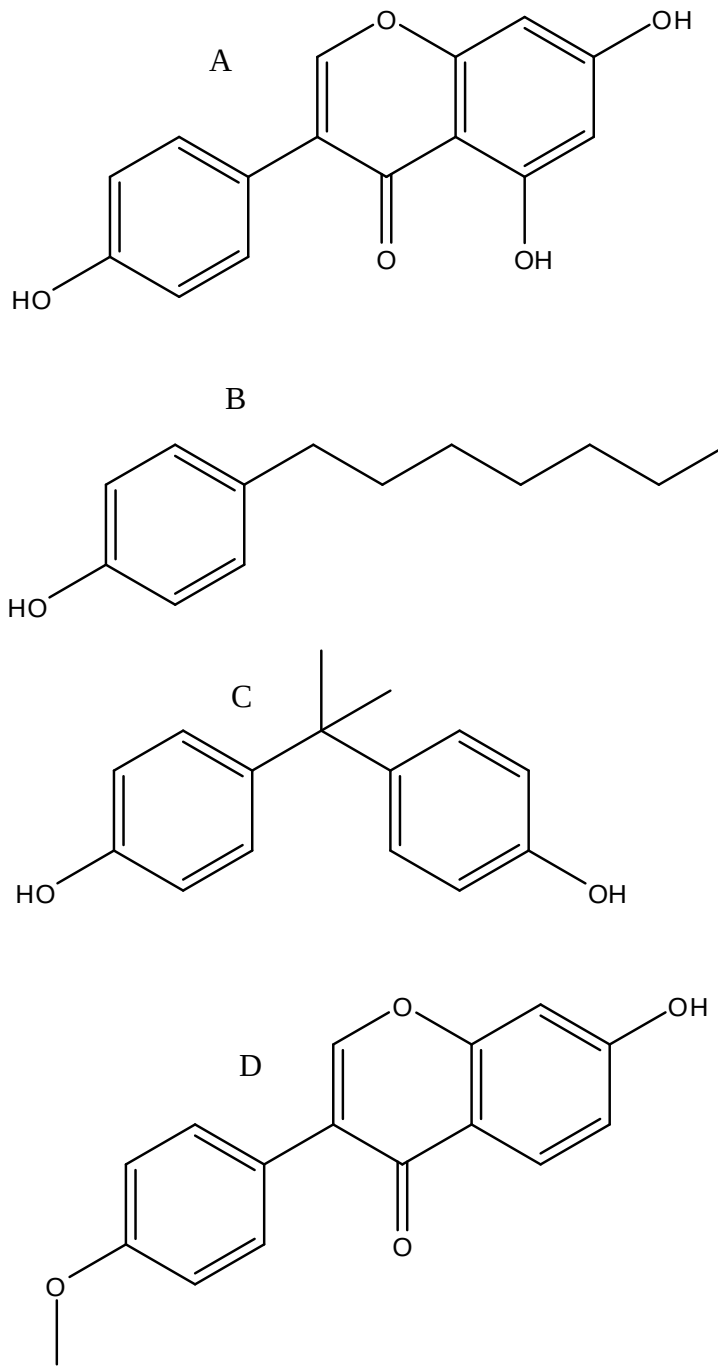

Figure 1.9: Genistein (A), nonylphenol (B), bisphenol-A (C) and formononetin (D)

1.5 Experimentally testing xenoestrogens

There are many different ways to test the potency of xenoestrogens, both *in vivo* and *in vitro,* each having its limitations. Many *in vitro* screening methods involve using cell lines which can be used in many different ways.MCF-7 cells have been used extensively since multiple E2-responsive end points can be determined, including cell proliferation and gene expression (Safe et al. 1998). Yeast is also used in a recombinant yeast cell estrogen screening assay, where yeast cells are transformed with plasmids encoding the human estrogen receptor and an estrogen responsive promoter linked to a reporter gene (Coldham et al. 1997). Another *in vivo* assay used is estrogen receptor competitive-binding assay using [H^3]-E2, which assesses a compounds ability to bind to the ER (Blair et al. 2000). *In vivo* testing methods are more time-consuming and expensive compared to *in vitro* methods, but provide more insight than *in vitro* methods, involving processes such as pharmacokinetics, metabolism and interactions with transport proteins that affect uptake into the cells such as SHBG (Safe et al. 1998). Most xenoestrogens have been screened using multiple methods to gain as much insight as possible into their mechanism of action and the effects they produce in the body.

1.6 MCF-7 cell line

MCF7 breast cancer cell line was first cultured in 1970 by the Michigan cancer foundation. It was derived from a pleural effusion from a 69 year old woman who had breast cancer (Soule et al. 1973; Levenson & Jordan 1997). It is widely used for research as it was the first ER-positive and estrogen-responsive breast cancer cell line to be documented (Sutherland et al. 1983).

MCF-7 is a cell line used in many studies due to its characteristics and being easy to culture. The MCF-7 cell line is adherent and grows in clumps. The cells adhere firmly to the culture vessel and to each other (Fig. 1.10).

MCF-7 cells are ERα positive and contain both membrane bound and intracellular receptors (Zivadinovic et al. 2005; White et al. 1994). One characteristic of the MCF-7 cell line is that it is ERα positive and grows rapidly when exposed to E2, making it a useful cell line for screening the effects of xenoestrogens (Brooks et al. 1973).

One concern noted in the1980's is the subtle differences in cultures from different laboratories. This could be a concern as MCF-7 cells are used all over the world. Given the passage number for each culture is likely to be different in each laboratory; this brings into question how much variation this could introduce into research results generated using this cell line (Villalobos et al. 1995; Lacroix & Leclercq 2004).

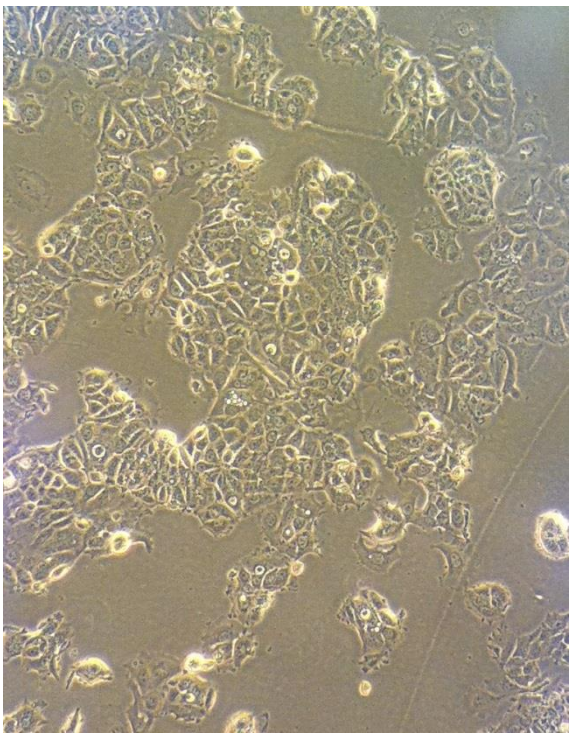

Figure 1.10: Confluent MCF-7 cell culture magnified 100x.

1.7 Parabens

1.7.1 What are parabens?

'Parabens' is a contraction of parahydroxybenzoates. They are esters of parahydroxybenzoic acid (Fig. 1.11). The ester group ranges from being a single chain hydrocarbon, to a branched chain or a benzyl group. They are hydrophobic and are sparingly soluble in water (Giordano et al. 1999; Itoe et al. 2005; Elder 1984).

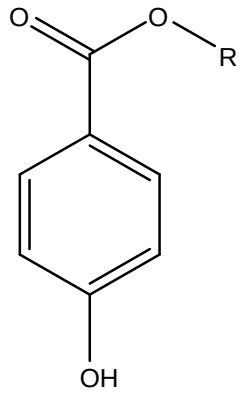

Figure 1.11: General structure of a p-hydroxybenzoic ester (paraben).

1.7.2 Uses of parabens

Parabens are preservatives that have been used in the pharmaceuticals and cosmetics industry for over 80 years to prevent microbial growth and extend the shelf life of formulae (Itoe et al. 2005). They have been used because they are cheap, have low toxicity and kill a variety of fungi and bacteria including both Gram positive and Gram negative (Charnock & Finsrud 2007). They have been used as preservatives since the 1920's when they were discovered as an alternative to p-hydroxybenzoic acid. Benzoic acid is only effective under acidic conditions whereas the ester form is effective up to pH 8.5 (Itoe et al. 2005). The parabens commonly used are the single straight chain derivatives, including methyl, ethyl, propyl and butyl (Fig 1.12). Occasionally benzyl and branched chain esters are used. The most

commonly used paraben is methylparaben, because it has the lowest toxicity of the parabens (LD50 [methylparaben, mouse, oral] = 8 g/kg) (Bingham et al. 2000). However, there is a correlation between the length of the side chain and how effective it is as an antimicrobial agent; the shortest chain (methyl) is the least effective, so requires a higher concentration (Charnock & Finsrud 2007). Studies show that parabens used in select combinations have greater antimicrobial activity compared to a single paraben at the same concentration (Charnock & Finsrud 2007). These combinations are selected to keep the paraben concentrations low, yet still maintaining sufficient antimicrobial activity.

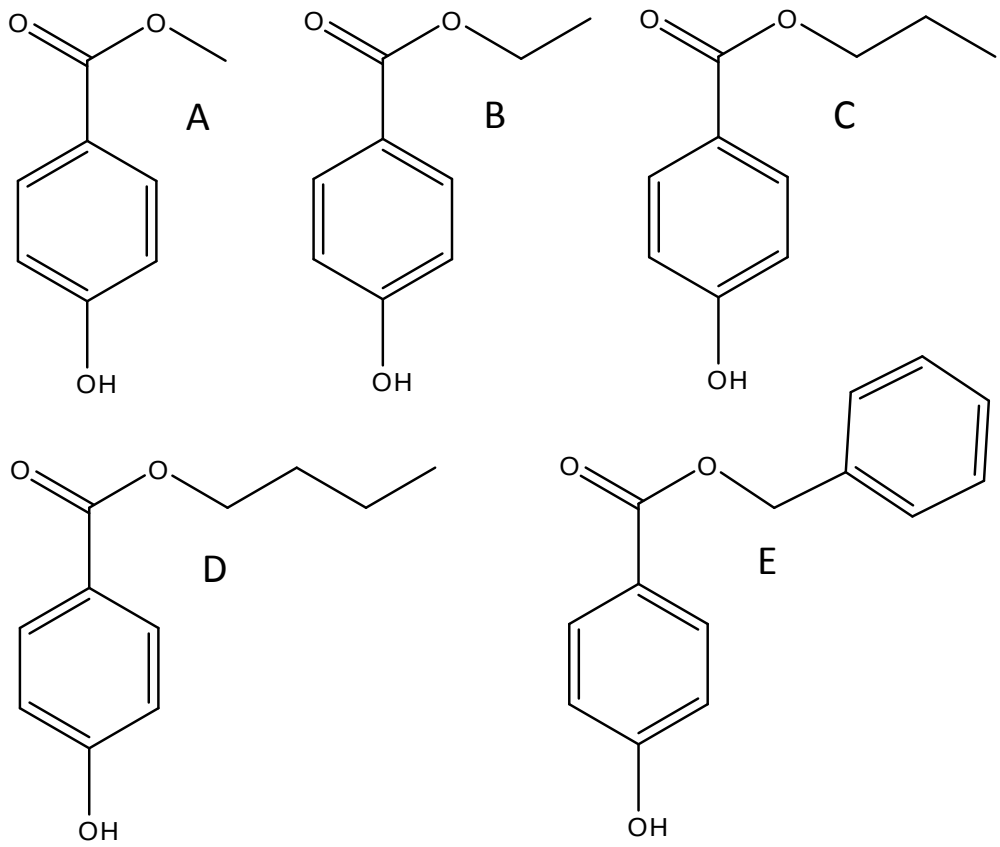

Figure 1.12: Methylparaben (A), ethylparaben (B), propylparaben (C), butylparaben (D) and benzylparaben (E).

1.7.3 Human exposure to parabens – are they safe?

The toxicity of parabens has been studied extensively and they have been described as safe for use (Elder 1984; Itoe et al. 2005). Currently the European Union cosmetic regulations permit parabens to make up 0.8% (w/w) of a cosmetic formula. It is estimated in the USA, that people are exposed to approximately 76 mg of parabens per day. This comes mostly from cosmetics, but also pharmaceutical preparations and food (Itoe et al. 2005).

Parabens have been brought into question recently because of their estrogenicity; it has been reported that parabens have low estrogenic activity (Routledge et al. 1998; Byford et al. 2002; Darbre 2008). Given the frequency of exposure to these compounds, it has been debated if we should allow the use of these compounds in our skin formulae, food and pharmaceutical preparations (Soni et al. 2005). Another trend with increasing chain length is increasing estrogenicity. The reasons for this are thought to be because of access into the cell and how well the paraben binds to the estrogen receptor binding site (Routledge et al. 1998). Parabens with longer or bigger carbon chains such as butylparaben and benzylparaben will be more efficient at penetrating the cell membrane to gain access, as suggested by their larger $LogP_{ow}$ values (Pedersen et al. 2007).

Topically applied parabens are hydrolysed by skin esterases to p-hydroxybenzoic acid (Darbre 2008; Pedersen et al. 2007; Routledge et al. 1998). Prior to being excreted, p-hydroxybenzoic acid is conjugated, typically with glycine and then excreted in urine or bile. Parabens that enter the body orally are hydrolysed by esterases in the small intestine or liver (Boberg et al. 2009). Studies show that parabens are not necessarily broken down immediately upon penetrating the skin (Darbre 2008), as detectable concentrations of parabens have been found in human breast tumours, showing some accumulation can occur (Barr et al. 2012; Darbre et al. 2004; Charles & Darbre 2013; Harvey & Everett 2012).

1.8 Xenoestrogen combinations

Every day we are simultaneously exposed to different xenoestrogens from a wide variety of sources, at different doses, producing an internal cocktail of estrogen mimicking compounds. For example, naturally occurring plant phytoestrogens might be eaten, bisphenol-A could be taken in through use of plastics with food and parabens could be absorbed through cosmetic and personal care product use. Many xenoestrogens have low potency, suggesting that their effects are negligible and therefore aren't concerning (Rajapakse et al. 2002). If the effect seen when multiple agents combined together is the same as predicted, based on the effects of the single agents, the combination effect is additive. If the effect is more effective than predicted, the combination effect is synergistic and if the effect is less effective than predicted, the combination effect is antagonistic (Kortenkamp & Altenburger 1999).

Studies on combinations of xenoestrogens started being published in the mid 1990's. In 1996, a study was published claiming synergistic effects between pairs of estrogenic pesticides, which was later retracted because the experimental results were unable to be reproduced by other laboratories (Ramamoorthy 1997; Arnold et al. 1996). This encouraged a lot of interest on the effects of combining xenoestrogens together, which lead to more studies around this concept. The need to know the effect produced when xenoestrogens are present together in combinations is growing, as new estrogenic compounds are being discovered all the time. There are nearly 800 chemicals that are known or suspected of interfering with hormone activity – many of these are xenoestrogens (Heindel et al. 2012).

Many common xenoestrogens have been characterised and their estrogenic effectiveness has studied using many techniques, both *in vivo* and *in vitro* (Shelby et al. 1996; Baker 2001; Safe et al. 1998). Assessing the effects of combinations of xenoestrogens has been shown to be challenging, as the results can be hard to interpret (Kortenkamp 2007). One study

combined DDT, genistein, 4-nonylphenol and 4-n-octylphenol together and showed the combined effect was additive compared to the effect produced from each of the xenoestrogens separately (Payne et al. 2000). Other studies have shown that when xenoestrogens are combined together at individual concentrations lower than the no-observable-effect concentration, additive combination effects could be seen. This suggests that the negligible effects of individual xenoestrogens become important when enough are present at once (Rajapakse et al. 2002).

1.9 Aims and objectives

The aim of this work is to investigate the effect of combinations of methylparaben and butylparaben on cultured ERα positive MCF-7 breast cancer cells to determine if their mechanisms of action are additive or synergistic. This will be studied by:

- Setting up a MCF-7 cell culture system and studying its growth properties.
- Developing a method to study effects of paraben exposure on MCF-7 cell proliferation.
- Exposing MCF-7 cells to combinations of methylparaben and butylparaben and measuring cell proliferation.
- Applying statistical methods to determine the significance of multiple compound exposure regimes.

2 Materials and methods

2.1 Chemicals

All chemicals used were standard laboratory grade except:

- β-Estradiol (BioReagent, suitable for cell culture, Sigma-Aldrich New Zealand Ltd., Auckland, New Zealand)
- Butyl 4-hydroxybenzoate (≥99.0% pure, Sigma-Aldrich New Zealand Ltd.)
- Methyl 4-hydroxybenzoate (ReagentPlus®, Sigma-Aldrich New Zealand Ltd.)
- Streptomycin sulphate salt (BioReagent, Sigma-Aldrich New Zealand Ltd.)
- Penicillin G sodium salt (BioReagent, Sigma-Aldrich New Zealand Ltd.)
- TrypLE™ Express (1X) (without phenol red; Invitrogen, Life Technologies New Zealand Ltd., Auckland, New Zealand)
- Dulbecco's Phosphate Buffered Saline (DPBS; Sigma-Aldrich New Zealand Ltd.)
- Minimum Essential Medium (Phenol red free; PRFMEM; Sigma-Aldrich New Zealand Ltd.)
- Minimum essential Medium (500 mL; Gibco, Life Technologies New Zealand Ltd.) - stored at +4°C
- Ethanol (AnalaR, Merck, Auckland, New Zealand)
- Fetal Bovine Serum (FBS; Gibco, Life Technologies New Zealand Limited)
- Charcoal, dextran coated (Sigma-Aldrich New Zealand Ltd.)
- Trypan Blue (BioReagent, Sigma-Aldrich New Zealand Ltd.)

2.2 Sterilisation

2.2.1 Autoclaving

All glassware, metal instruments and Eppendorf micropipette tips were autoclaved at 121°C, 15 psi for 20 min.

2.2.2 Filtration

Culture media and DPBS were filter sterilised using Steritop™ Filter Units (Millipore 0.22 μm, Merck Millipore Merck Ltd., Auckland, New Zealand). Antibiotic solution was filter sterilised using a syringe filter (0.2 μm Minisart®, Sartorius Stedim, supplied by Micronz Ltd., Martinborough, New Zealand) and a 5 mL sterile syringe.

2.2.3 70% ethanol (v/v) aerosol

Sterilisation of laboratory work surfaces and the inside of laminar flow cabinet was carried out using 70% ethanol (v/v) spray. Bottles containing sterile media, autoclaved canisters containing pipettes and autoclaved boxes containing micropipette tips were sprayed with 70% ethanol (v/v) before being transferred to the laminar flow cabinet.

2.2.4 Cell procedures

Procedures involving MCF-7 cell cultures were carried out in a laminar flow cabinet (Cytogard CG2000 Series, Clyde-Apac, Minto, New South Wales, Australia). This includes manipulations involving sterile solutions, e.g. culture media, DPBS, antibiotics and FBS.

2.3 Preparation of antibiotic solution

Streptomycin sulphate (2.8 g) and benzylpenicillin sodium salt (3 g) was made up to 100 mL in MilliQ water (18.2 MΩ/cm, Q-Guard 1 purification pack, Merck Millipore Merck Ltd.,

New Zealand). The mixture was stirred with a magnetic stirrer overnight to form a solution. The solution was divided into 5 mL aliquots using a 5 mL sterile plastic syringe and a 0.2 μm Minisart® filter into sterile glass vials (25 mL). The vials were stored at +4°C. One vial of antibiotic solution (5 mL) was sufficient for 500 mL of medium.

2.4 Inactivation of fetal bovine serum

FBS was heat inactivated to destroy complement proteins; using the method of Davis and Hsueh (Davis & Hsueh 2002). A bottle of FBS (500 mL) was removed from the -18°C freezer and thawed at +4°C overnight. It was transferred to a 37°C water bath with the water level above the FBS level in the bottle to complete the thawing process. The contents were mixed by inversion every 10 min. The thawed FBS was incubated in a 37°C water bath for an additional 15 min until the FBS's temperature was 37°C. The temperature of the bath containing the FBS was raised to 56°C; this required approximately 35 min. The FBS was inverted every 10 min to ensure the FBS was heated evenly. Once the water temperature of the bath was 56°C, the FBS was incubated for 30 min and inverted every 10 min. The FBS was removed from the water bath and allowed to cool for 30 min to room temperature. It was aliquotted into five 100 mL Schott bottles and stored at -18°C.

2.5 Charcoal stripping of fetal bovine serum

Dextran-coated charcoal (127±2 mg) was weighed into a sterile 50 mL conical centrifuge tube (LabServ®, supplied by Thermo Fisher Scientific New Zealand Ltd., Auckland, New Zealand). HEPES (50 mL, 10 mM) containing 0.25 M sucrose and 1.5 mM $MgCl_2$ was added; the tube was incubated overnight at +4°C. Following incubation, the mixture was centrifuged (10 min, 500 x g) to allow the charcoal to form a pellet. The supernatent was removed by aspiration and replaced with 50 mL inactivated FBS. The tube was inverted 20

times to mix the charcoal and FBS thoroughly before being incubated at 4°C for 24 hours. The mixture was centrifuged (10 min, 1700 x g) to allow the charcoal to form a pellet. The FBS was aliquotted into a Schott bottle and stored at -18°C.

2.6 Preparation of culture medium

MEM 500 mL bottles were used for maintaining stock cell cultures. Inactivated FBS (55 mL) and antibiotic solution (5 mL) was added and the solution was stored at +4°C.

2.7 Preparation of Minimum Essential Medium (phenol red free)

Approximately 900 mL of MilliQ water was added to a sterile 1L Schott bottle. Powdered medium (PRFMEM) was added and the bottle shaken to dissolve the powder. A small amount of MilliQ water was used to dissolve any remaining powder in the package and this was added to the Schott bottle. Sodium bicarbonate (2.2 g) was added and dissolved by shaking gently. The pH was adjusted to pH 7.1-7.3 using 1 M HCl. The resulting solution was made up to 1 L with MilliQ water. Antibiotic solution (10 mL) and stripped FBS (110 mL) were added to the solution. The final solution was sterilised by filtration using a Steritop™ Filter Unit (0.22 μm). The solution was aseptically aliquotted into 250 mL sterile Schott bottles (approximately 200 mL per Schott bottle) and stored at +4°C.

2.8 Preparation of Dulbecco's Phosphate Buffered Saline

Approximately 900 mL of MilliQ water was added to a 1 L Schott bottle. Powdered DPBS was added and the bottle shaken to dissolve the powder. A small amount of MilliQ water was used to dissolve any remaining powder in the package and added to the Schott bottle. The solution was made up to 1 L with MilliQ water. The final solution was sterilised by filtration

using a Steritop™ Filter Unit (0.22 μm). The solution was aseptically aliquoted into 100 mL sterile Schott bottles (approximately 80 mL per Schott bottle) and stored at +4°C.

2.9 Cell Culture

2.9.1 MCF-7 Cells

MCF-7 cells were kindly provided by Dr John Lewis, Canterbury District Health Board Laboratories, Christchurch.

2.9.2 Maintaining MCF-7 cell cultures

Growing MCF-7 cells were maintained in MEM medium in culture flasks (25 cm^2; Corning®, Sigma-Aldrich New Zealand Ltd.) at 37°C, 5% CO_2 atmosphere.

2.9.3 Cryopreservation of MCF-7 cells

Confluent MCF-7 cells in 5 culture flasks were treated with TrypLE™ Express (1X), no phenol red (TrypLE; see section 2.11.4). The cells were re-suspended in MEM (600 μL) supplemented with 5% dimethyl sulfoxide (DMSO) as recommended by American Type Culture Collection (ATCC). MEM/DMSO (100 μL) was added to 6 cryovials (1 mL Nunc®, CryoTube®, Thermo Fisher Scientific New Zealand Ltd., Auckland, New Zealand). Each cryogenic vial was labelled then wrapped in cotton wool and placed in a small polystyrene box (13 cm x 9 cm x 5 cm). The box containing the vials was placed in a -80°C freezer overnight and then transferred to the liquid N_2 vapour phase in a Dewar flask (50 L, Air Liquide New Zealand Ltd., Auckland, New Zealand) for approximately 6 hours before being fully submerged in the liquid N_2. After each freezing step, the contents of 1 vial were thawed and transferred into a sterile flask containing warm MEM (10 mL) to check viability by seeing if the cells attached to the bottom of the culture flask within 24 hrs.

2.9.4 Passaging MCF-7 cell cultures

The medium was removed from within a culture flask containing confluent cells by aspiration using a sterile Pasteur pipette. Cold DPBS (3 mL) was added to the flask and then aspirated to remove any residue of MEM. TrypLE (1 mL) was added to the flask, the flask was incubated at 37°C, 5% CO_2 atmosphere for 30 min to allow the cells to detach. MEM (10 mL) was added to deactivate the TrypLE. The contents of the flask were transferred to a 50mL sterile centrifuge tube using a sterile glass pipette (10 mL). The tube was centrifuged for 5 min at 4000 x g to allow the cells to form a pellet. The MEM-containing TrypLE was removed by aspiration using a sterile Pasteur pipette and the cells were re-suspended in MEM (10 mL). Cell suspension (2 mL) was added to each of 5 sterile culture flasks. MEM (8 mL) was added to each flask. The flasks were incubated at 37°C, 5% CO_2 atmosphere until confluent (approximately 5-7 days).

2.9.5 Counting cells

2.9.5.1 Manually counting cells

Cells treated with trypan blue were counted using a cytometer. The cells were prepared for counting as follows; cell suspension (10 μL) was added to 40 μL of trypan blue (0.4% w/v) and thoroughly mixed. The cell suspension/dye mixture (20 μL) was placed on a cytometer and covered with a coverslip. The cytometer was checked for an even coverage of cells and the cells within 8 x 16 square grids were counted at 100x magnification using an inverted microscope (CKX41, Olympus, Christchurch, New Zealand).

Cell concentration = (5/8 x T) x 10^4 cells/mL.

Where T = the number of cells counted in 8 x 16 square grids.

The factor 5/8 corrects for dilution of the cell suspension with trypan blue and gives the average number of cells per grid.

The factor 10^4 accounts for the fraction of original sample counted (1 x 16 square grid has 0.1 μL volume of cell/dye suspension). The final value has the unit cells/mL.

2.9.5.2 Countess® Automated Cell Counter

Cells treated with trypan blue were counted using Countess® Automated Cell Counter. The cells were prepared for counting as follows; cell suspension (10 μL) was mixed with 10 μL trypan blue (0.4% w/v) twice in 2 small vials. The cell/dye mixture (10 μL) was pipetted from each vial into 2 chambers of a Countess® Automated Cell Counter slide. The slide was inserted in the cell counter and the cells auto-counted.

2.9.6 Seeding[1] a well plate

A flask containing confluent MCF-7 cells was treated with trypsin as described in section 2.11.4. The cell pellet was re-suspended in PRFMEM and mixed thoroughly by being drawn in and out of a micropipette tip (1 mL) to ensure the cells were evenly suspended with as little clumping as possible. The cell suspension (2 x 10 μL) was removed and counted to determine the cell concentration using a cytometer as outlined in section 2.11.5.1. PRFMEM was added to the suspension to give 10^5 cell/mL. Cell suspension (220 μL) was added to each well of a 24 well cell culture plate (Costar®, 24 well, flat bottom, Sigma-Aldrich New Zealand Ltd.) using a 1 mL micropipette. Cell suspension (2 x 10 μL) was removed from each well for counting to determine the starting cell concentration. The volume of each well was made up to 2 mL with PRFMEM or 1.99 mL of PRFMEM followed by 10 μL of a treatment in ethanol.

[1] Seeding refers to taking a small number of cells, placing them in a new vessel and allowing them to grow.

2.9.7 Method development – determining a method for effective dispersal of MCF-7 cells using TrypLE™ Express

MCF-7 cells were seeded into well cell culture plates (36 wells in 2 x 24 well cell culture plates, 18 wells per plate; see section 2.9.6, except no initial cell concentration was determined). Ethanol containing E2 (10 μL; 2 nM) was added to each well to give a final concentration of 0.01 nM E2. The plates were incubated at 37°C, 5% CO_2 atmosphere for 7 days (confluence was reached). The PRFMEM was removed from 6 wells from each plate by aspiration using a sterile Pasteur pipette. DPBS (1 mL) was added to each well to remove any residual PRFMEM and immediately aspirated using a sterile Pasteur pipette. TrypLE (150 μL) was added to 3 wells in each plate. TrypLE (0.5 mL) was added to the remaining 3 wells in each plate and the plates were incubated at 37°C, 5% CO_2 atmosphere for 5 minutes to allow the cells to detach. The first plate was left undisturbed and the second plate was disturbed by being gently rocked. The wells were checked for dispersal using an inverted microscope (100x magnification). PRFMEM (350 μL for wells containing TrypLE (150 μL) and 0.5 mL for wells containing TrypLE (0.5 mL)) was added to each well to deactivate the TrypLE. The TrypLE/PRFMEM containing cell suspension was mixed well to break up cell clumps by drawing the mixture in and out of a 1 mL micropipette 10 times. The mixture was then transferred to a 1.7 mL Eppendorf tube. This dispersal process using TrypLE was repeated twice, the first allowing 10 min for cell dispersal, the second allowing 30 min for cell dispersal. For the cells exposed for 10 min, the disturbed plate was gently rocked after 5 min and then 10 min, and for the cells exposed for 30 min, the disturbed plate was gently rocked after 15 min and then 30 min.

2.9.8 Removing cells from plate wells for counting

The PRFMEM was removed from each well by aspiration using a sterile Pasteur pipette. DPBS (1 mL) was added to the well to remove any residual PRFMEM and immediately aspirated using a sterile Pasteur pipette. TrypLE (150 μL) was added to the well, incubated at 37°C, 5% CO_2 atmosphere for 5 min to allow the cells to detach. The well plate was then gently rocked and incubated at 37°C, 5% CO_2 atmosphere for another 5 min to allow the cells to detach. PRFMEM (350 μL) was added to each well to deactivate the TrypLE. The TrypLE/MEM containing cell suspension was mixed well to break up cell clumps by drawing the mixture in and out of a 1 mL micropipette 10 times. The mixture was then transferred to a 1.7 mL Eppendorf tube.

2.9.9 MCF-7 growth curve

2.9.9.1 MCF-7 growth curve with estrogen present (10 pM)

Well cell culture plates were seeded (30 wells in 2 x 24 well cell culture plates; see section 2.9.6). Half the wells were seeded in one step using a culture flask containing confluent MCF-7 cells to minimise re-clumping, then the remaining wells were seeded with a second flask containing confluent MCF-7 cells. Ethanol containing E2 (10 μL; 2 nM) was added to each well to give a final concentration of 0.01 nM. The plates were incubated at 37°C, 5% CO_2 atmosphere for 10 days. Each day, the PRFMEM was removed from 3 wells by aspiration using a sterile Pasteur pipette. Cold DPBS (1 mL) was added to the wells and immediately removed by aspiration using a sterile Pasteur pipette to remove any residual PRFMEM. TrypLE (150 μL) was added to each well and the plate was incubated at 37°C, 5% CO_2 atmosphere for 5min to allow the cells to detach. PRFMEM (350 μL) was added to each well and the contents were mixed to facilitate even distribution of the cells. The cell suspension from each well was transferred to a 1.7 mL Eppendorf tube. The contents of each

tube was mixed again using a 1mL micropipette; the contents were drawn up and ejected 10 times. Cell suspension (2 x 10 μL) from each tube was counted (see section 2.9.5.1).

2.9.9.2 MCF-7 control growth curve

See section 2.9.9.1; exchanging ethanol containing E2 (10 μL; 2 nM) for ethanol (10 μL).

2.9.9.3 MCF-7 growth curve in PRFMEM

Well cell culture plates were seeded (30 wells in 2 x 24 well cell culture plates) using a flask containing confluent MCF-7 cells. The method in section 2.9.6 was followed, except the cell suspension concentration was determined using the automated cell counter (see section 2.9.5.2) and adjusted to 5 x 10^3 cells/mL, a starting cell count was not carried out and each well was made up to 2 mL using PRFMEM. The plates were incubated at 37°C, 5% CO_2 atmosphere for 10 days. Each day, the PRFMEM was removed from 3 wells by aspiration using a sterile Pasteur pipette. Cold DPBS (1 mL) was added to the wells and immediately removed by aspiration using a sterile Pasteur pipette to remove any residual PRFMEM. Trypsin solution (150 μL) was added to each well and the plate was incubated at 37°C, 5% CO_2 atmosphere for 5mins to allow the cells to detach. PRFMEM (350 μL) was added to each well and the contents were mixed to facilitate even distribution of the cells. The cell suspension from each well was transferred to a 1.7 mL Eppendorf tube. The contents of each tube was mixed again using a 1mL micropipette; the contents were drawn up and ejected 10 times. Cell suspension (2 x 10 μL) from each tube was counted using the Countess® Automated Cell Counter (see section 2.9.5.2).

2.9.9.4 MCF-7 growth curve in MEM

See section 2.9.9.3, exchanging PRFMEM for MEM.

2.9.9.5 MCF-7 growth curve with estrogen present (18.3 pM)

See section 2.9.9.3, except each well was only made up to 1.99 mL of PRFMEM and ethanol containing E2 (10 μL; 3.67 nM) was added to each well to give a final concentration of 18.3 pM.

2.9.9.6 MCF-7 growth curve with estrogen present (3.67 pM)

See section 2.9..3, except each well was only made up to 1.99 mL of PRFMEM and ethanol containing E2 (10 μL; 734.3 pM) was added to each well to give a final concentration of 3.67 pM.

2.9.9.7 MCF-7 growth curve with estrogen present (0.367 pM)

See section 2.9.9.3, except each well was only made up to 1.99 mL of PRFMEM and ethanol containing E2 (10 μL; 73.4 pM) was added to each well to give a final concentration of 0.367 pM.

2.9.10 MCF-7 cell exposure experiments

Cell culture well plates were seeded (24 well cell culture plate; see section 2.9.6) with the number of cells per well, chosen prior, in a total volume of 1.99 mL. A sufficient number of wells were seeded to allow 3 wells per treatment. Ethanol (10 μL) containing the compound of interest at a known concentration was added to each well. For each exposure, ethanol (10 μL) was added to 3 control wells and ethanol containing E2 (10 μL; 2nM) was added to 3 wells to give a final concentration of 0.01nM per well. The plates were incubated at 37°C, 5% CO_2 atmosphere for 9 days.

2.9.10.1 Exposing MCF-7 cells to 17β-estradiol

MCF-7 cells were exposed to different concentrations of E2 (see section 2.11.9) except the cells were exposed for 6 days instead of 9 days. The cells were exposed to estrogen at 36.7 pM, 18.3 pM, 9.15 pM, 3.66 pM, 2.745 pM, 1.83 pM, 0.915 pM and 0.367 pM. This exposure also contained 3 wells that didn't contain any treatment. After 6 days the cells were removed from the wells and counted (see section 2.9.8) except using the Countess® Automated Cell Counter (see section 2.9.5.2).

2.9.10.2 Exposing MCF-7 cells to methylparaben and butylparaben

MCF-7 cells were exposed to methylparaben and butylparaben (see section 2.9.10). The cells were exposed to methylparaben at 10 μM and 100 μM and butylparaben at 10 μM and 0.1 μM. After 9 days, the cells were removed from the wells and counted (see section 2.9.8 and 2.9.5.1).

2.9.10.3 Exposing MCF-7 cells to methylparaben and butylparaben combinations

MCF-7 cells were exposed to methylparaben and butylparaben (see section 2.9.10). The cells were exposed to both single parabens and mixtures; methylparaben at 10 μM and 100 μM, butylparaben at 10 μM and 0.1 μM and mixtures of methyl and butylparaben (50 μM, 5 μM and 5 μM, 5 μM). After 9 days, the cells were removed from the wells and counted (see section 2.9.8 and 2.9.5.1).

2.10 Statistical analysis using Analysis of Variance

The paraben exposure data was analysed to determine if there was any significant difference between treatments using analysis of variance (ANOVA). A one-way stacked ANOVA (95% confidence) was carried out using Minitab.

2.11 UV spectroscopy of methylparaben and butylparaben cocktails

Methylparaben in MilliQ water (5 mL; 10 μg/mL) and octanol (5 mL) was pipetted into a 50 mL glass-stoppered separating funnel. The glass stopper was replaced and the contents were inverted 10 times and then left to separate for 10 min. The water fraction (lower; labelled methylparaben water fraction) and octanol fraction (upper; labelled methylparaben octanol fraction) were collected separately in glass vials. The same procedure was repeated for butylparaben in octanol (5 mL; 10 μg/mL) and MilliQ water (5 mL) with the collected fractions labelled butylparaben octanol fraction and butylparaben water fraction. The same procedure was repeated again for butylparaben in octanol (5 mL; 5 μg/mL) and methylparaben in MilliQ water (5 mL; 5 μg/mL) with the collected fractions labelled butylparaben and methylparaben octanol fraction and butylparaben and methylparaben water fraction. Another fraction was made (labelled butylparaben and methylparaben in octanol 1:1) by pipetting butylparaben octanol fraction (0.5 mL) and methylparaben octanol fraction (0.5 mL) into a glass vial using a micropipette. All fractions were analysed by a UV spectrophotometer (Agilent Technologies (5301 Stevens Creek Blvd, Santa Clara CA95051, United States) Cary 100 UV-Vis) between λ190 nm and λ300 nm. Methylparaben in MilliQ water (10 μg/mL), methylparaben in octanol (10 μg/mL), butylparaben in octanol (10 μg/mL), MilliQ water and octanol were also analysed by UV spectroscopy to obtain their curve profiles.

3. Results

3.1 Method development – use of stripped FBS

MCF-7 cells grow rapidly when cultured in MEM (10% FBS) compared to when grown in PRFMEM (10% stripped FBS) (Fig 3.1). Interestingly, cells grown in PRFMEM had a significantly extended lag phase compared to their growth in MEM. This difference in growth rate and growth characteristics is likely due to the growth stimulatory effects of estrogens released from proteins in the FBS and the presence of phenol-red in MEM, known to be a weak xenoestrogen (see section 4.4). Since future studies were to investigate the estrogenic effect of xenoestrogens, PRFMEM containing stripped FBS was used for all experiments to remove the growth stimulating effects of FBS protein-associated estrogenic contaminants. These findings confer with previous findings (Blom et al. 1998; Soto et al. 1995).

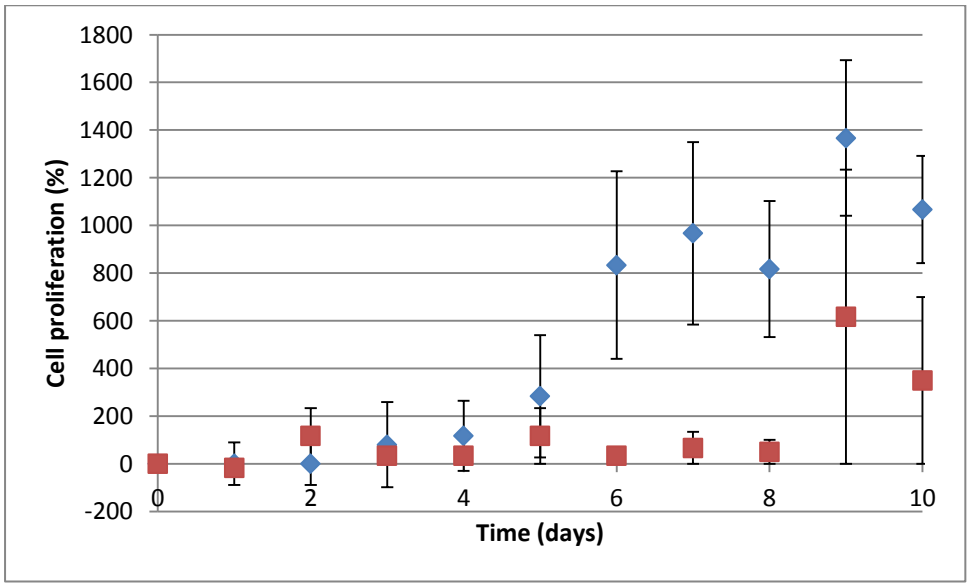

Figure 3.1: Percentage proliferation (± SD) of MCF-7 cells in culture showing the difference between cells cultured in MEM (10% FBS) and cells cultured in PRFMEM (10% stripped FBS). ◆ *= cells cultured in MEM* ■ *= Cells cultured in PRFMEM. Starting cell concentration approximately 5 x* 10^3 *cells/mL.*

3.2 Method development – determining a method for effective dispersal of MCF-7 cells using TrypLE™ Express

MCF-7 cells were exposed for 5 min, 10 min or 30 min to TrypLE (150 μL or 500 μL; Table 1). Cells exposed to TrypLE (150 μL and 500 μL) for 5 min were not detached and still remained adhered to the bottom of the flask. Gently rocking the plate to disturb the cells did not encourage detachment in this short timeframe. Cells exposed to TrypLE (150 μL and 500 μL) for 10 min had begun to detach, though many cells still remained adhered to the bottom of the flask. Gently rocking the plate to disturb the cells helped detachment, but clumping was seen in the wells containing the larger volume of TrypLE. Cells exposed to TrypLE (500 μL) for 30 min were completely detached from the flask, but white clumps were seen floating in the wells. Cells exposed to TrypLE (150 μL) for 30 min were completely detached when the plate was gently rocked to disturb the cells. Minimal cell clumping was observed in these wells. Cells exposed to TrypLE (500 μL) for 30 min were completely detached in wells where the plate was rocked gently to disturb the cells or not disturbed. Cell clumping was observed in wells that were both disturbed and not disturbed by gentle rocking. The clumping was further reduced in almost all wells when the cell suspension was drawn in and out of a 1 mL micropipette, except the wells with severe clumping.

Incubation time	TrypLE™ Express - just covering bottom of well (150 µL)		TrypLE™ Express – excessive (500 µL)	
	Plate disturbed*	**Plate not disturbed***	**Plate disturbed***	**Plate not disturbed***
5 min	Cells not detached	Cells not detached	Cells not detached	Cells not detached
10 min	Cells partially detached	Cells starting to detach	Cells somewhat detached, some clumping seen	Cells somewhat detached, some clumping seen
30 min	Cells detached and mostly dispersed	Cells mostly detached and mostly dispersed	Cells detached, floating clumps seen	Cells detached, floating clumps seen

Table 1: Observation of MCF-7 cells exposed to TrypLE™ Express for different times at different volumes, with and without being disturbed.*
**In this context disturbed means gently rocking the well plate to mix the contents*

3.3 Method development – estrogen exposure and the use of Countess® Automated Cell Counter

MCF-7 cells have a variable growth rate when exposed to different concentrations of E2 (Fig 3.2 and 3.3). The cells were counted using the Countess® Automated Cell Counter after being exposed to each E2 treatment for 6 days. The variation was exceptionally large across all treatments bringing into question the source of variability. An attempt to reduce variability was made by counting cells each day instead of after a set amount of time (see section 4.2.2).

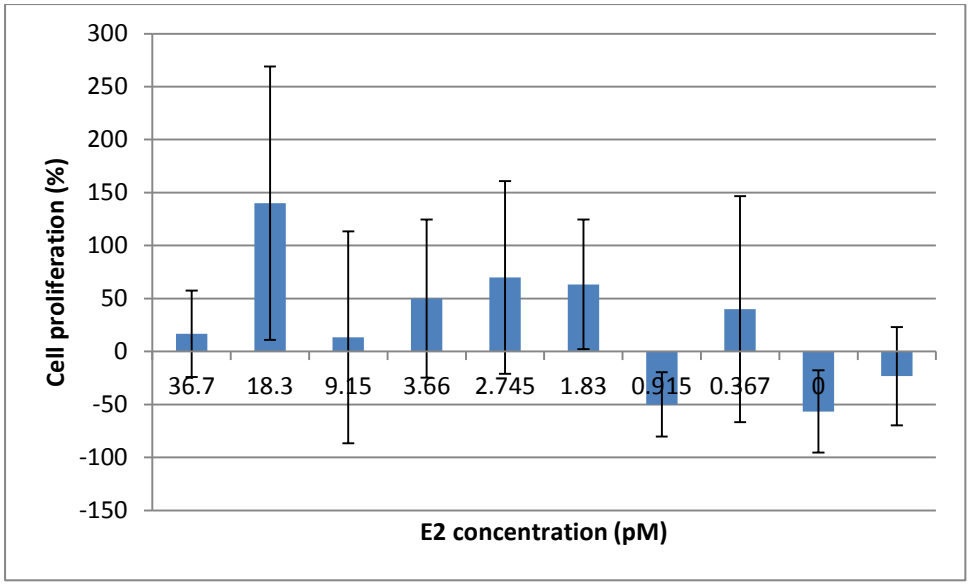

Figure 3.2: Percentage proliferation (± SD) of MCF-7 cells following exposure to E2 after 6 days. Starting cell concentration approximately 5 x 10^3 cells/mL. NB: E2 concentrations were determined by serial dilution of 37 mM E2. The values represent the theoretical concentrations achieved by the dilution sequence. They do not reflect the accuracy of the experiment.

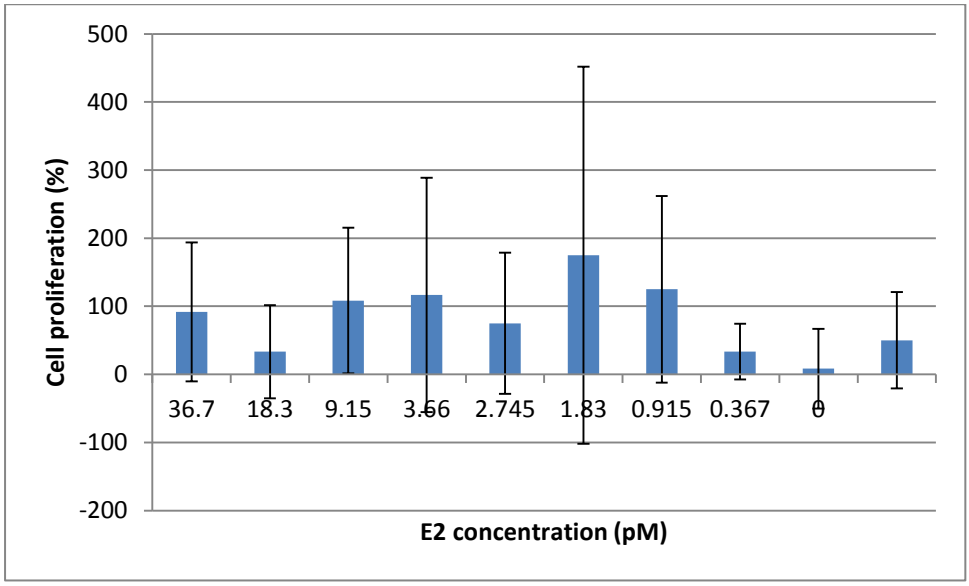

Figure 3.3: Percentage proliferation (± SD) of MCF-7 cells following exposure to E2 after 6 days. Starting cell concentration approximately 5 x 10^3 cells/mL.

3.4 Method development – growth curves in the presence of 17β-estradiol to reduce variability using the Countess® Automated Cell Counter

MCF-7 cells were exposed to 18.3 pM, 3.67 pM and 0.367 pM E2 (Fig. 3.4, 3.5 and 3.6) and counted each day to investigate if the growth rate variability could be improved within triplicate wells compared to cells exposed for 6 days and then counted (Fig. 3.2 and 3.3). Although not statistically different due to the variability within triplicate wells being great, there is an increasing trend in cell proliferation when exposed to 18.3 pM and 0.367 pM. The cell number from each well was determined using Countess® Automated Cell Counter. The initial number of cells per well was approximately 10^4. Due to the variation within triplicate wells which was found to be caused by the Countess® Automated Cell Counter (see section 4.2) manual counting using a cytometer was used for further studies.

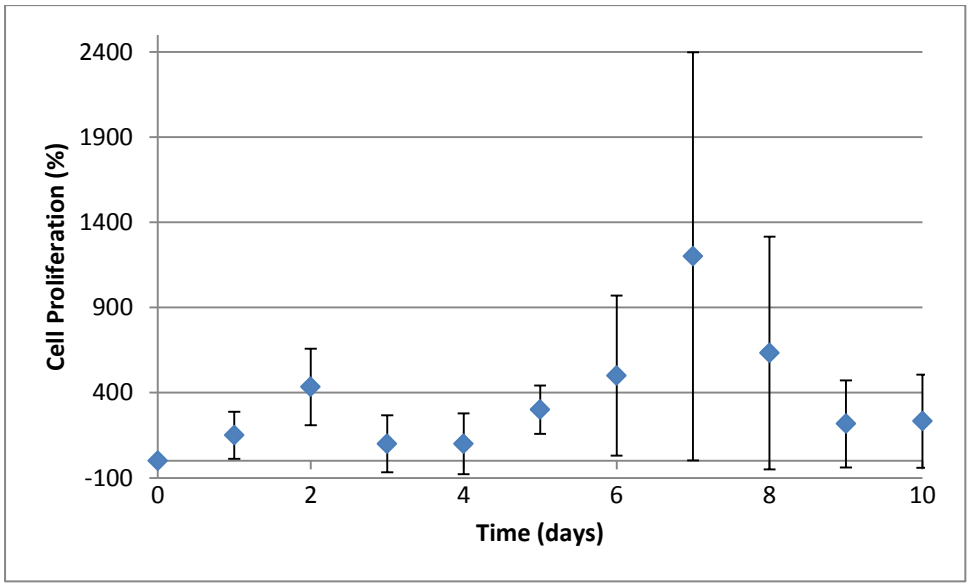

Figure 3.4: Percentage proliferation (± SD) of MCF-7 cells exposed to 18.3 pM E2 over 10 days. Starting cell concentration approximately 5 x 10^3 cells/mL.

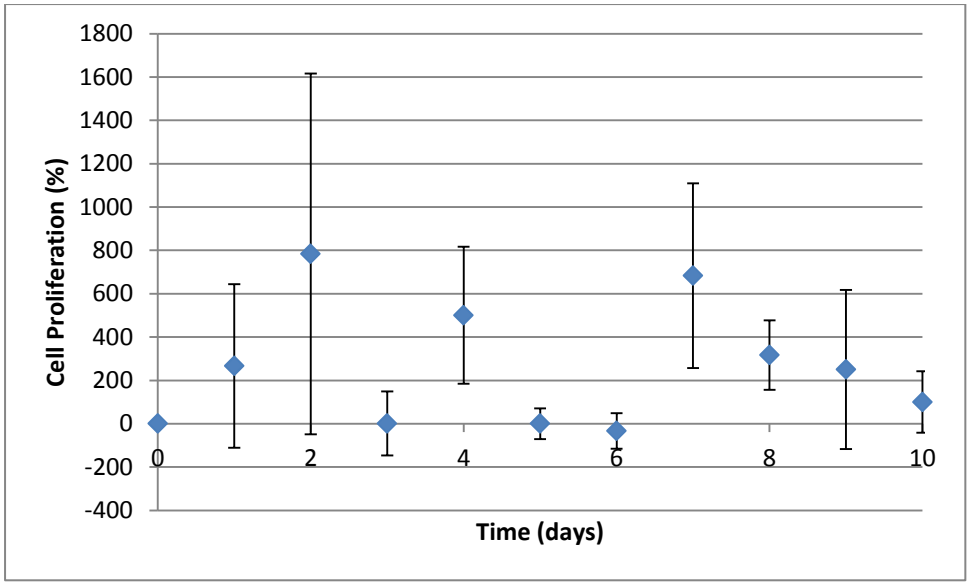

Figure 3.5: Percentage proliferation (± SD) of MCF-7 cells exposed to 3.67 pM E2 over 10 days. Starting cell concentration approximately 5 x 10^3 cells/mL.

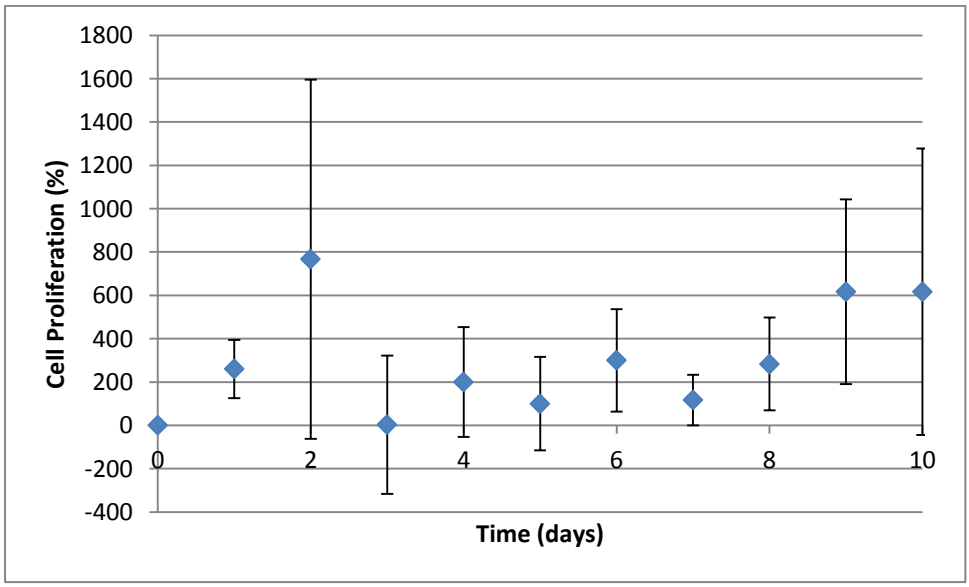

Figure 3.6: Percentage proliferation (± SD) of MCF-7 cells exposed to 0.367 pM E2 over 10 days. Starting cell concentration approximately 5 x 10^3 cells/mL.

3.5 MCF-7 growth curve in the presence of 10 pM 17β-Estradiol – initial cell concentration determined by counting cells

MCF-7 cells grow when exposed to 10 pM E2 (Fig 3.7). The lag phase was quite long, with a large increase in growth at day 9. The initial concentration of cells in each well was determined using a cytometer and used to calculate the proliferation once harvested. MCF-7 cells in the ethanol control were still in the lag phase after 10 days. The variable proliferation of cells exposed to 10 pM E2 within triplicate wells increased during the log phase of growth. Since this experiment shows that counting cells using a cytometer reduces variation compared to counting cells using the Countess® Automated Cell Counter, further experiments looking at effects on cell proliferation can be carried out by exposing cells for a set length of time using this counting method. Based on this result, 9 days was selected as the exposure time for future exposure studies.

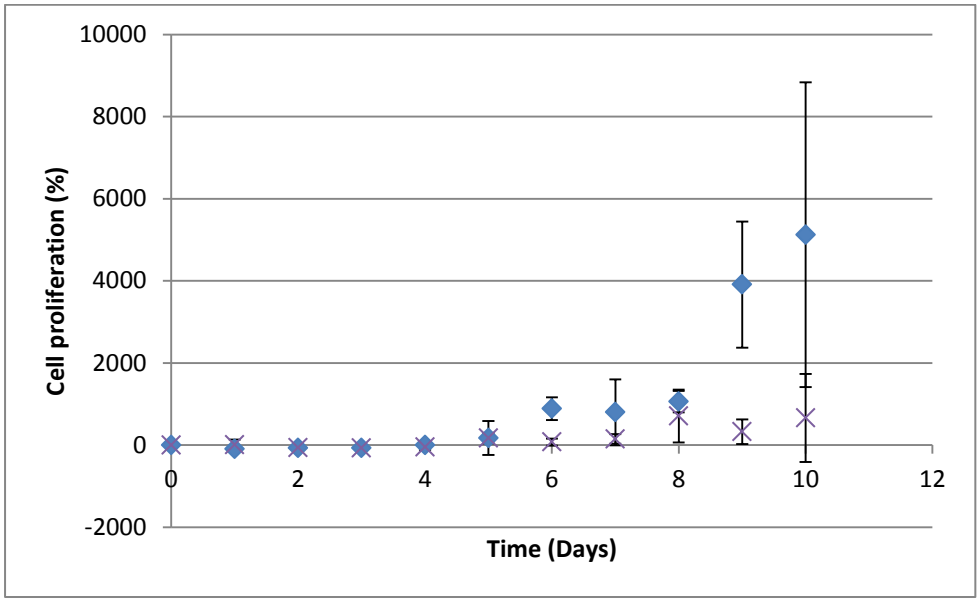

Figure 3.7: Percentage proliferation (± SD) of MCF-7 cells over 10 days. ◆ *= 10 pM E2, X = control (ethanol). Starting cell concentration approximately 1.5 x* 10^4 *cells/mL (determined by counting cells; see section 4.2.4).*

3.6 Paraben exposure experiments – initial cell concentration determined by counting cells

MCF-7 cells grow rapidly when exposed to methylparaben and butylparaben, (Fig 3.8 and 3.9).The cells were exposed to two different concentrations of each paraben which showed dose dependant proliferation (fig 3.8). The cell proliferation was greater when exposed to butylparaben compared to methylparaben (Fig. 3.8 and 3.9). The variability within some treatments was large, despite counting by cytometer. To get around this issue, the starting cell number was altered to investigate if overall proliferation could be increased to achieve the maximum difference between treatments. The starting cell concentration was increased to 1.5 x 10^4 cells/mL and MCF-7 cells were exposed to the same treatments as Fig. 3.8 and Fig. 3.9, but also butylparaben and methylparaben combinations (Fig. 3.10 and Fig. 3.11).

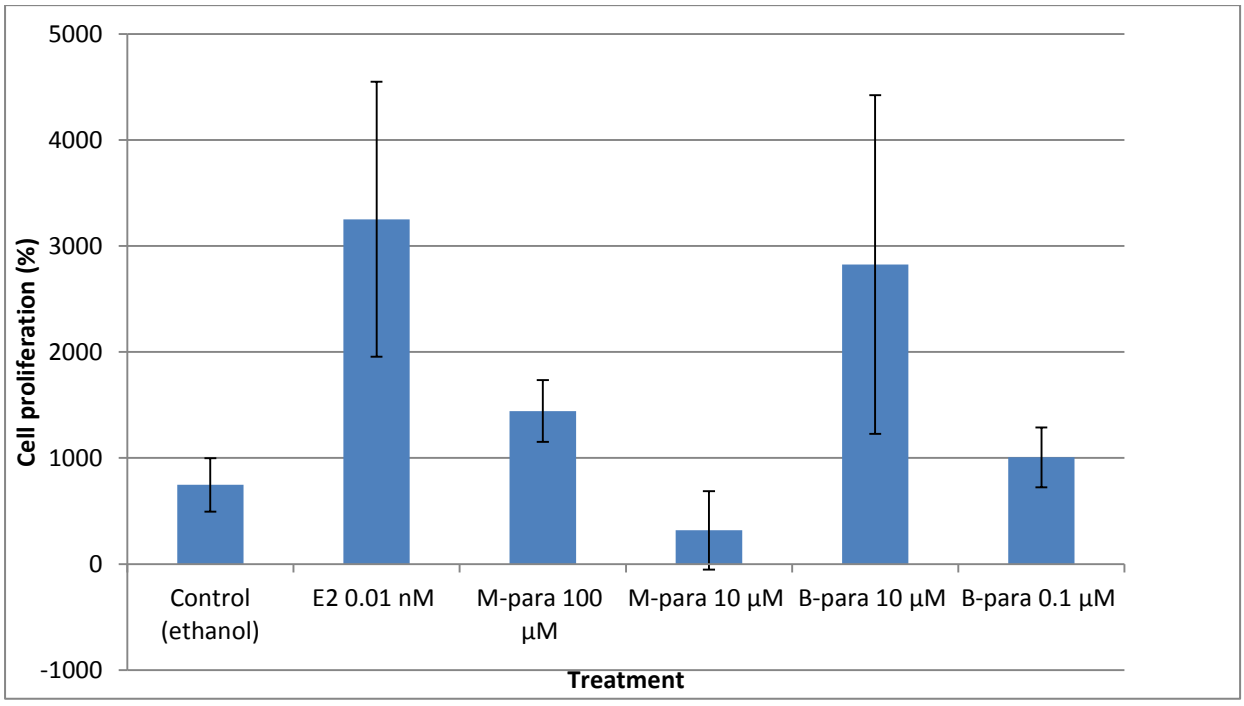

Figure 3.8: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1 x 10^4 cells/mL (determined by counting cells; see section 4.2.4).

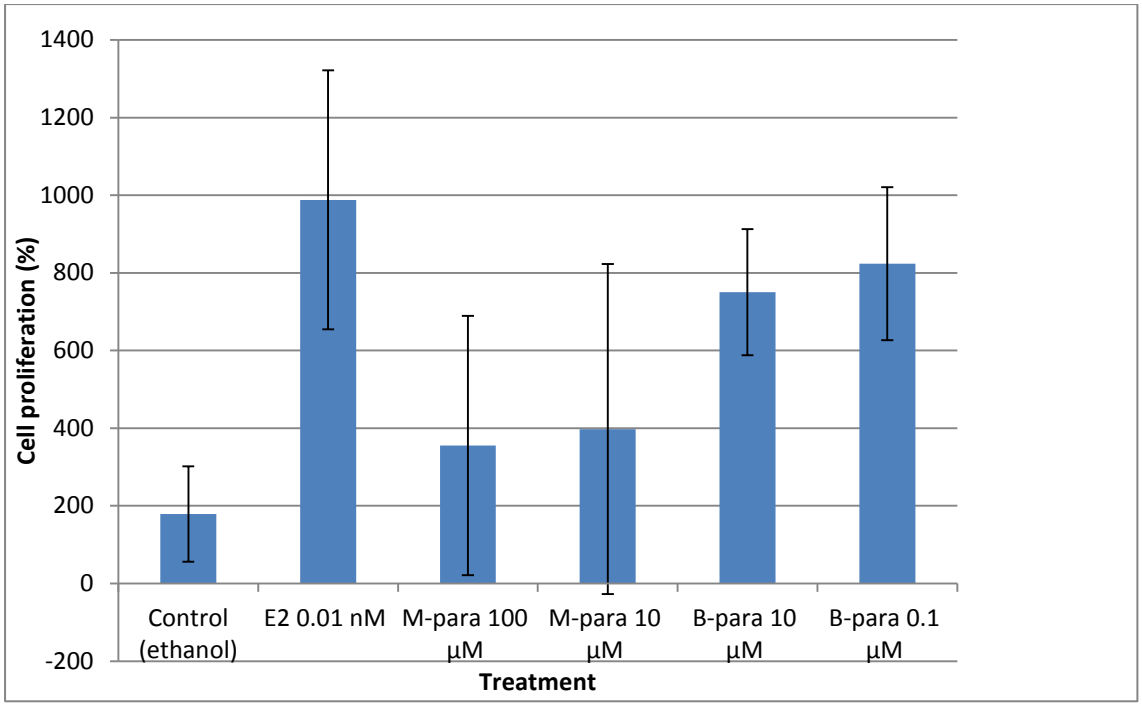

Figure 3.9: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1 x 10^4 cells/mL (determined by counting cells; see section 4.2.4).

3.7 Paraben combination exposure experiments – initial cell concentration determined by counting cells

3.7.1 Paraben combination exposure experiments – initial cell concentration determined by counting cells (approximately 1.5 x 10^4 cells/mL)

When MCF-7 cells were exposed to a combination of butylparaben and methylparaben, they appear to grow the same, if not more than one of the individual paraben components at a higher concentration (Fig 3.10 and Fig. 3.11); however large variation makes this uncertain. The starting cell concentration was altered again and increased to 5 x 10^4 cells/mL to see the effect this would have on variation.

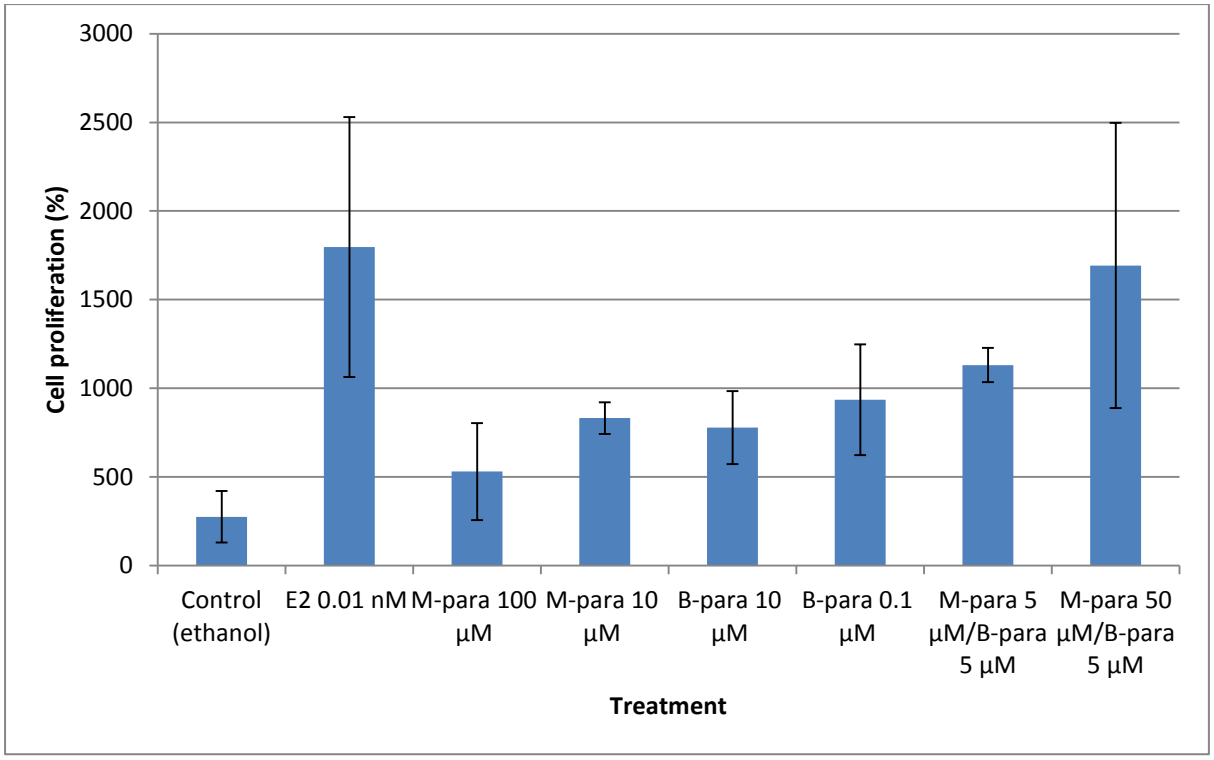

Figure 3.10: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1.5 x 10^4 cells/mL (determined by counting cells; see section 4.2.4).

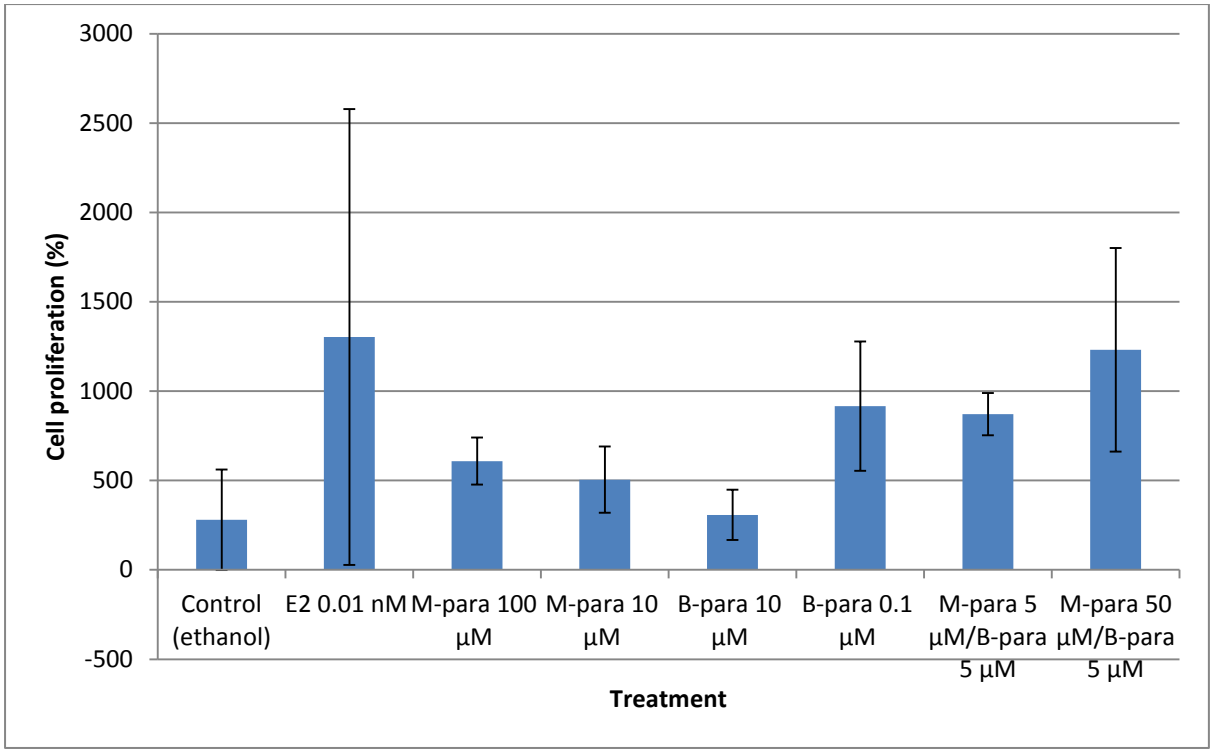

Figure 3.11: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1.5 x 10^4 cells/mL (determined by counting cells; see section 4.2.4).

3.7.2 Paraben combination exposure experiments – initial cell concentration determined by counting cells (approximately 5 x 10^4 cells/mL)

MCF-7 cells grow slowly over 9 days when the starting cell concentration was 5 x 10^4 cells/mL (Fig. 3.12 and 3.13) and they were exposed to the same treatments described previously. This shows that if too many cells are present initially, the cells reach confluence too quickly and the proliferation is low for the 9 day exposure period because there isn't enough space for growth to continue. All of the treatments show a similar proliferation including E2, making this experiment inadequate for looking at treatment effect.

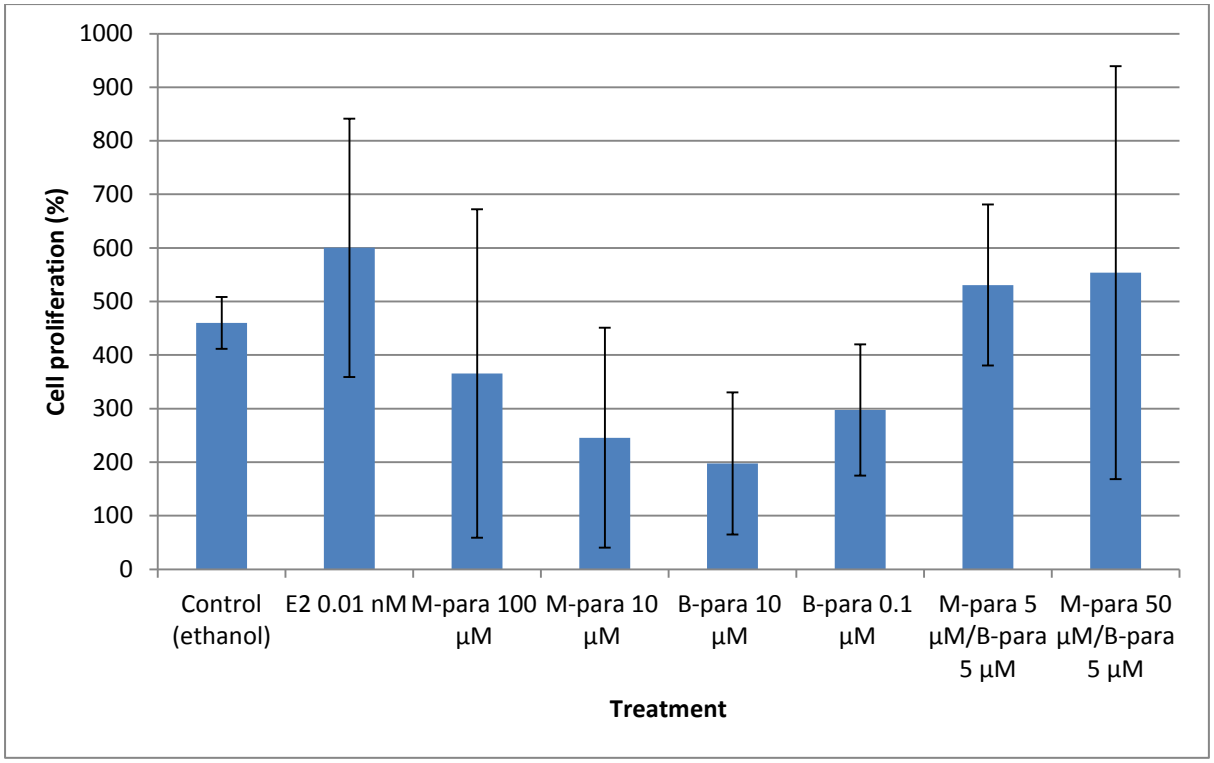

Figure 3.12: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 5 x 10^4 cells/mL (determined by counting cells; see section 4.2.4).

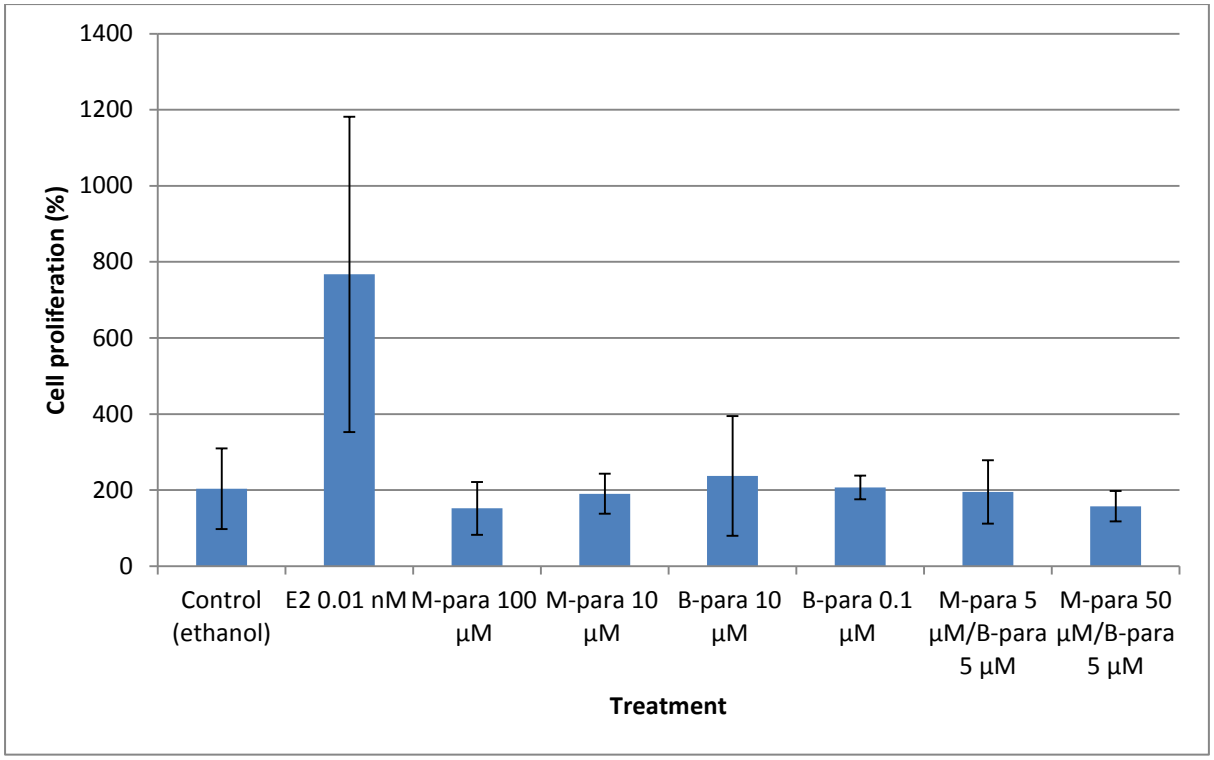

Figure 3.13: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 5 x 10^4 cells/mL (determined by counting cells; see section 4.2.4).

The results of the MCF-7 growth curve in the presence of E2, paraben exposure experiments and paraben combination exposure experiments were examined to identify any ways of minimising the variation within treatments. Using the starting cell concentration determined by dilution instead of by initial count showed overall less variation within treatments (Fig. 3.14 – 3.20) and is thought to be more accurate than initially counting each well (see discussion section 4.2.4).

3.8 MCF-7 growth curve in the presence of 10 pM 17β-Estradiol – initial cell concentration determined by dilution

When the cell proliferation is calculated using the initial cell concentration determined by dilution (Fig. 3.14) instead of by initial cell count (see section 3.4), the overall variation is lower. The shape of the growth curve of MCF-7 cells exposed to 10 pM E2 calculated using the diluted cell concentration is different, showing a steeper log phase that occurs sooner than the curve generated using the initial cell count method. If this curve had been used to determine the length of time to expose the cells in the exposure experiments (see section 3.5 – 3.6), a different number of days might have been selected.

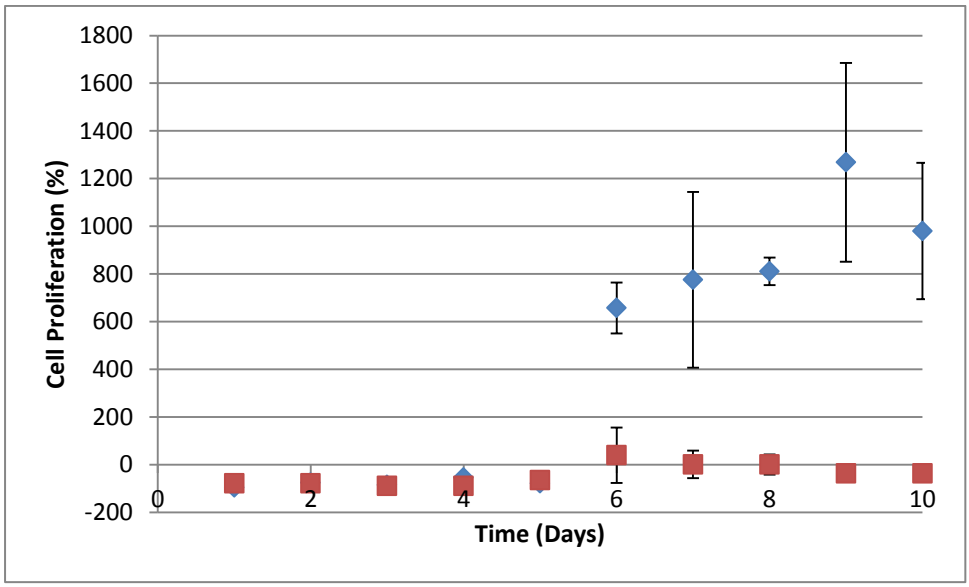

Figure 3.14: Percentage proliferation (± SD) of MCF-7 cells over 10 days. ◆ = 10 pM E2, X = control (ethanol). Starting cell concentration approximately 1.5 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

3.9 Paraben exposure experiments – initial cell concentration determined by dilution

When the cell proliferation is calculated using the initial cell concentration determined by dilution (Fig. 3.15 and 3.16) instead of by initial cell count (see section 3.5), the overall variation is lower. Dose dependence is still shown (Fig. 3.15) and butylparaben still causes greater cell proliferation than methylparaben (Fig. 3.15 and 3.16).

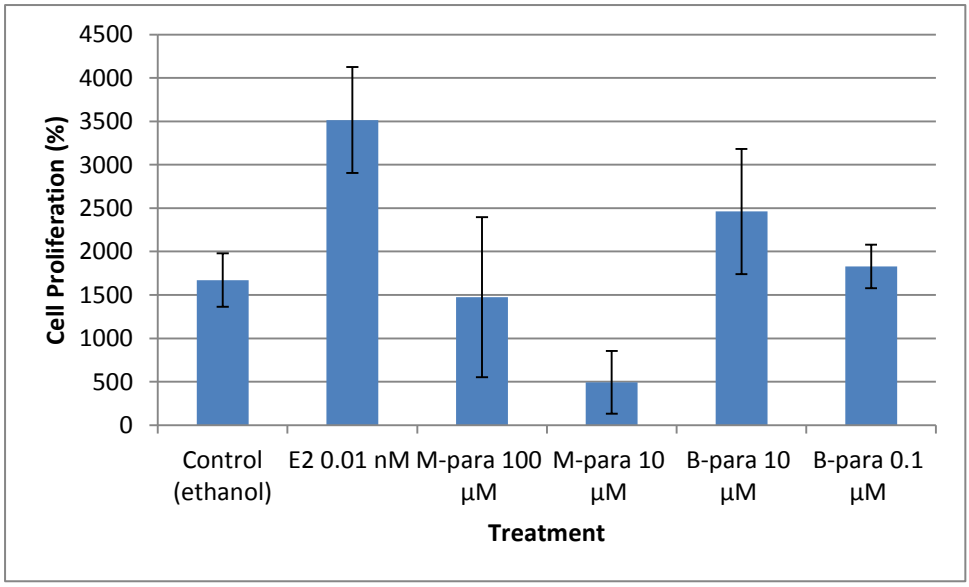

Figure 3.15: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

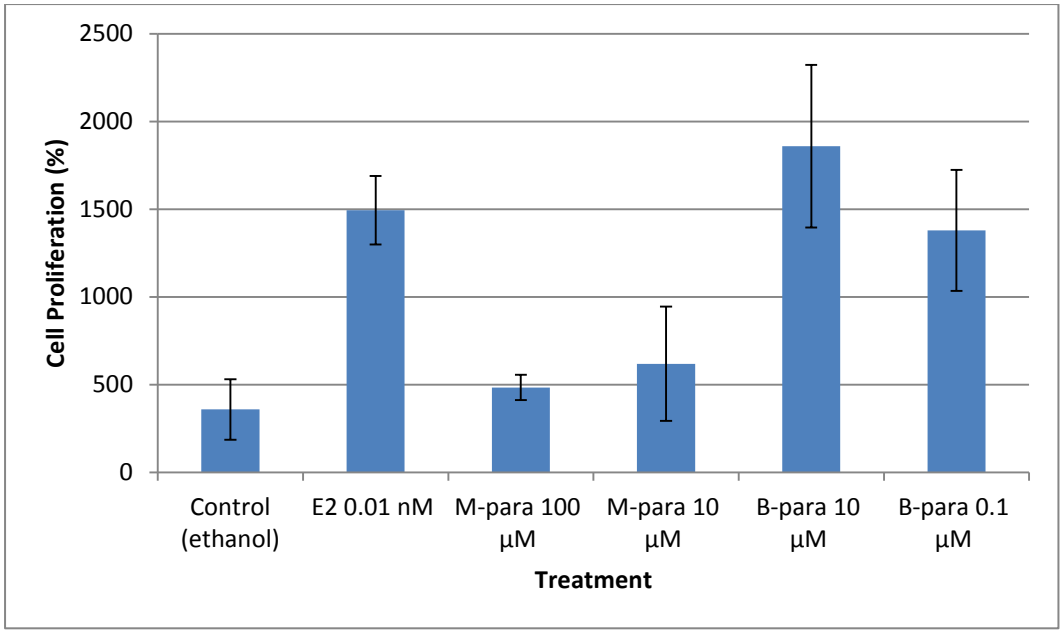

Figure 3.16: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

3.10 Paraben combination exposure experiments – initial cell concentration determined by dilution

3.10.1 Paraben combination exposure experiments – initial cell concentration 1.5 x 10^4 cells/mL

When the cell proliferation is calculated using the initial cell concentration determined by dilution (Fig. 3.17 and 3.18) instead of by initial cell count (see section 3.7.1), the overall variation is lower. The effects of the individual paraben treatments were indistinguishable from each other and the proliferation was low compared to the same treatments presented in Figures 3.15 and 3.16. However, when methylparaben and butylparaben were combined together at lower concentrations, the rise in proliferation was significant when comparing butylparaben (10 µM) and butylparaben (5 µM)/methylparaben (5 µM) (P= 0.000; Fig. 3.17

and 3.18), and butylparaben (10 µM) and butylparaben (5 µM)/methylparaben (50 µM) (P=0.001; Fig. 3.17 and 3.18).

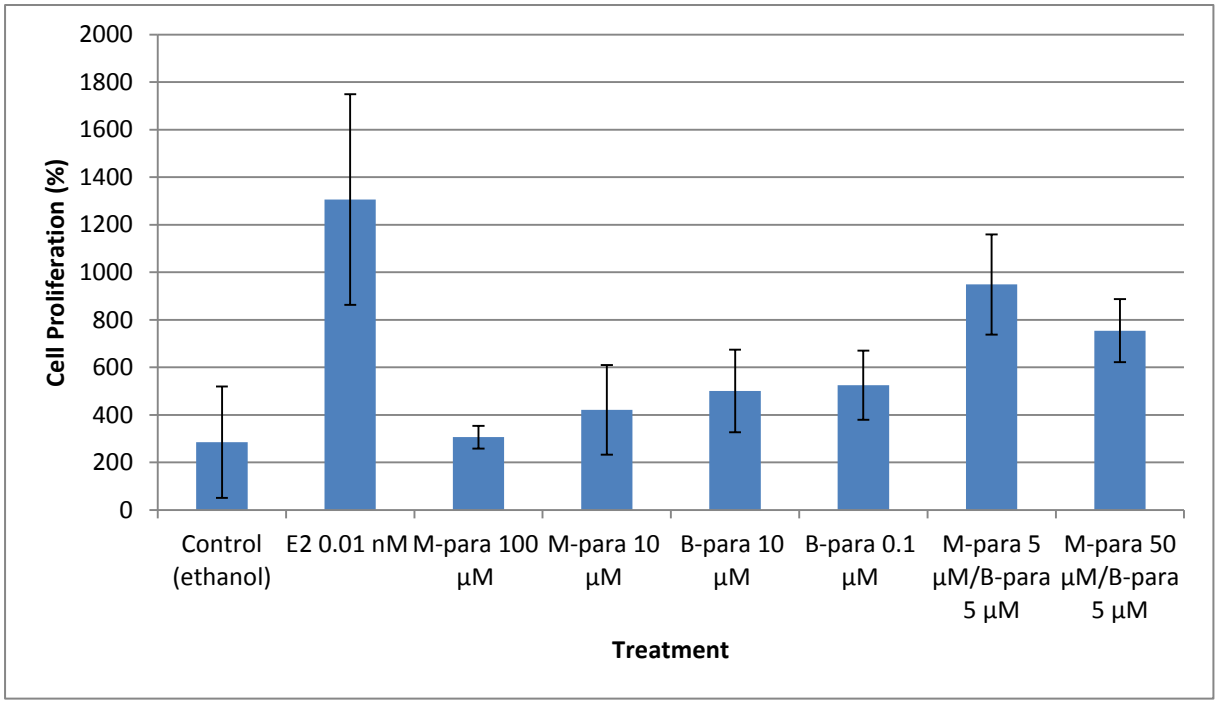

Figure 3.17: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 1.5 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

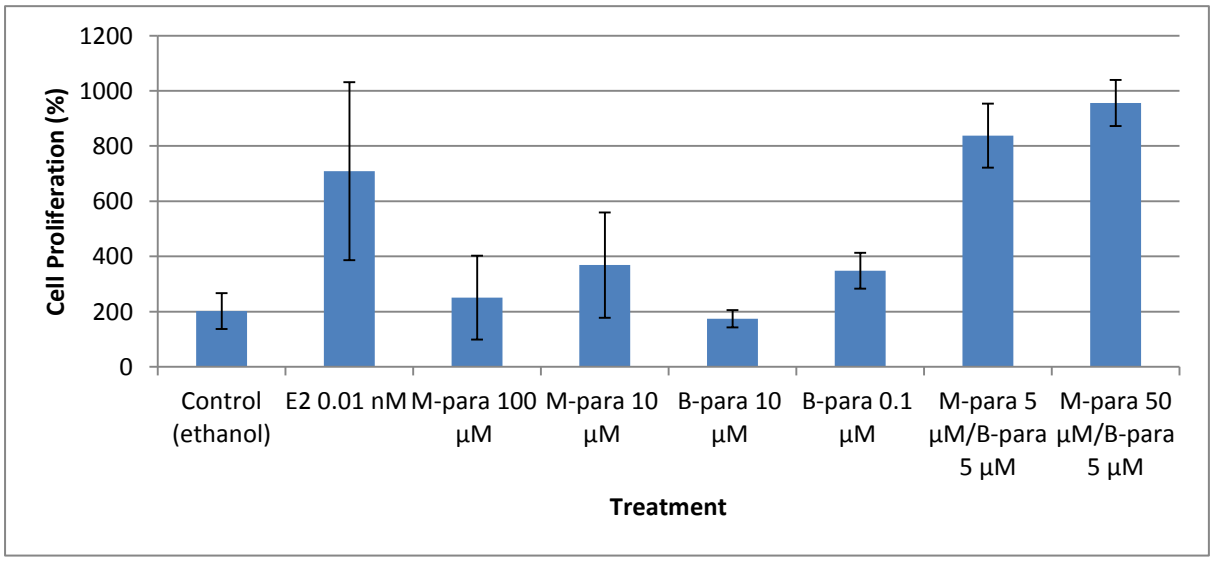

Figure 3.18: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. E2 = 17β-Estradiol, M-para = methylparaben and B-para = butylparaben.

Starting cell concentration approximately 1.5 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

3.10.2 Paraben combination exposure experiments – initial cell concentration 5 x 10^4 cells/mL

When the cell proliferation is calculated using the initial cell concentration determined by dilution (Fig. 3.19 and 3.20) instead of by initial cell count (see section 3.7.2), the overall variation is lower. The proliferation was low across all treatments still, due to the initial cell concentration being too high; therefore this result was not included in statistical analysis.

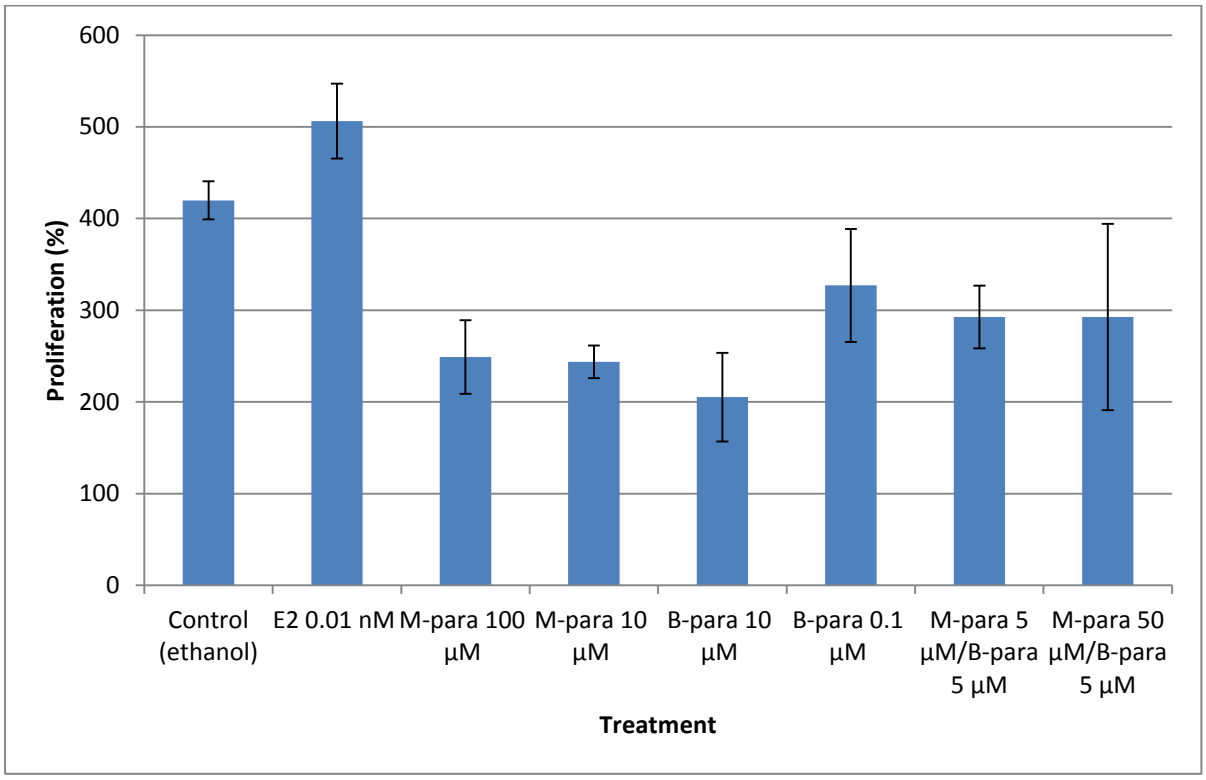

Figure 3.19: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 5 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

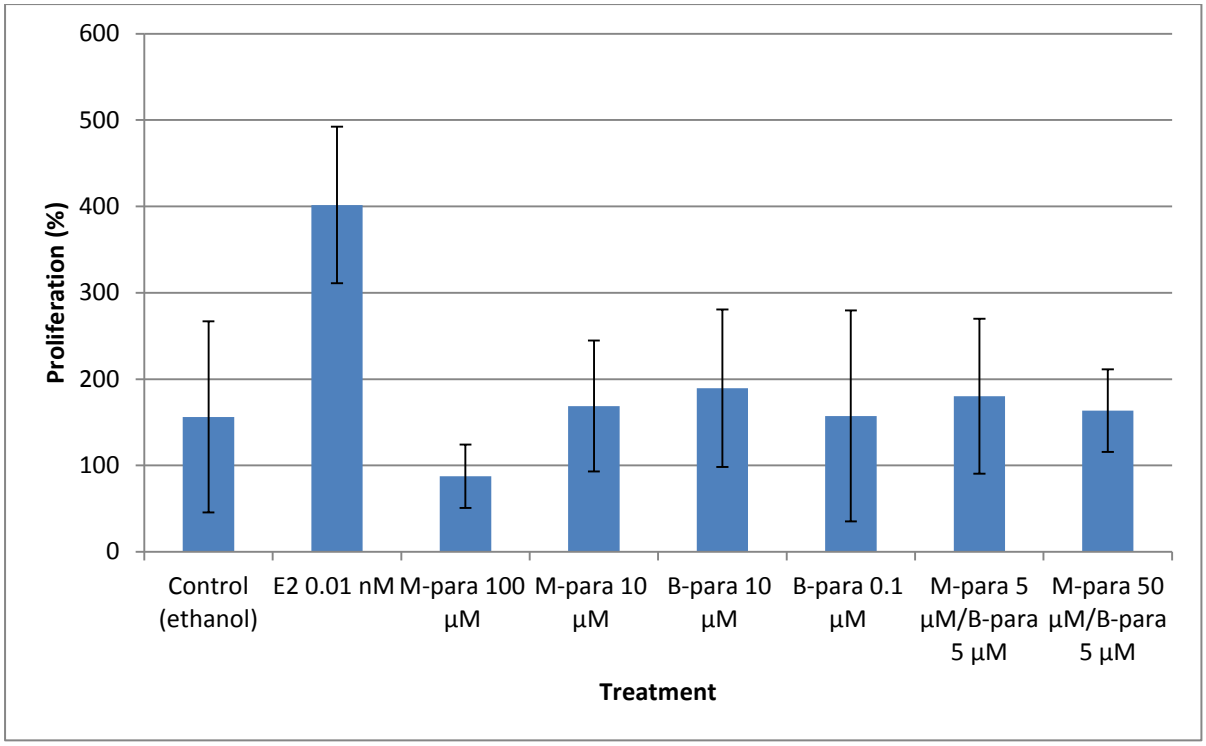

Figure 3.20: Percentage proliferation (± SD) of MCF-7 cells following exposure to parabens after 9 days. M-para = methylparaben and B-para = butylparaben. Starting cell concentration approximately 5 x 10^4 cells/mL (determined by dilution; see section 4.2.4).

3.11 Statistical analysis using Analysis of Variance

3.11.1 Statistical analysis of paraben exposures using Analysis of Variance

Paraben exposures (see section 3.8) were statistically analysed to determine if treatments differed using ANOVA (Fig. 3.21). Fisher's method showed that E2 and butylparaben 10 µM were statistically different than ethanol, methylparaben 100 µM and 10 µM at a 95% confidence level.

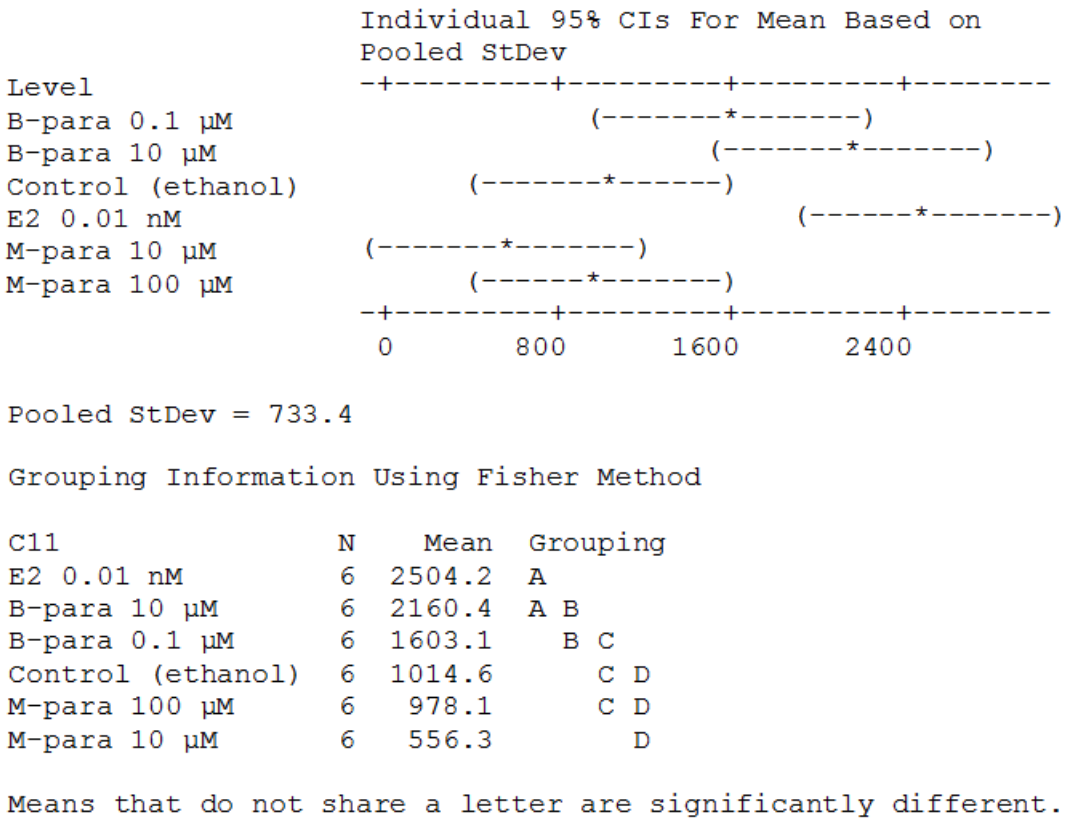

Figure 3.21: One-way analysis of variance (ANOVA) of cell proliferation data (Fig 3.15 and 3.16) grouped using Fisher's method. B-para = butylparaben and M-para = methylparaben.

3.11.2 Statistical analysis of paraben combination exposure experiments using Analysis of Variance

Paraben combination exposures (see section 3.10.1) were statistically analysed to determine if treatments differed using ANOVA (Fig. 3.22). Fisher's method showed that E2 and both methylparaben/butylparaben combinations were significantly different than the remainder of the treatments at a 95% confidence level.

```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level                     --------+---------+---------+---------+-
B-para 0.1 µM                   (------*-----)
B-para 10 µM                 (-----*-----)
Control (ethanol)         (-----*-----)
E2 0.01 nM                                      (------*-----)
M-para 10 µM                   (-----*-----)
M-para 100 µM               (-----*-----)
M-para 5 µM/B-para 5 µM                      (-----*-----)
M-para 50 µM/B-para 5 µM                   (-----*------)
                          --------+---------+---------+---------+-
                                300       600       900      1200

Pooled StDev = 225.4

Grouping Information Using Fisher Method

C8                        N    Mean  Grouping
E2 0.01 nM                6  1007.6  A
M-para 5 µM/B-para 5 µM   6   893.1  A
M-para 50 µM/B-para 5 µM  6   854.9  A
B-para 0.1 µM             6   436.5    B
M-para 10 µM              6   394.8    B
B-para 10 µM              6   337.5    B
M-para 100 µM             6   278.5    B
Control (ethanol)         6   243.8    B

Means that do not share a letter are significantly different.
```

Figure 3.22: One-way analysis of variance (ANOVA) of cell proliferation data (Fig 3.17 and 3.18) grouped using Fisher's method. B-para = butylparaben and M-para = methylparaben.

3.12 UV Studies on the spectroscopy of methylparaben and butylparaben combinations

Butylparaben in octanol had 2 absorption peaks; λ213 nm and λ259 nm (Fig. 3.23) and methylparaben in water had 2 absorption peaks; λ194 nm and λ256 nm (Fig. 3.24). Methylparaben in octanol (10 µg/mL) absorbed at λ259 nm (data not shown). Butylparaben is very water insoluble so a peak for butylparaben in water was not collected.

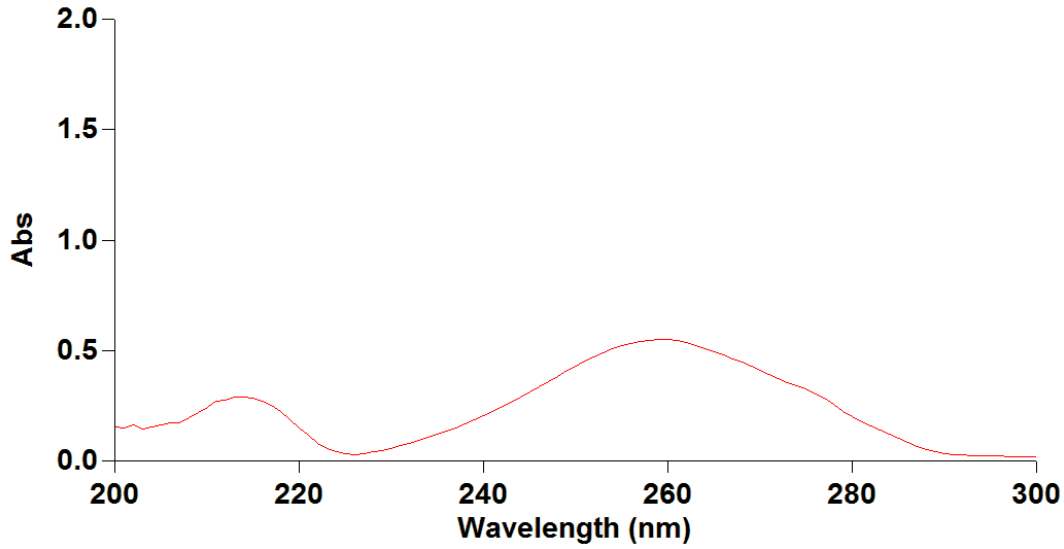

Figure 3.23: Butylparaben in octanol (10 µg/mL).

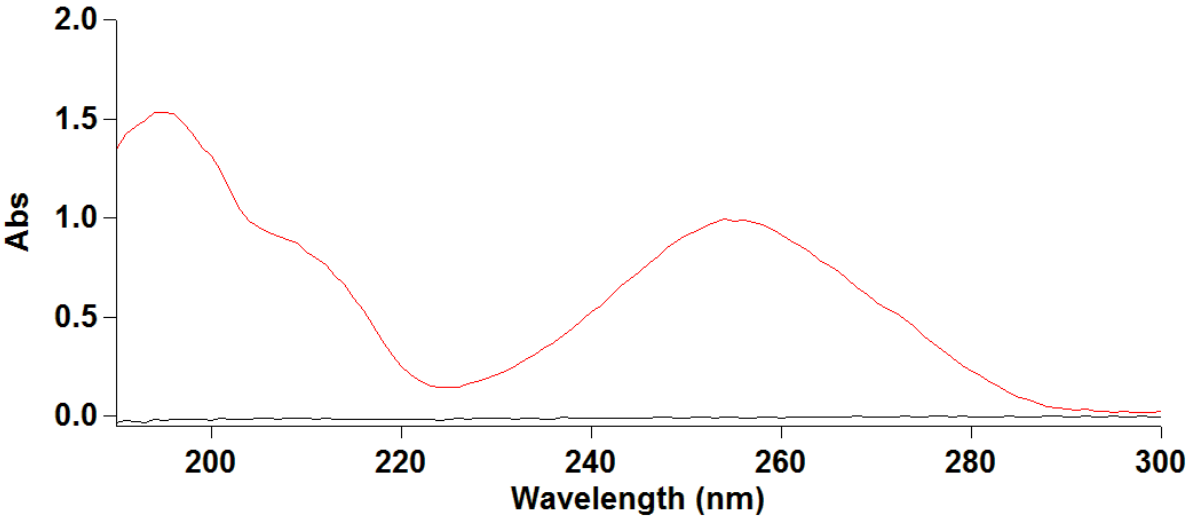

Figure 3.24: UV spectra of methylparaben in water (red; 10 µg/mL) and water (black).

Both methylparaben in octanol (methylparaben octanol fraction) and butylparaben in octanol (butylparaben octanol fraction) had an absorbance peak at λ259 nm, but with different absorbance values (Fig 3.25). The methylparaben octanol fraction absorbed at $A_{\lambda 215} = 0.562$ and $A_{\lambda 259} = 1.044$[2] and the butylparaben octanol fraction absorbed at $A_{\lambda 214} = 0.336$ and $A_{\lambda 259} = 0.539$. The butylparaben and methylparaben in octanol fraction absorbed nearly halfway

[2] Absorbance value above 1.0 isn't used quantitatively as Beer's Law doesn't apply.

between the methylparaben in octanol fraction and the butylparaben in octanol fraction at $A_{\lambda 259}$= 0.731.

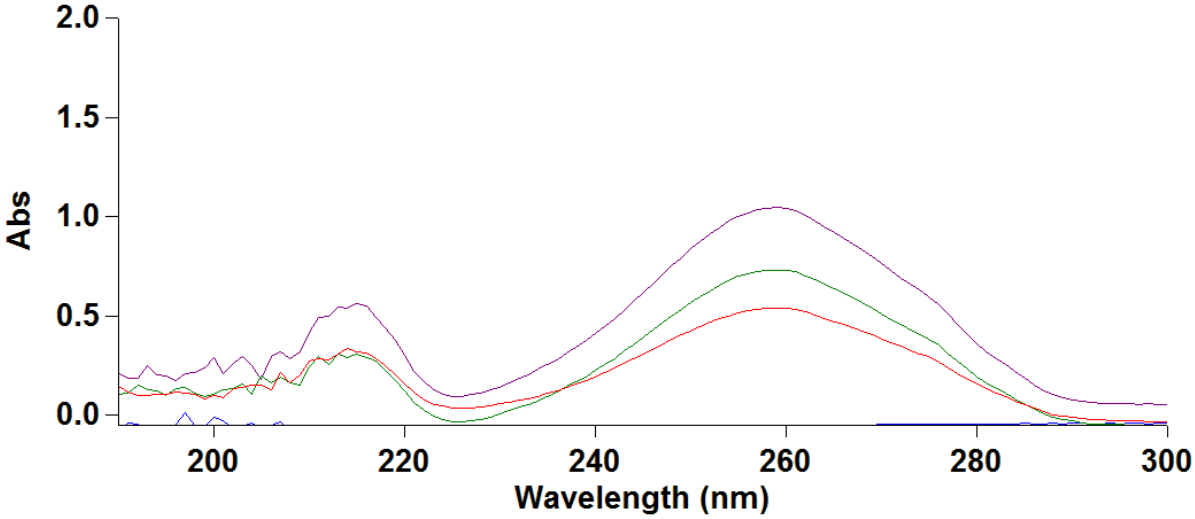

Figure 3.25: UV spectra of fractions methylparaben in octanol (purple), butylparaben in octanol (red), methylparaben and butylparaben in octanol (green) and octanol (blue).

The corresponding water fractions had very different spectra profiles (Fig 3.26). The water spectrum had an unknown contamination between λ190 – 230 nm. The methylparaben in water fraction, butylparaben in water fraction and butylparaben and methylparaben in water fraction had peaks <λ190 nm.

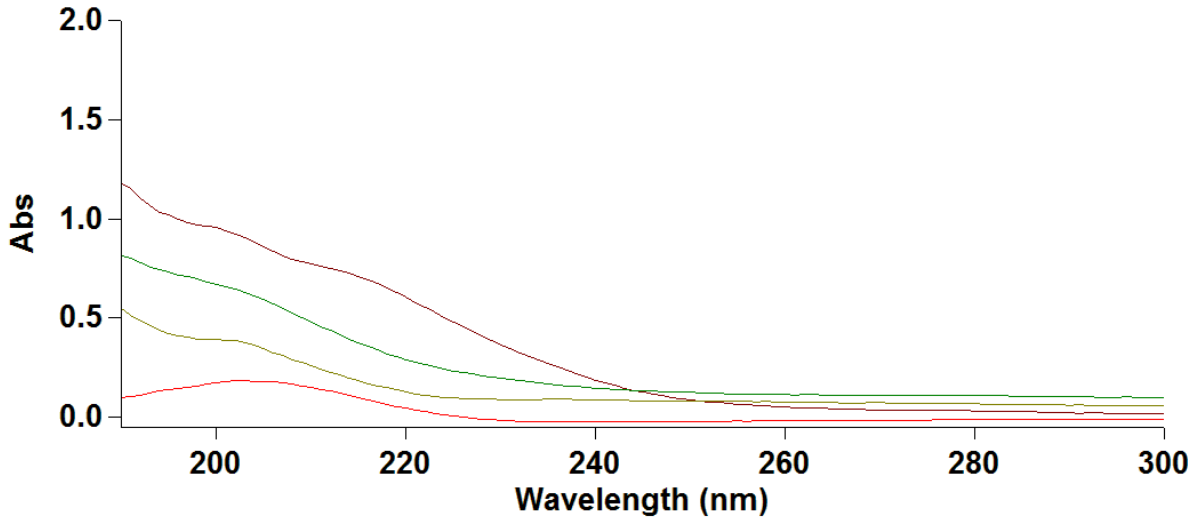

Figure 3.26: UV spectra of fractions aq-met (purple), aq-but (green), aq-but-met (yellow) and water (red).

A fraction containing an equal amount of the methylparaben in octanol fraction and the butylparaben in octanol fraction (labelled butylparaben and methylparaben in octanol 1:1) absorbed at $A_{\lambda 215}$= 0.581 and $A_{\lambda 260}$= 0.829 (Fig. 3.27).

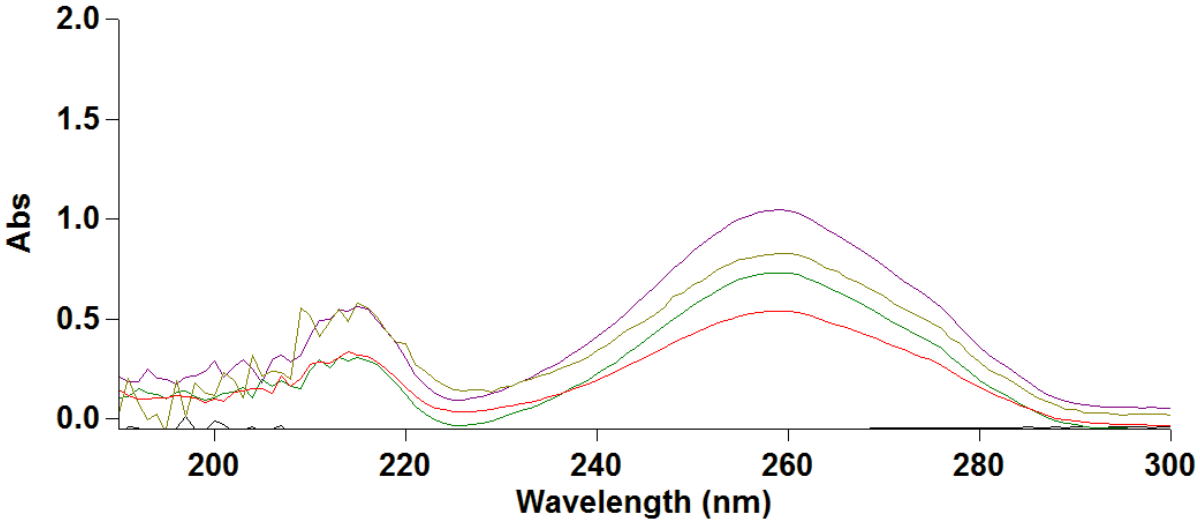

Figure 3.27: UV spectra of fractions methylparaben in octanol (purple), butylparaben in octanol (red), methylparaben and butylparaben in octanol (green), methylparaben and butylparaben in octanol 1:1 (yellow) and octanol (black).

4. Discussion

4.1 The estrogenic effect of butylparaben and methylparaben alone and in combination

As stated in the introduction, one aim of this study was to determine the effect combinations of paraben have on the growth of MCF-7 cells in culture. Given that parabens are almost always present together in combinations in personal care products and cosmetics, it is important to investigate the effect of adding parabens together, compared to the effect produced by single parabens. The results of this study show that MCF-7 cells proliferate in the presence of butylparaben and methylparaben, providing further evidence that they are xenoestrogens as previously shown by Darbe and others (Byford et al. 2002; Routledge et al. 1998; Darbre 2008). Cells exposed to 10 μM and 0.1 μM butylparaben showed greater cell proliferation compared to cells exposed to 10 μM methylparaben (Fig. 3.15 and 3.16). A very important observation was made when MCF-7 cells were exposed to butylparaben (5 μM) and methylparaben (5 μM) in combination (Fig. 3.17 and 3.18). The resulting cell proliferation observed was greater than either butylparaben alone at 10 μM or methylparaben alone at 10 μM. This observation was also made when MCF-7 cells were exposed to butylparaben (5 μM) and methylparaben (50 μM) in combination (Fig. 3.17 and 3.18). The resulting cell proliferation observed was also greater than either butylparaben alone at 10 μM or methylparaben alone at 100 μM. These differences are statistically significant according to ANOVA at 95% confidence (section 3.11.2). This evidence shows that combining these parabens together appears to be more than just an additive effect and could potentially be synergistic, giving a better understanding of the cocktail effect. The relevance of this to the *in vivo* situation is significant given exposure to parabens and other xenoestrogens often occurs simultaneously and frequently. The experimental procedure, which resulted in this finding, will be discussed - including method development related to variability issues. Since

variation was a persistent issue in the studies carried out, a part of the study involved method development to address this. This involved determining the most accurate method for measuring proliferation using cell counting, by comparing Countess® Automated Cell Counter and manual counting using a cytometer, and finding a successful way of dissociating cell clumps using TrypLE to obtain an even cell suspension.

4.2 Sources of variation: Countess® Automated Cell Counter vs cytometer

4.2.1 Countess® Automated Cell Counter – determining cell concentration using imaging

The cell counter uses imaging software that detects roundness of an appropriate size determined by the user (10 uM – 60 uM). The cells were stained with trypan blue 0.4%, allowing the counter to distinguish between live cells and dead cells (dark circle with a bright centre, compared to a dark circle; fig 4.1). The automated counter counts in 10^4 increments within the 10^4 and 10^7 cells/mL range and being most accurate between 10^5 and 10^6 (Invitrogen 2013a).

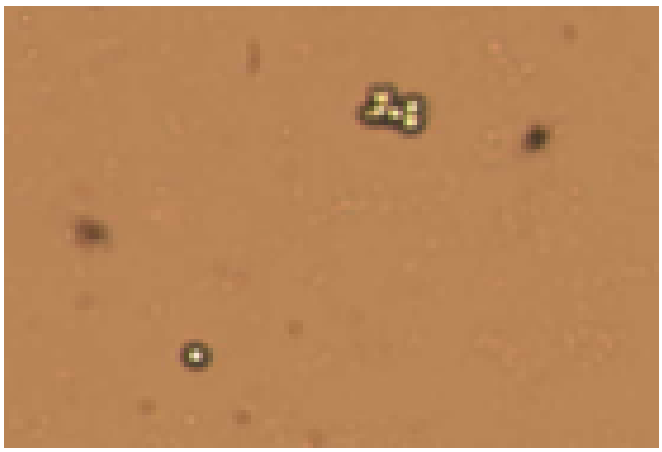

Figure 4.1: MCF-7 cells stained with trypan blue (0.4%).

4.2.2 Variation using Countess® Automated Cell Counter

The cell proliferation in the studies in section 3.1, 3.2 and 3.3 was measured using the Countess® Automated Cell counter and all experiments had large variation, in some cases obscuring effects between sets of triplicate wells (Fig. 3.2 – 3.6). When the variability was examined in the estrogen exposure experiments (Fig. 3.2 and 3.3) it was thought that small variations between MCF-7 daily growth, compounded over the course of an exposure, could be causing the observed variation. Because of this, 3 of the estrogen concentrations that were used in the exposure were selected and growth curves were generated. Triplicate wells were counted each day for 10 days and the resulting curves were compared. A trending increase in proliferation could be seen in cells exposed to 18.3 pM and 0.367 pM E2 (Fig. 3.4 and 3.6). There was no increase in trend in cell proliferation in the cells exposed to 3.67 pM E2. Despite seeing an increasing trend, there was no statistical significance as the variation was too great. This provides evidence that the variation was caused by the cell counter software failing to count the correct number of cells present. Upon looking into this further, it was found that the cell counter cannot distinguish clumped cells, despite the clumps being small and each cell being clearly distinguishable. The counter either undercounts the number of cells present (Fig. 4.2) or discounts the clumps entirely (4.3). The amount of debris present in the cell counting mixture affects the dead cell concentration as the counter identifies some debris as dead cells (Fig. 4.4; lower left corner). These results show that the Countess® Automated Cell Counter is not suitable for counting MCF-7 cells using the dispersal method used in these studies due to their clumping nature. Following this finding, a manual counting method was used in further studies.

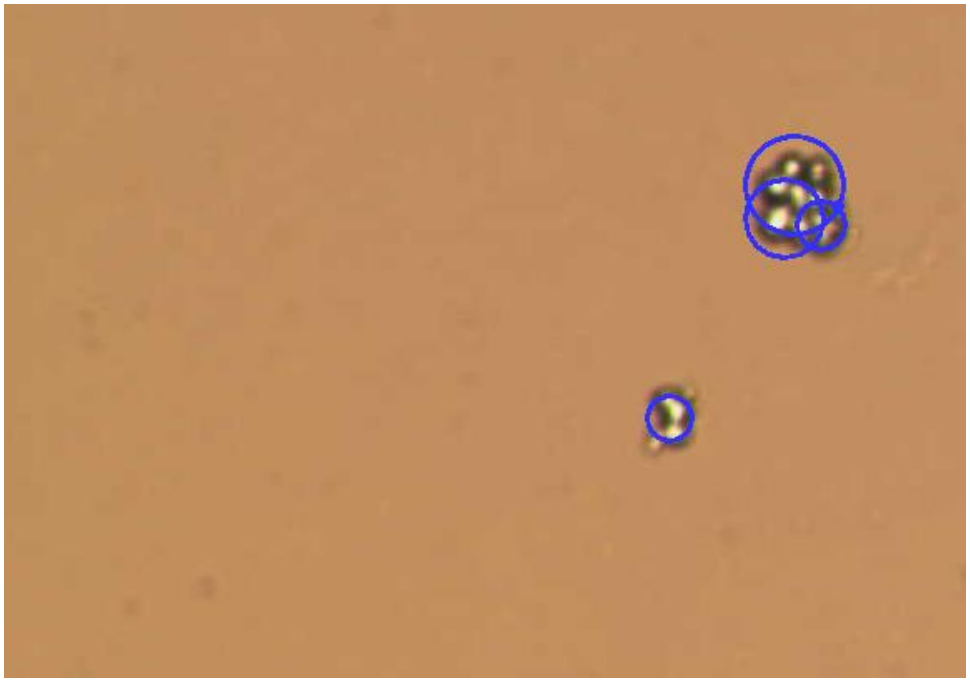

Figure 4.2: Countess® Automated Cell Counter analysis showing counted cells. Blue circle: 1 counted live cell.

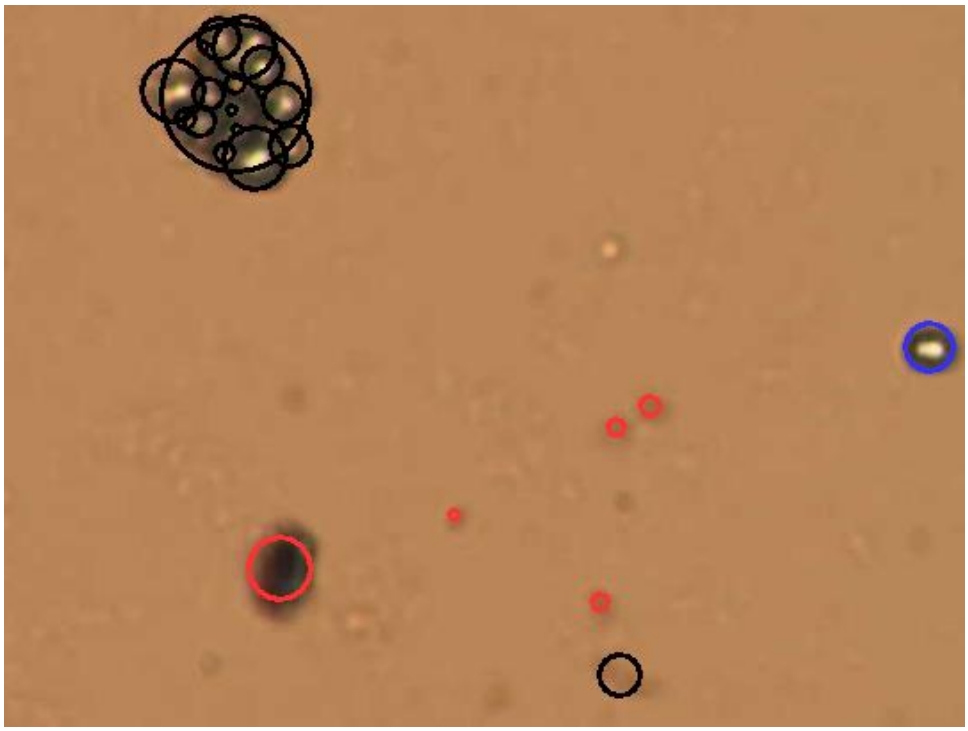

Figure 4.3: Countess® Automated Cell Counter analysis showing counted cells. Blue circle: 1 counted live cell, red circle: 1 counted dead cell, black circle: discounted from analysis.

4.2.3 Counting cells manually using a cytometer

Some advantages of counting cells manually using a cytometer are that a practiced operator can distinguish between live and dead cells, the microscope's focus can be adjusted to observe badly positioned cells and cells present in clumps can be carefully counted. One disadvantage is human error, in both preparation of a slide and visually counting it. Counting using a cytometer is most accurate when cells are uniformly spread and not clumped (Freshney 2010).

4.2.4 Accurately determining low cell concentration

In the studies which involved seeding a well plate, the starting cell concentration was chosen so that the lag phase of cell growth was not too long, yet proliferation was still sufficient to observe the difference between the effects of treatments. The starting cell concentration was determined in two ways – by counting a dense cell suspension and then diluting it to the required concentration (referred to as concentration determined by dilution) and by counting the final diluted suspension (referred to as concentration determined by counting). A flask of cells prepared and ready to use for seeding (see section 2.9.6) has a concentrated cell suspension. Determining the desired cell concentration by dilution involves counting this suspension and diluting it with medium to the concentration needed. This method was thought initially not to be accurate enough as it did not take into account if a significantly different number of cell clumps ended up in each well within a well plate, as was observed in some wells. Because of this, a sample of diluted suspension from each well was removed for counting to give an individual concentration to use for calculating the cell proliferation. When counting cells with a cytometer, if the overall cell number present on the grid is too low ($<100/mm^2$), the count is not as accurate (Freshney 2010). The concentration determined by dilution method is more accurate in relation to having a larger number of cells to count,

but does not address the issue of clumps. The concentration determined by counting method takes into account clumps, but the count is more inaccurate due to lower cell numbers. The concentration determined by counting method was carried out (sections 3.4 – 3.6) and compared to applying the concentration determined by dilution method to the same data (sections 3.7 – 3.9) and overall variation for the concentration determined by dilution method was lower, showing that the accuracy of counting more cells initially outweighs any effect some cell clumps would have on the final cell numbers after 9 days of exposure. The data generated using the diluted method was used for statistical analysis (section 3.10 and 3.11).

4.3 Sources of variation: dissociation of clumped cells

The MCF-7 cell line is adherent; the cells attach firmly to plastic culture vessels and each other (Fig. 1.10). This makes changing the culture medium easy, but can cause problems when sampling cells for passaging or counting. This adhesion is caused by proteins present on outer surface of the cells binding with the plastic. Cancer cells grown *in vitro* somewhat lose contact inhibition that regular cells employ, which leads to signals being disordered and results in the cells growing clumped together (Hirohashi 1998). The standard way to detach cells that adhere is by using trypsin (or another protease) to hydrolyse the cell surface adhesion proteins that bond to the surface of the culture vessel and to other cells, freeing the cells. The potential issue with this is trypsin isn't a specific protease that only hydrolyses the proteins that adhere; trypsin acts by hydrolysing a peptide bond typically on the carboxyl side of arginine or lysine. Other vital cell surface proteins and membrane traversing proteins could be affected too, causing loss of function and disruption to other vital cell processes. Given MCF-7 cells express estrogen receptor-α on their cell membrane surface, any modification of the receptor caused by trypsinisation would have a negative effect on any experiments involving estrogen or the estrogen receptor. Because of this, cell dispersal using trypsin needs

to be carried out with caution. Cells can be over trypsinised by being treated with trypsin with an activity that is too high, or by being exposed for too long. The correct exposure time will result in cell dispersal with little-to no clumping without compromising viability. The most accurate concentration obtained by counting cells involves having a suspension that is as clump-free and uniform as possible (Freshney 2010). TrypLE was used to dissociate cells prior to counting to measure proliferation and for routinely maintaining the cell cultures (see sections 2.9.4 and 2.9.8). TrypLE which contains a pure recombinant enzyme was chosen because it is gentler than some trypsin extracts (Invitrogen 2013b). Throughout the studies involving cell counting, obtaining a uniform clump-free suspension was challenging and unpredictable. Some factors influencing cell dispersal included the volume of TrypLE used, exposure time to TrypLE and whether cells were disturbed while exposed to TrypLE (see section 3.2, Table. 1). The cell growth phase also had an effect; cells that were confluent were harder to disperse, possibly due to larger adhered cell clumps. Minor cell disturbance by gentle rocking aided dispersal, but too much rocking encouraged clump formation, which drawing the suspension in and out of a micropipette tip did little to reduce.

4.4 Creating an estrogen-free environment – the use of stripped FBS and phenol red-free MEM

4.4.1 Dextran-coated charcoal stripped FBS

The results of the study comparing the proliferation of MCF-7 cells grown in MEM (10% FBS) to PRFMEM (10% stripped FBS) proved that there is enough estrogen present in MEM (10% FBS) to have a significant effect on cell proliferation (section 3.1). Being a blood product from a biological system (i.e. fetal), FBS contains sex hormone binding globulin

which binds the majority of the steroid hormones present in the serum. These hormones, including estrogens are then available to the cells when FBS is added to the cell medium. This is why it is crucial to strip out the hormones in FBS using dextran-coated charcoal, using one of the methods described in the literature (Soto et al. 1995; Blom et al. 1998). The method used here by Blom et al. (1998) was successful at providing a serum for estrogen related experiments that did not induce proliferation as shown by the results in section 3.1.

4.4.2 Use of phenol red indicator

Phenol red indicator is added to cell culture media to help monitor the health of the cells through observing changes in pH. As waste products produced by the cells build up, the pH slowly decreases and becomes orange and eventually yellow, indicating the medium needs changing. A change from red to yellow can also indicate the presence of bacterial or yeast contamination (Freshney 2010). In 1986 Berthois et al. published a paper showing phenol red was estrogenic. Phenol red has structural similarities to 17β-estradiol (Fig. 4.4). The concentrations of phenol red present in culture media (15 – 45 μM) were high enough to cause proliferation in MCF-7 cells (Berthois et al. 1986). This is problematic if the effects of estrogen are being studied using estrogen sensitive cells such as MCF-7. After this discovery, media formulations without phenol red were developed and used for culturing cells used in studies involving estrogen and xenoestrogens. Minimum essential medium without phenol red was used in this study for all estrogen and paraben related experiments for these reasons.

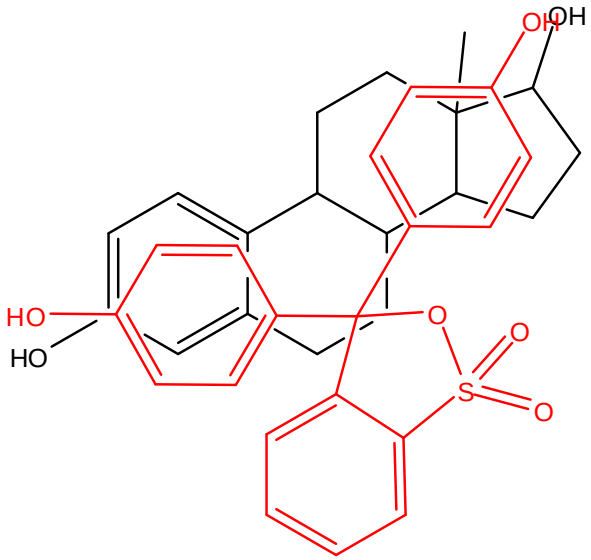

Figure 4.4: E2 (black) and phenol red (red) superimposed to show molecular analogies.

4.5 The absorption of parabens through the skin

Parabens present in cosmetics and personal care products when applied topically are absorbed into the body via the skin. How easily a paraben penetrates the skin depends on its water solubility and the type of emulsion and formulation it is used in (e.g. ethanol which is commonly found in formulae can increase permeation of parabens) (Pedersen et al. 2007). As the alkyl chain length increases, so does hydrophobicity and the ability of the paraben to cross the cell membrane and enter the cell increases (Boberg et al. 2009; Pedersen et al. 2007). *In vitro* studies have shown that permeability coefficients correlate with octanol/water partition coefficients (Pedersen et al. 2007). Given that parabens are present in combinations in many formulae, it is important to know if the ability of one paraben to penetrate the cell membrane is affected by the presence of another e.g. chaperoning; one hydrophobic molecule aiding another less hydrophobic molecule to move across the cell membrane. An octanol/water partition coefficient experiment with methylparaben and butylparaben present

together was carried out to investigate if this might be the case (see section 3.11). Methylparaben was dissolved in water (10 μg/mL) as it is the paraben with the highest water solubility and butylparaben was dissolved in octanol (10 μg/mL). The absorption spectra for these solutions were determined by UV spectroscopy. Methylparaben in water had absorbance peaks at λ194 nm λ256 nm (Fig. 3.24) and butylparaben in octanol had absorbance peaks at λ213 nm λ259 nm (Fig. 3.23). Methylparaben in water was shaken with an equal volume of octanol and left to separate to determine how much methylparaben absorbed into the organic phase without butylparaben present. The results of this showed that all of the methylparaben moved from the aqueous phase to the organic phase due to the presence of a peak in the methylparaben in octanol fraction (Fig. 3.25) and the absence of a peak of the same profile shape in the methylparaben in water fraction (Fig. 3.26). The methylparaben in water fraction spectrum had a different profile shape, showing the presence of a water soluble contaminant. Butylparaben in octanol was shaken with and equal volume of water and left to separate to determine how much if any would absorb into the aqueous phase without methylparaben present. The results showed there was no butylparaben was present in the butylparaben in water fraction as to be expected as butylparaben is very water insoluble. As with the methylparaben in water spectrum, the butylparaben in water spectrum had a different profile shape (Fig 3.26). A fraction containing equal parts of the butylparaben in octanol fraction and the methylparaben in octanol fraction was analysed to show where the absorbance would occurred if both were present in the organic layer. The absorbance of this methylparaben in octanol and butylparaben in octanol 1:1 fraction was $A_{\lambda 259}$= 0.824. Comparing this absorbance to the methylparaben and butylparaben in octanol fraction (0.731; Fig. 3.27), they both absorb approximately halfway between the methylparaben in octanol fraction (1.044) and the butylparaben in octanol fraction (0.539). This shows that if there is any “chaperone effect” where the presence of butylparaben is having an effect on the

movement of methylparaben into the octanol phase or vice versa, it is minor, suggesting that any potential elevated growth produced by combining methylparaben and butylparaben together is not caused by butylparaben chaperoning methylparaben into the cell. Methylparaben and butylparaben both have high octanol/water partition coefficients ($LogP_{ow}$) of 1.66 and 3.24 respectively (Tavares et al. 2009), showing both are hydrophobic and dissolve in the octanol phase more readily than the water phase. Despite this, the literature suggests that one reason for methylparaben's lower estrogenic activity is due to poorer absorption into the cell (Harvey 2003). Evidence is conflicting, as some studies report increasing absorption with increasing chain length, while others report the opposite with methylparaben being the most easily absorbed and the emulsions present have an effect on the degrees of absorption of other parabens when applied to human skin (Soni et al. 2005; Pedersen et al. 2007).

4.6 The estrogenicity of parabens – single parabens and in combinations

4.6.1 The estrogenicity of parabens

When exposed to 10 μM butylparaben, MCF-7 cells proliferate at a similar rate to MCF-7 cells exposed to 0.01 nM E2. MCF-7 cells exposed to 100 μM and 10 μM methylparaben had no effect compared to the control (Fig. 3.15 and 3.16). Previous studies show that 100 μM methylparaben causes proliferation of MCF-7 cells. (Byford et al. 2002)

The concentrations of butylparaben and methylparaben used in this study were chosen based on the results of the study carried out by Byford and colleagues, which involved exposing MCF-7 cells to parabens at different concentrations and measuring their proliferation (Byford et al. 2002). In their study, MCF-7 cells exposed to 10 μM butylparaben showed a large

proliferation compared to MCF-7 cells exposed to 0.1 μM butylparaben. This was the same for MCF-7 cells exposed to 100 μM methylparaben compared to MCF-7 cells exposed to 10 μM methylparaben.

4.6.2 The estrogenicity of parabens in combinations

In this study, the combinations of butylparaben and methylparaben were chosen based on the proliferation observed when MCF-7 cells were exposed to single parabens at the chosen concentrations (section 3.9). Since the proliferation of MCF-7 cells exposed to butylparaben (10 μM) was large compared to methylparaben (100 μM), a combination that contained butylparaben (5 μM) and methylparaben (50 μM), which is half of these concentrations was chosen. If the combination effect between butylparaben and methylparaben is additive, the proliferation of MCF-7 cells exposed to the combination should be halfway between the two single doses containing twice the concentration of each paraben concentration contained in the mixture. The other combination containing butylparaben (5 μM) and methylparaben (5 μM) was also chosen for the same reasons stated above. The proliferation of MCF-7 cells observed when exposed to each of these combinations was greater than the proliferation of MCF-7 cells exposed to either paraben component at twice the concentration (Fig. 3.17 and 3.18). The difference was statistically significant when analysed by ANOVA. The combinations were also analysed by Fisher method which concluded that each combination treatment was different from the single paraben treatments (Fig. 3.22). From this statistical analysis, it appears that combining methylparaben and butylparaben together has a synergistic effect; however there are discrepancies that need to be addressed.

Dose response was not evident in many of the exposure experiments – including the relevant paraben combination exposure experiments (section 3.10.1). Both treatments of methylparaben and both treatments of butylparaben caused MCF-7 cells to proliferate the

same amount as the control (Fig. 3.17 and 3.18). This is interesting, given the large differences in dose, especially between 10 μM butylparaben and 0.1 μM butylparaben. This does not support results found previously by others (Byford et al. 2002).

The variation present in all exposure experiments was still great, despite finding ways to reduce it; however the paraben combination treatments were still significantly different from the paraben single treatments.

Given different parabens are present together in cosmetics and pharmaceutical preparations, it is logical to investigate their combined estrogenic effect. This became a need when studies published showed multiple parabens were found in breast tumours, demonstrating that intact parabens can present in the breast. (Darbre et al. 2004) This followed a review by Darbre outlining the use of underarm cosmetics containing parabens and a possible link to breast cancer (Darbre 2003). Since these publications, further studies have been carried out looking at concentrations of parabens in human breast tissue. One study measured 5 different parabens at different locations within the breast and found that the levels of methylparaben and propylparaben were highest compared to other parabens measured (Barr et al. 2012). One conclusion from this is the intact parabens were present from dermal exposure, rather than oral exposure as they were not in the presence of the higher concentration of esterases found in the gut or the liver, producing the metabolite p-hydroxybenzoic acid, compared to the concentration of esterases found in the skin (Harvey & Everett 2012).

Following this, a study was carried out that exposed MCF-7 breast cancer cells to combinations of parabens at the concentrations found in human breast tissue (Charles & Darbre 2013). The results from this study show that combining parabens together at the concentrations found in human breast tissue causes an increase in proliferation of MCF-7 breast cancer cells. The results were interpreted as all 5 parabens having an additive effect.

Figure 2; (F) in this article shows a large increase in cell proliferation compared to the proliferation produced by each individual paraben, which appears to be synergistic though no mention of this is made. This finding is important in the context of testing parabens and using parabens in personal care products. One point the study doesn't address is how accessible the parabens are to bind with the estrogen receptor and cause estrogenic effects. Given the low water solubility of parabens, as discussed earlier, is it possible they will sit in within the cell membrane and not be accessible to the estrogen receptor to cause an estrogenic effect?

4.7 Conclusion

This study found evidence for potential synergistic effects on the proliferation of MCF-7 cells in culture when butylparaben and methylparaben are present together in combination. Because of large the variation and dose dependant discrepancies within these results, this is not certain and more studies need to be carried out to draw a more definite conclusion.

4.8 Further study possibilities

Given the results of this study, more work needs to be carried out to determine if the effect observed when butylparaben and methylparaben are present together is synergistic or just additive. A method that shows clear dose dependence with small variability needs to be used to test the same combinations again, possibly with a more appropriate selection of paraben concentrations that produce a large difference in MCF-7 cell proliferation. Another concept that was not addressed in this study was the possible breakdown of parabens by esterases produced by MCF-7 cells. This might have been relevant in this study given the MCF-7 cells present in the exposure experiments were exposed to the same medium containing the paraben treatment for the whole exposure, allowing time for the paraben to be metabolised and therefore not contribute to causing cell proliferation.

Lastly, given multiple parabens have been found in breast tissue (Barr et al. 2012), it is important to investigate how accessible they are to the estrogen receptor and if they cause an increase in the incidence of breast cancer.

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