Nonylphenol and Perfluorooctanoic Acid Stimulate Proliferation of MCF-7 Breast Cancer Cells via Activation of Estrogen Receptor Alpha

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NONYLPHENOL AND PERFLUOROOCTANOIC ACID STIMULATE PROLIFERATION OF MCF-7 BREAST CANCER CELLS VIA ACTIVATION OF ESTROGEN RECEPTOR ALPHA

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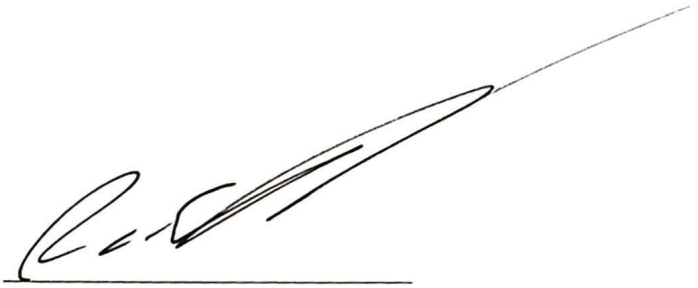
Signature 

Date 4/15/16

DEDICATION

This work is dedicated to my parents and brother whose unwavering support and love never faltered during the tenure of this project.

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ABSTRACT

AHMED SAID. Nonylphenol and Perfluorooctanoic Acid Stimulate Proliferation of MCF-7 Breast Cancer Cells via Activation of Estrogen Receptor α (Under the direction of Dr. Sarah Lundin-Schiller).

Endocrine disrupting compounds (EDC) have been persistent throughout the environment since the industrialization of the modern world. This study examines two lesser known EDC's and their estrogenic and anti-estrogenic effects using the estrogen receptor positive breast cancer cell line, MCF-7. MCF-7 cells proliferate in response to estrogen receptor activation and some studies support a role for increased intracellular Ca^{++} concentrations in the proliferation of these cells. In this study, perfluorooctanoic acid (PFOA, 10^{-8} M or 10^{-7} M, dependent on passage), nonylphenol (NP, 10^{-8}M or 10^{-7}M, dependent on passage), and estradiol-17β (E2, 10^{-10}M) significantly increased proliferation of MCF7 cells compared to control as determined by bioreduction of resazurin dye. This proliferation was inhibited by ICI 182,780 (10^{-7}M), a high affinity estrogen receptor antagonist for the estrogen receptor α (ERα). Proliferation was not inhibited by G36 which selectively inhibits g-protein coupled estrogen receptor (GPER) but not ERα. Neither NP nor PFOA when added to cells in combination with E2 inhibited the E2 proliferative effect. However, the combination of PFOA and E2 resulted in greater proliferation than either alone. The combination of NP and E2 did not consistently stimulate proliferation greater than E2 alone. Alone, E2, PFOA, and NP failed to induce a detectable Ca^{++} flux in MCF-7 cells. However, when coupled with ATP, which triggers a transitory Ca^{++} flux in MCF-7 cells, these estrogenic compounds appear to modulate the recovery phase of ATP induced Ca^{++} release. We conclude that NP and PFOA, by activating ERα, stimulate the proliferation of MCF-7 cells and are thus estrogenic EDCs. This action is not dependent upon stimulating increases in

intracellular Ca^{++} but the modulation of Ca^{++} reuptake may be a secondary mode of action of these estrogenic compounds.

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CHAPTER I INTRODUCTION

Endocrine disrupting compounds (EDC) are persistent throughout the environment since the industrialization of the modern world with a well-known compound being dichlorodiphenyltrichloroethane (DDT) which was used widely in the United States in the Mid-Twentieth Century (Tauber, 1950). Dichlorodiphenyltrichloroethane use was banned in the United States in 1972 following the establishment of the Environmental Protection Agency (EPA) (REGULATION (EC) No 850/2004, 2004) (Environmental Protection Agency, 2011). The term "endocrine disruptor" is a relatively new term, gaining widespread attention in the early 1990's (Colborn & Saal, 1993). It was not until 2001 with the formation of the Stockholm Convention when the international community, with the exception of a few nations, began to ban the widespread use of EDCs such as dioxins and polychlorinated biphenyls (PCBs). An accumulating body of evidence indicates exposure to EDCs leads to health effects such as obesity, neurological disorders, and various types of cancer (Soto & Sonnenschein, 2010, Rezg & Fazaa, 2014, Diamanti-Kandarakis, et al, 2009). Exposure to EDC's during fetal development is of particular concern due to the heightened sensitivity of developing tissues. Exposure *in utero* is associated with long-lasting effects with delayed onset such as non-developing primary and secondary sex characteristics, increased body weight with age, and increased risk of certain cancers (Newbold, R. R, et al, 2008, Diamanti-Kandarakis, et al, 2009).

Endocrine disrupting compounds operate through a variety of mechanisms to disturb multiple body systems such as: mimicking or blocking endogenous hormones at the receptor, altering downstream components of signal transduction pathways, changing enzyme expression or activity, and changing the epigenome resulting in transgenerational effects (Diamanti-Kandarakis, et al, 2009, Wetherill et al., 2007).

MCF-7 cells are a human breast adenocarcinoma cell line derived in 1970. A notable characteristic of this cell line is its proliferation when exposed to estrogens making it a useful cell line for EDC research (H. Soule et al, 1973). Estrogenicity of a compound can be measured indirectly by quantifying proliferation of MCF-7 cells in the absence and presence of said compound. The proliferative effects of a particular compound can be quantified by measuring an increase in the number of MCF-7 cells over the approximate 29 hour doubling time (T. Wiese et al., 1992). Estradiol acts on MCF-7 cells via the classical nuclear receptor pathway in which activated receptors bind to response elements of target genes and act as transcription factors; these transcriptions factors promote the production of proteins necessary for cell replication, such as tyrosine kinase, p21/ras and MAP kinases (Migliaccio et al, 1996). Additionally, estradiol activates g-protein coupled estrogen receptors (GPER) (T. Improta-Brears et al, 1999, Prossnitz, 2011). The GPER activation elicits an increase in free intracellular calcium ion concentration which activates the mitogen-activated protein kinase (MAPK) pathway leading to proliferation in MCF-7 cells. It is assumed estrogen mimicking compounds operate through the aforementioned intracellular pathways by binding to either or both types of receptors (T. Improta-Brears et al, 1999, Prossnitz, 2011).

This study will examine the effects of increasing concentrations of NP and PFOA on MCF-7 cell proliferation and free, intracellular calcium ion concentrations in the presence and absence of the nuclear receptor antagonist ICI 182, 780 and the GPER antagonist, G36. This study will establish the relationship PFOA and NP have on proliferation and release of intracellular calcium levels in MCF-7 cells as well as determining which ER subtype these EDC's bind if any.

The significance if this research is that it will aid in screening for EDCs that present themselves in household goods. This type of study has a direct and immediate effect on public health. Additionally, this study will provide critical missing information on the mechanisms by which PFOA and NP operate in terms of receptors. Furthermore, this study will deliver data on Ca^{++} release, a missing link in determining modes of action in these compounds.

The results of this study will add to the current knowledge of EDC's, particularly NP and PFOA and its effects on a human cell line. Testing poorly understood EDC's on a human cell line could prompt public health agencies to investigate the topic further and possibly issue warnings or even ban the use of high concentrations of the compound. The results can aid in the explanation of the molecular mechanisms behind the activity of NP and PFOA.

Specific Aims-

1. Establish a method for using the Biospectrum Multispectral Imaging System to measure MCF-7 cell proliferation via resazurin reduction assay (bioreduction).
2. Establish method for measuring increased intracellular calcium ion levels in MCF-7 cells using Fura-2AM labeling and the Nikon Ti Eclipse and NIS Elements software.
3. Measure MCF-7 proliferation in the presence and absence of nonylphenol and perfluoroctanoic acid plus and minus estrogen receptor antagonist ICI 182,780 and G36, using estradiol 17β as a positive control.
4. Measure free intracellular calcium concentration in MCF-7 cells before and after addition of nonylphenol, perfluoroctanoic acid and estradiol 17β, using adenosine triphosphate as a positive control.

CHAPTER II LITERATURE REVIEW

2.1 Estrogen

Estrogen is a broad term for the four isomers of the steroidal female sex hormone. Estrogens are eighteen carbon long molecules originating from cholesterol. Of the 4 types, estrone, estradiol, estriol, and estetrol, the most common is 17β estradiol, otherwise known as estradiol. Figure one illustrates the molecular differences between the first three types while Figure two demonstrates estetrol's structure. Estrogens are comprised of a benzene ring, two cyclohexanes and cyclopentane with various combinations of attached hydroxide groups. Because of this structure, estrogens, like other steroids, can diffuse through cell membranes to bind to intracellular receptors. Estradiol is identified from the other estrogens by the ending 5 carbon ring with a single hydroxide group attached rather than double bonded oxygen or two hydroxide groups as seen in the other isomers of estrogen (J. Yager & J. Leihr, 1996).

Estradiol originates from the 21 carbon steroid, cholesterol. After a cleavage enzyme, cholesterol is converted to pregnenalone followed by several intermediate compounds to testosterone then finally converted by the enzyme aromatase to estradiol (B. McEwen et al., 1977). The steroidogenesis pathway is demonstrated in Figure three. The conversion of testosterone to estradiol is found in several tissues of the body and is crucial in sexual differentiation during development in both male and female (B. McEwen, et al 1977, K. Ryan, 1982, V. Pentikäinen, et al 2000).

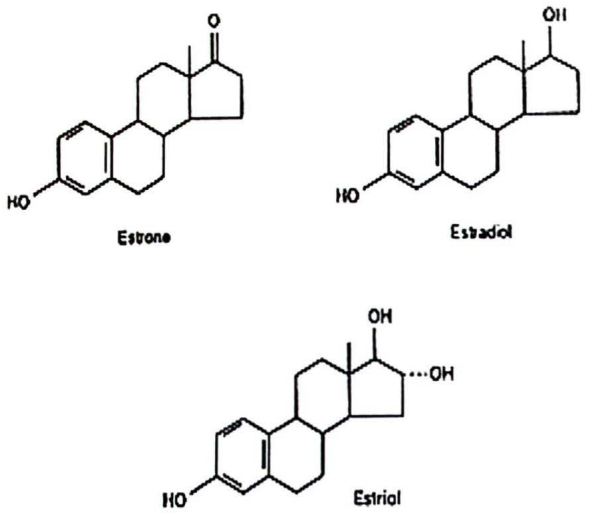

Figure 1. The structural differences between the 3 most common isomers of estrogen (AMA dataset)

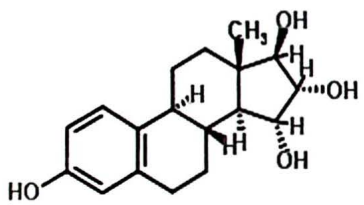

Figure 2. Molecular structure of estetrol. (ChemSpider)

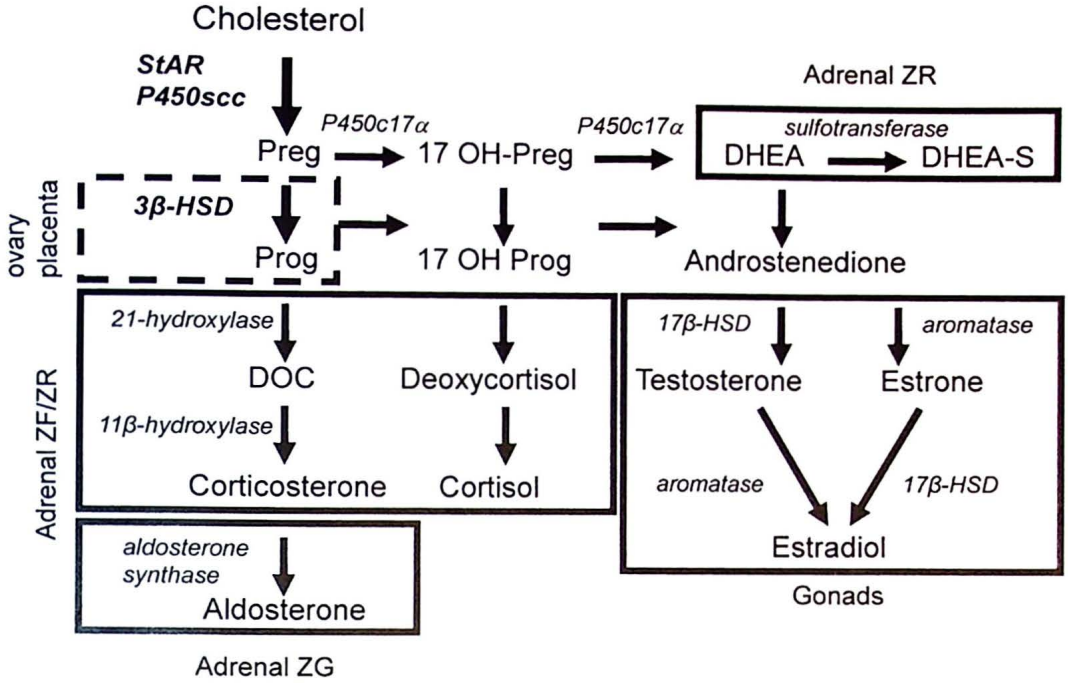

Figure 3. The steroidogenesis conversion pathway from cholesterol to estradiol (H. LaVoie & S. King, 2009)

2.2 Estrogen receptors

There are three main types of estrogen receptors (ER): ERα, ERβ, and g-protein coupled estrogen receptor (GPER) (R. Prossnitz & M. Barton., 2011). The ERα and ERβ are most alike in structure and function while the GPER is different in its overall structure and more importantly, function (R. Prossnitz & M. Barton., 2011). The genetic sequence for ERα and ERβ are 96% similar while the ligand binding region is 56% similar in structure (Z. Papoutsi et al., 2009). The binding domains of these structures are apparently not particularly selective as several compounds other than estrogens can bind to these receptors with ease such as flavons, isoflavones and flavonoids (L. Coward et al., 1993, K. Korach et al, 1988). The GPER is similar to other g-protein coupled receptors (GPCR) in that they are both seven pass transmembrane proteins that promote activation of adenylyl cyclase leading to protein kinase A (PKA) activation

(E. Filardo, & P. Thomas, 2012, E. Prossnitz & M. Barton, 2009). The GPER mode of action is analogous to GPCR action (E. Filardo, & P. Thomas, 2012).

ERα and ERβ activation can have very different effects on target cells (K. Dahlman-Wright et al., 2006). ERα functions mainly as a transcription factor for up-regulation of genes needed for cell growth (S. Hayashi et al, 2003). Conversely, ERβ agonists have been shown to slow or even stop cell growth (N. Heldring et al, 2007). However, they can work with one another by forming a heterodimer to activate genes or aid in the proliferation of breast cancers (E. Fox et al., 2008, E. Powell & W. Xu, 2008). Both ERα and ERβ, when bound to their ligand, can bind to control elements on the target genes and initiate transcription (N. Heldring et al, 2007). Activation of transcription is prompted when coregulators bind to the ER to form a protein complex that modifies the structure of the DNA and assists in the enlistment of RNA polymerase II to the transcription site (N. Heldring et al, 2007). Target genes include nuclear receptor interacting protein 1 (NRIP1), growth regulation by estrogen in breast cancer 1 protein (GREB1) and ATP binding cassette A3 protein (ABCA3) (C. Lin et al., 2004).

ERα is mostly found in the nucleus of the cell but is also found, albeit in significantly lower numbers, on the cell membrane (M. Razandi et al., 1999, S. Kocanova et al., 2010). Classically, it operates by binding to estradiol which causes a conformational change (dimerization and release of repressors) in the protein's structure and leads to binding to control elements on its target genes, up-regulating their expression (S. Kocanova et al., 2010). ERα regulates the expression of target genes through its site-specific interaction with DNA and with other co-regulatory proteins. The estradiol activated ER dimer binds to estrogen response elements (ERE), which are DNA specific sequences within the promoter (R. Kumar et al., 2011). The ERE regulates transcription through the recruitment of general transcription machinery (R.

Kumar et al., 2011). Overall, ERα binding causes an increase in cell proliferation and is one of the most upstream elements in the nuclear activation pathway in estrogen responsive tumor cells (S. Catalano et al., 2004). ERα also down-regulates its own receptors when bound to estrogen in a negative feedback loop in estrogen responsive tumor cells (S. Catalano et al., 2004).

As mentioned, ERα is also found on the cell membrane and can act as a rapid cell signaling receptor (M. Razandi et al., 1999, M. Barton., 2012, S. Catalano et al., 2004, R. Song et al., 2002). Membrane bound ERα has been shown to elicit rapid changes in the intracellular mediator concentrations such as cAMP and calcium ions. These rapid effects have been called nongenomic to distinguish them from classical ERα action as a transcription factor (R. Song et al., 2002). Estrogen stimulation of membrane associated ERα in MCF-7 cells is a precursor to activation of the mitogen-activated protein kinase (MAPK) pathway. Activation of the MAPK pathway leads to a plethora of biological and morphological changes such as formation of membrane ruffles, pseudopodia, increases in nitric oxide (NO) synthesis, and increased intracellular calcium ion levels (Z. Zhang et al., 2002). The MAPK pathway can also lead to changes in gene expression.

As mentioned ERβ is found mainly in the nucleus of the cell but like ERα is also found at the cell membrane; however, its roles are very different from ERα (M. Razandi et al., 1999). As stated before, estrogen bound to ERβ drives cells into an apoptotic state through the genomic MAPK pathway (F. Acconcia et al., 2005). ERβ can also affect ERα activity as it has been shown to reduce ERα transcription in bones in mice as shown by mRNA quantification verified by Western Blot analysis. (A. Börjesson et al., 2010). Similar to ERα, ERβ may also have rapid cell signaling activity (M. Barton., 2012). Overall, the ERα and ERβ relationship can be

summarized as "ying-yang" where one counteracts the activities of the other (M. Lindberg et al., 2003). However, the MCF-7 cells show only low levels of ERβ (E. Vladusic et al., 2000)

A third type of ER, g-protein coupled estrogen receptor (GPER), formerly called GPR30, is a transmembrane receptor that induces rapid cellular signaling (R. Prossnitz & M. Barton., 2011, M. Barton., 2012). GPERs are not only found on the cell membrane but are also active on the endoplasmic reticulum (C. Revankar et al., 2005). These membrane bound receptors are known to activate the MAPK, PKA, adenylyl cyclase and phosphatidylinositide 3-kinase (PI_3K) pathways when bound to 17β estradiol, allowing a variety of actions to occur including an increase of intracellular calcium and rising levels of nitric oxide (NO) (R. Prossnitz & M. Barton., 2011, M. Barton., 2012). The results of these events are otherwise considered non-genomic, rapid cellular signaling functions of the receptor (M. Razandi et al., 1999, T. Improta-Brears et al., 1999). Activation of GPER can change the metabolism in the short term and ultimately change gene expression in the long term (M. Maggiolini, A. Vivacqua, G. Fasanella, 2004, R. Lappano et al., 2012)

The pure anti-estrogen ICI 182, 780 and anti-cancer drug tamoxifen, act as antagonists of the classical ERs but agonists on the GPER, quite possibly reducing the overall effectiveness of these therapies, by activating "non-genomic cellular signaling effects" (C. Revankar et al., 2005, E. Prossnitz & M. Barton, 2009). Additionally, EDC's have been shown to bind to the GPER as agonists as well (N. Chevalier, A. Bouskine, & P. Fenichel., 2012). GPER expression is apparent in several cancer cell lines including MCF-7 cells and its overexpression is closely associated with more aggressive and invasive cancers (SKBR3 and HL-60). (R. Prossnitz & M. Barton., 2011, C. Blesson & L. Sahlin, 2012). GPER also play roles in the nervous, immune, reproductive, and cardiovascular systems (R. Prossnitz & M. Barton., 2011).

2.3 Physiological effects of estrogens

Present in both male and female alike, estrogens play an important role in reproduction, sexual development, libido and sexual differentiation (R. Hess et al., 1997, J. Warnock et al., 2005, V. Rochira & C. Carani., 2009). Estrogen during female development is essential to development of the reproductive tract, and secondary sex characteristics. In mature females estrogens regulate the reproductive cycle and play a role in increasing bone density. In terms of sexual development, estradiol is responsible for feminization of the brain, degeneration of the Wolffian duct system and progression of the Mullerian duct system (J. Bakker et al., 2003, M. Mccarthy., 2008). It has been shown that estradiol is essential during development and in adulthood for normal sexual behaviors in female mice (J. Bakker et al., 2002). Female mice that did not produce or were injected with estradiol, did not pick up on receptivity cues during reproductive phases with male mice (J. Bakker et al., 2002). During female development, the brain expresses high levels of receptors for estradiol. Furthermore, during brain development, the effects of estradiol are generally permanent. (M. Mccarthy, 2008).

Estrogens also play a vital role in mental health, particularly in females (S. Douma et al., 2005, G. Lasiuk, & K. Hegadoren., 2007). Estradiol's effects on mental health are complex and change longitudinally across ages groups (Rasgon et al., 2003). The relationship of estradiol oral contraceptives to major depressive disorders appears to be dose dependent as early generation oral contraceptives showed a greater risk to disorders than the newer lower estrogen mixtures (S. Robinson et al., 2004). However, some studies report positive results with estradiol treatment for women with postpartum depression, perimenopausal or post-menopausal depression (Rudolph et al., 2004). Additionally, estradiol has been shown to have protective effects on the brain particularly during hypoxia/ischemia (M. McCarthy., 2008).

In males, estrogen is produced by the brain and testis through the enzyme aromatase (C. Roselli, S. Abdelgadir & J. Resko., 1997). During sexual development, studies suggest that estrogen plays a role in testosterone production from Sertolli cells and possibly cell adhesion in developing testis as there a significant concentration of ERβ located in the male reproductive tract (B. Akingbemi et al., 2003, L. O'Donnel et al., 2001, C. MacCalman et al., 1997, R. Hess., 2003). It has been suggested that estradiol plays a role in seminiferous tubule volume, in the absence of androgens (F. Ebling et al., 2000). ERα also is important in reproduction as mice with ERα genes knocked out were rendered infertile due to a lack of reabsorption of fluid in the epididymal ductal lumen resulting in increased testicular weight (R. Hess et al., 1997, R. Hess., 2003). This appears to be the main function of estrogen in the male reproductive tract (R. Hess., 2003).

2.4 Xenoestrogens

Xenoestrogen is a broad term used to classify compounds, both natural and synthetic, that imitate molecular estrogen. Phytoestrogens are the name used for naturally occurring estrogen-like compounds found in plants such as flavons, isoflavones and flavonoids (J. Turner et al., 2007, Z. Dang & C. Lowik, 2005). Synthetic xenoestrogens are those that are/were industrially produced such as polychlorinated biphenyls (PCB), plasticizers, phthalates, alkylphenols, and fluorosurfactants (Diamanti-Kandarakis, et al, 2009).

Naturally found phytoestrogens such as flavons, isoflavones and flavonoids are generally found in soy, fava beans and coffee (L. Coward et al., 1993, P. Kaufman et al., 1997, R. Alves et al., 2010). These estrogen mimicking compounds share a structural similarity to endogenous estradiol and can bind to estrogen receptors acting as agonists or antagonists (S. Mueller., 2004).

Although the relative binding affinities (RBA) of these compounds may be low relative to endogenous estradiol, they are found widespread in foods ingested by humans on a daily basis rendering them just as potent as xenoestrogens found in the environment (G. Kuiper et al., 1998).

Xenoestrogens also generally have a low RBA yet their persistence in the environment makes them a constant threat to the living organisms (N. Kudo, & Y. Kawashima, 2003). Industrially and commercially used synthetic xenoestrogens i.e., BPA, nonylphenol, DDT and others are also widespread throughout the environment. Some xenoestrogens such as PFOA are so widespread that nearly 99% of the Americans sampled between 2003 and 2004 had a detectable level of 4 parts per billion (ppb) (A. Calafat et al, 2007).

A well-studied estrogen mimicking substance is nonylphenol. It is an organic xenoestrogenic alkylphenol found accumulating in sewer sludge and river sediment (A. Soares et al, 2008). Nonylphenol is a metabolite of nonylphenol ethoxylate and is used as an industrial surfactant (A. Soares et al, 2008). Nonylphenol is amphipathic and is used extensively as a solubilizing agent. For example, in the United States 380 million pounds were produced in 2010 alone (Environmental Protection Agency, 2010). Nonylphenol makes its way into household goods via paints, laundry detergents and oil additives. A European Union directive has restricted "the sale and use of products containing more than 0.1% NPE [nonylphenol ethoxylate] or NP" since 2003 (European Union, 2007). Because of its long 60-year biodegradation period, bioaccumulation is evident, particularly in aquatic organisms (A. Soares et al, 2008). Feminization of males and decreases in male fertility are among some of the effects found in aquatic environments where NP levels are detectable (A. Soares et al, 2008). Nonylphenol mimics endogenous estradiol by binding to estrogen receptors *in vitro* (M. Maras et al, 2006) (S. Laws et al, 2000) (R. Blair et al, 2000). As an estrogen mimicker, NP has been shown to increase

proliferation in MCF-7 cells by binding to the estrogen receptors (H. Shen et al, 2003) (M. Soto et al, 1991) (L. Ren et al, 1997). Li et al, 2006 has further demonstrated that NP stimulates MCF-7 proliferation by activation of the MAPK pathway.

Perfluorooctanoic acid (PFOA) is a less well-known endocrine disruptor and has been shown to have detrimental developmental effects on mice (White et al., 2011; (Wei, et al 2007; (M. Ylinen et al, 1985) and more recently has been shown to interfere with estrogen and thyroid hormone signaling in a variety of bioassays (Du et al, 2013). An eight carbon long synthetic compound, PFOA is used widely in hundreds of industrial and manufacturing applications and is a major component of Gore-Tex® and Teflon®. Uses range from nonstick cookware to waxed paper to carpet cleaning solutions because of its excellent water and oil repelling properties due to a hydrophobic and lipophobic fluorocarbon tail (M. Ylinen et al, 1985). Nonetheless, the strong polarity between the carbon and fluorine atoms creates a very stable compound that becomes persistent in nature (R. Renner, 2008) (N. Kudo, & Y. Kawashima, 2003). Because it resists various natural degradation processes, PFOA stays in the environment at relatively high concentrations (N. Kudo, & Y. Kawashima, 2003). The persistence of PFOA in the environment is reflected in the fact that most people in industrialized nations carry a burden of PFOA that is measurable in blood serum. Between 2003 and 2004, nearly 99% of the Americans sampled had a detectable level of 4 parts per billion (ppb) (A. Calafat et al, 2007). Most common exposure to PFOA for humans is through food and water (R. Renner, 2007). These results are supported by the EPA which states it is persistent in the environment, found in the blood of the general U.S. population, and causes developmental and other adverse effects in laboratory animals (EPA).

Perfluorooctanoic acid (PFOA) interacts with organisms mainly through the activation of the peroxisome proliferator-activated receptor alpha (PPARα) which affects gene expression in

cells; one of its endogenous ligands is oleoylethanolamide (OEA), a fatty-acid ethanolamide (G. Kennedy et al, 2004) (J. Fu et al, 2005). The PPARα plays a key role in inflammation, immunity, nutrient metabolism and energy homeostasis as well as the management of energy stores during fasting or a high fat diet, particularly in the liver (S. Kersten et al, 1999) (M. Rakhshandehroo et al, 2010). A ligand-activated transcription factor, PPARα shows the highest expression in hepatocytes, cardiomyocytes, enterocytes, and the proximal tubule cells of kidney (O. Braissant et al, 1996).

Since the MCF-7 cell line expresses the PPARα receptor as well as estradiol receptors, it may seem unclear which receptor PFOA is binding to; however, it is understood that the PPARα activation is responsible for the up-regulation of estradiol receptors in certain instances such as the anti-inflammatory activity of estradiol as was demonstrated when mice that were ovariectomized showed their anti-inflammatory activity is weakened than the wild type. (K. Suchanek et al, 2002, C. Crisafulli et al, 2009).

As a PPARα agonist, PFOA increases the expression of regulatory activities including lipid metabolism and cellular differentiation. Perfluorooctanoic acid mimics the fatty acid ligand for the PPARα. Liver, Leydig, and acinar cell cancers are a result of PFOA exposure through the PPARα activation mechanism, studies suggest (G. Kennedy et al, 2004). However, since these studies were conducted on rodents, the same physiological implications on humans are not necessarily the same (G. Kennedy et al, 2004). Nonetheless, further research is needed to confirm whether the same effect is applied to humans. Other mechanisms in which PFOA operates in humans are not well known (White et al., 2011, E. Hood, 2008).

2.5 E-Screen assay

The E-Screen assay was developed to determine the estrogenicity of possible EDC's with estrogen like properties by utilizing the proliferative effect of estradiol on estrogen responsive cells as a quantitative measurement (A. Soto et al., 1995). It is a high throughput technique which concurrently tests a compound's agonism and antagonism to estrogen receptors. Additionally, the E-screen assay could determine effective inhibitors. It also replaced the rodent bioassays as they were not feasible for screening large numbers of chemicals (A. Soto et al., 1995). The E-screen assay was much more practical, simpler, and cost effective (A. Soto et al., 1995). This assay compares the number of cells treated with no estradiol to those treated with various concentrations of E2 to a range of concentrations of chemicals with possible estrogenic properties (A. Soto et al., 1995).

2.6 Calcium ratioing

Calcium ratioing is a technique where intracellular calcium can be calculated from the fluorescence ratio at certain wavelengths (O. Barreto-Chang & R. Dolmetsch, 2009). One of the most common florescent dyes is Fura-2; its excitation peak is 340nm when calcium is unbound and 380nm when calcium is bound (O. Barreto-Chang & R. Dolmetsch, 2009). A release of intracellular calcium would result in a higher 340/380 ratio than when bound. (O. Barreto-Chang & R. Dolmetsch, 2009). This technique is invaluable for determining which mode of action NP and PFOA operate through.

CHAPTER III MATERIALS AND METHODS

3.1 Chemicals

Dulbecco's Modified Eagle Medium (DMEM; 11058-021) with L-Glutamine and HEPES, heat inactivated fetal bovine serum (FBS), phosphate buffered saline (PBS; 10010-023), Antibiotic/antimycotic 100X (AbAm) was purchased from Life Technologies, Carlsbad, CA. 17β Estradiol (E2; 044K1027), Resazurin sodium salt (Resazurin/Alamar blue; R7017-1G), Perfluoroctanic acid (PFOA; 171468-5G), trypsin-EDTA (trypsin; T4174), Trypan blue (T8154) and 4-nonylphenol (NP; 46405) were purchased from Sigma-Aldrich, St. Louis, MO. Charcoal stripped heat inactivated fetal bovine serum (CS FBS) Atlanta Biologicals, Lawrenceville, GA. The inhibitor G36 (AZ0001303) was purchased from Azano Biotech, Albuquerque, NM. The inhibitor ICI 182-780 (ICI; 1047) was purchased from Tocris Bioscience, Minneapolis, MN. Eagles Minimum Essential Media (EMEM; 30-2003) with phenol red, HEPES, and L-Glutamine obtained from American Type Culture Collection (ATCC), Manassas, VA.

3.2 E-Screen assay

Culture of MCF-7 cells

Cells used for this experiment were estrogen responsive adenocarcinoma breast cancer MCF-7 (HTB-22) and were purchased from ATCC, Manassas, VA. Cells were maintained in 25 cm^2 tissue culture flasks (Becton-Dickinson; 353109) at 5% CO_2 and 37°C in EMEM with 2% antibiotic/antimycotic and 10% fetal bovine serum. Cells were split Monday and fed Wednesday and Friday. Near 80% confluence, flasks are split by loosening cells using trypsin (0.25% w/v)-EDTA (0.03% w/v) solution and resuspended in complete DMEM. After 7 days cells are split into a "withdrawn" flask, devoid of all estrogens by using DMEM (phenol -) media with 5% CS

FBS and 2% antibiotic/antimycotic. Cells are seeded in the withdrawn flask at 1.0 x 10^5 cells/mL and allowed to incubate for another 7 days. Cells are fed again on Wednesday and Friday. Cells numbers were determined using a hemocytometer (American Optical, Buffalo, NY) with trypan blue staining using the Sigma-Aldrich protocol for trypan blue.

Plating and treatment

After seven days in withdrawn flask, cells were trypsinized and collected from the flask and plated onto a flat bottom 96-well plate (Costar; 3596). Cells are plated at a density of 1.8 x 10^5 cells/mL by placing 100µL of cell suspension is micropipetted into each well and allowed to incubate for 24 hours. Culture mediam was then removed and cells were treated with their respective treatments in DMEM. *See Appendix 1.* Seventy-two hours later old media/treatment were removed and retreated with fresh media/treatment. Cells are allowed to incubate for an additional 72 hours.

Experimentation

On the day of experimentation (Monday), 10µL of 5% resazurin was added to each well on the plate and allowed to incubate for an additional 2 hours. After the 2-hour incubation time, the plate was then transferred onto the UVP Biospectrum 810 plate reader (UVP, Upland, CA). The UV light was turned on and the emission filter was set to 570-640nm.

A region of interest (ROI) was selected for each well. The blank wells were used as a "blank" to set up a background baseline fluorescent level. Each ROI was analyzed and quantified for its mean density measured by fluorescence as shown by Fig. 4.

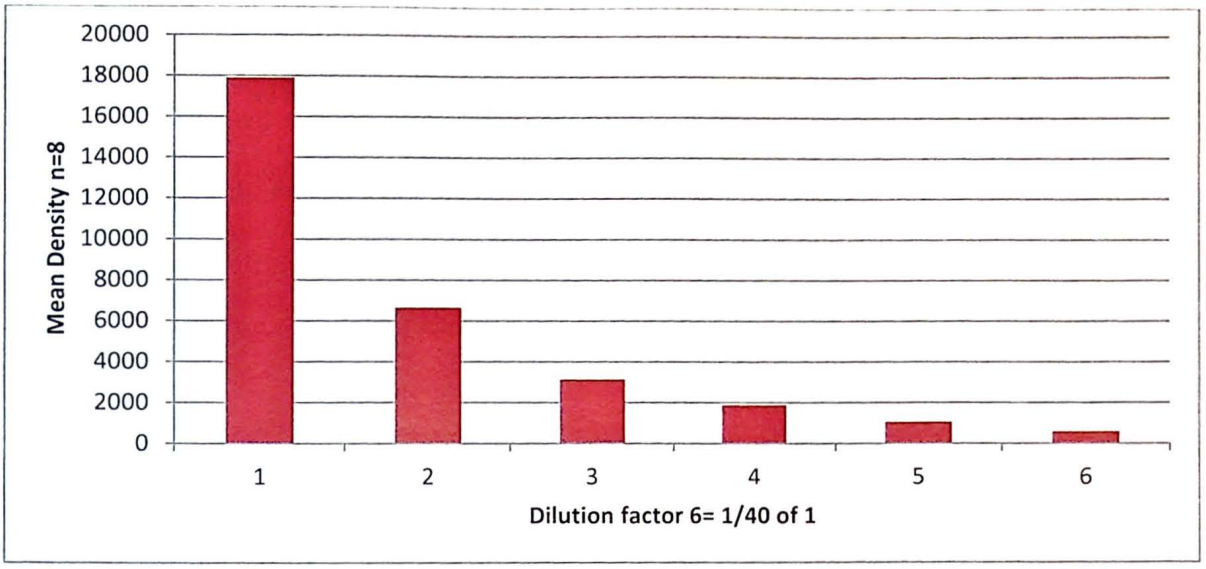

Figure 4. Mean density of MCF-7 cells as measured by fluorescence on the BioSpectrum 810 plate reader. Dilutions of 1:10 were made in serial dilutions six times to demonstrate the increasing fluorescence correlating with the increasing cell concentration.

3.3 Calcium ratioing

Cell culturing

Cells were maintained in T-25 seed flasks at 5% CO_2 and 37°C in EMEM with 2% antibiotic/antimycotic v/v and 10% fetal bovine serum v/v. Cells were split Monday and fed Wednesday and Friday. Cells were allowed to grow for 7 days before splitting into another seed flask. After 7 days cells are split into a "withdrawn" flask, devoid of all estrogens by using DMEM media with 5% CS FBS and 2% antibiotic/antimycotic. Cells were seeded in the withdrawn flask at 1.0 x 10^5 cells/mL and allowed to incubate for another 7 days. Cells were fed again on Wednesday and Friday. Cells were counted with a hemocytometer using the Sigma-Aldrich protocol for Trypan blue.

Plating

After 7 days in withdrawn flask, cells were trypsinized and collected from the flask. Approximately 1mL of cell suspension was pipetted and plated onto a 35mm glass bottom dish. Cells were suspended in DMEM and allowed to incubate. Plates were fed Wednesday, Friday and the following Monday. Glass bottom 35mm dishes were obtained from MatTek Corporation (Ashland, MA).

Experimentation

On the day of experimentation (Tuesday), cells were washed with 3 times with Hanks Balanced Salt Solution (HBSS) pre-warmed to 37°C. Plate was then removed of all HBSS and replaced with HBSS .05% bovine serum albumin (BSA). Afterwards, 24μL of 1.2% pluronic/400μM Fura-2 AM solution was added to the plate and swirled to distribute the solution evenly. Plate was then placed in an incubator away from light at 5% CO_2 and 37°C for 45 minutes. After incubation, plate was removed from incubator and washed 3 times with HBSS. The plate was then replaced with 1.8mM $CaCl_2$ in HBSS + .05% BSA and allowed to incubate again for 15 minutes. After incubation, plate was then placed on the Nikon Ti Eclipse microscope (Nikon Instruments, Melville, NY) for visualization using excitation at 340nm and 380nm with emission of 510 nm captured by Andor camera and analyzed by NIS Element software (Nikon). Once all parameters were set up on the software, the experimentation was started for 20 minutes. At the 1:15 minute mark, 200μL of the respective treatment added to the plate. After the full 20 minutes of experimentation and detection, region of interest (ROI's) were selected on individual cells on the video and analyzed for changes in calcium ratio (340/380). In developing the method to measure Ca^{++} release in the MCF-7 cells, two ionophores, Ionomycin

(Life Technologies; I-24222) and Calcimycin (A-23187) (Invitrogen; A-1493) were tested before finally using ATP as the positive control in subsequent experiments. ATP was selected because it is a known physiological agent that causes calcium release (A. Rossi et al., 2002).

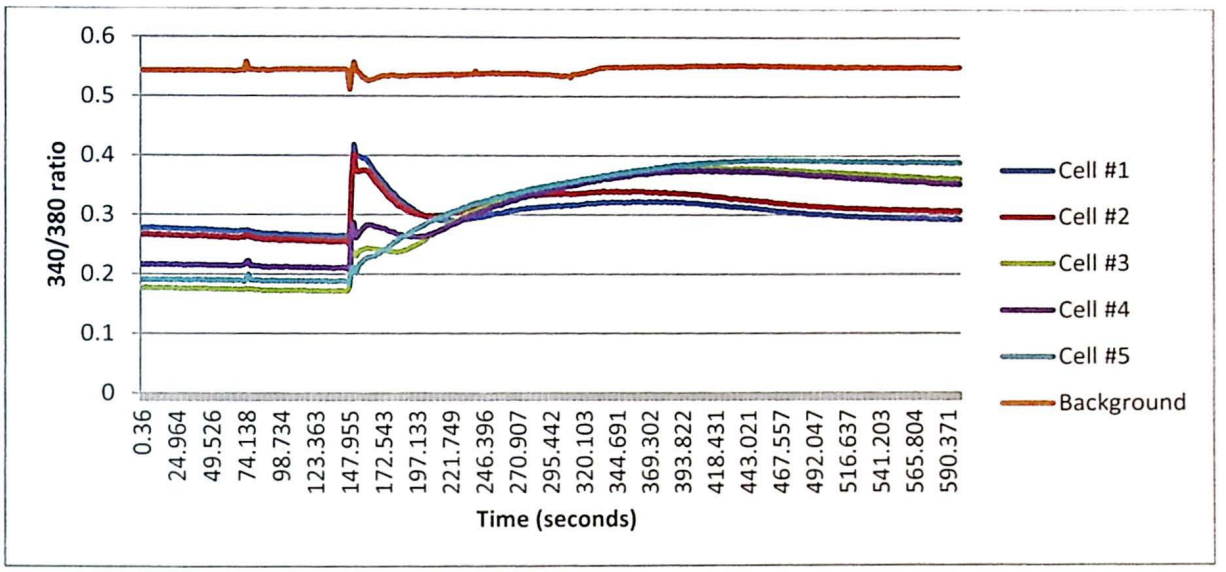

Figure 5. The effect of Ionomycin ionophone on MCF-7 cells.

3.4 Statistical Analysis for E-Screen Assay

Each treatment concentration had four replicates on each 96 well plate; a total of four 96 well plates were tested. Data were analyzed using a one-way analysis of variance (ANOVA) with mean density as a product of treatment. All data were analyzed using JMP 11 Pro software (SAS Institute, Inc., Cary, NC). A $p \leq 0.05$ specified statistically significant results. Data were compared using Tukey Kramer HSD connecting letters report.

3.5 Statistical Analysis for Calcium Ratioing

All ROI's were evaluated for maximum change in the 340/380 ratio. A minimum of 5 ROI's were selected for each treatment plate. The ROI's were then averaged for each treatment

plate. Data were analyzed using a one-way ANOVA. A $p \leq 0.05$ specified statistically significant results. Data were compared using Tukey Kramer HSD connecting letters report.

CHAPTER IV RESULTS

4.1 E-screen assay

Replicates

Each 96 well plate had four replicates of each treatment. *See appendix I* for experiment layout. A total of four 96 well plates were run during the length of the entire experiment. The results below are displayed as mean density measured by fluorescence. Each well was defined as a specific ROI and measured according to its area. Representative experiments are shown in Figures 6-12. Results from all passages may be seen in *Appendix II.*

In this study perfluorooctanoic acid (PFOA, 10^{-8}M or 10^{-7}M, dependent on passage), nonylphenol (NP, 10^{-8} or 10^{-7}M, dependent on passage), and estradiol-17β (E2, 10^{-10}M) significantly increased proliferation of MCF7 cells compared to the control determined by bioreduction of resazurin dye. This proliferation was effectively inhibited by ICI 182, 780 (10^{-7}M), a high affinity estrogen receptor antagonist for estrogen receptor α. Proliferation was not inhibited by G36 which selectively inhibits GPER but not ERα. Neither NP nor PFOA when added to cells in combination with E2 inhibited the E2 proliferative effect. However, the combination of PFOA and E2 resulted in greater proliferation than either alone. The combination of NP and E2 did not consistently stimulate proliferation greater than E2 alone.

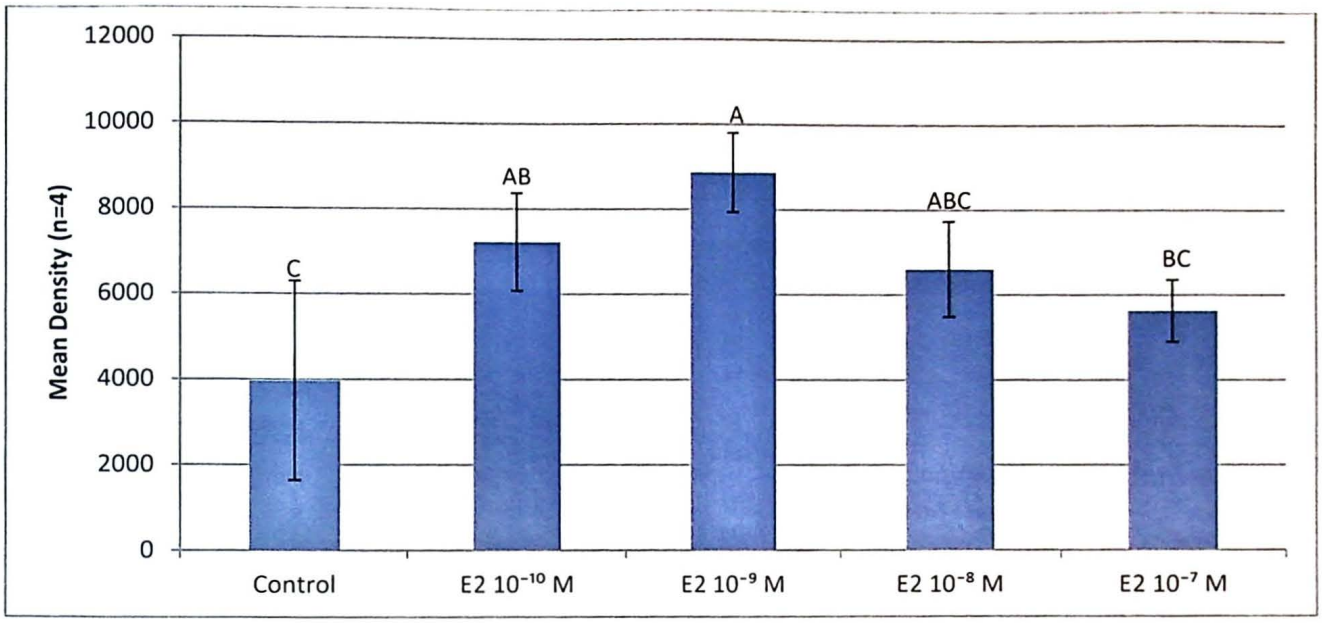

Figure 6. The effect of E2 on proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M and E2 10^{-9} M treatments were statistically different than the control, contrary to the E2 10^{-8} M and E2 10^{-7} M treatments which were not. This suggests that at least two concentrations of E2 had a proliferative effect on the cells.

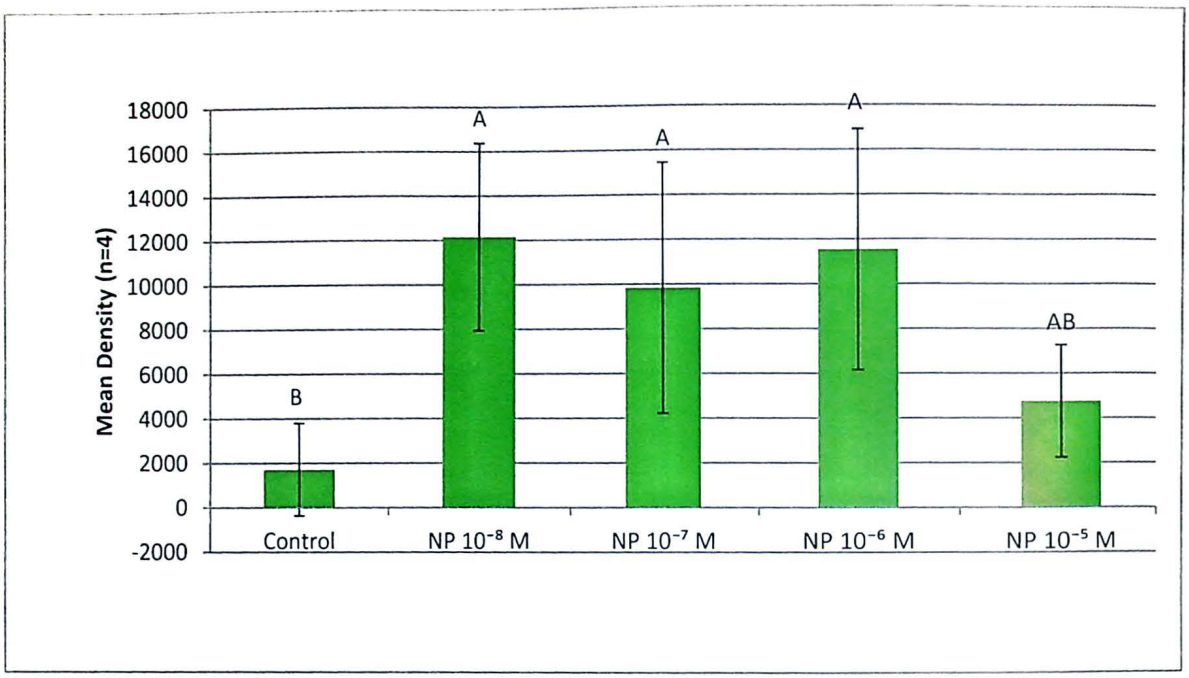

Figure 7. The effect of NP on proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. All concentrations of NP were significantly different than the control except NP 10^{-5} M. This suggests that at least three concentrations of NP induced a proliferative effect on the cells.

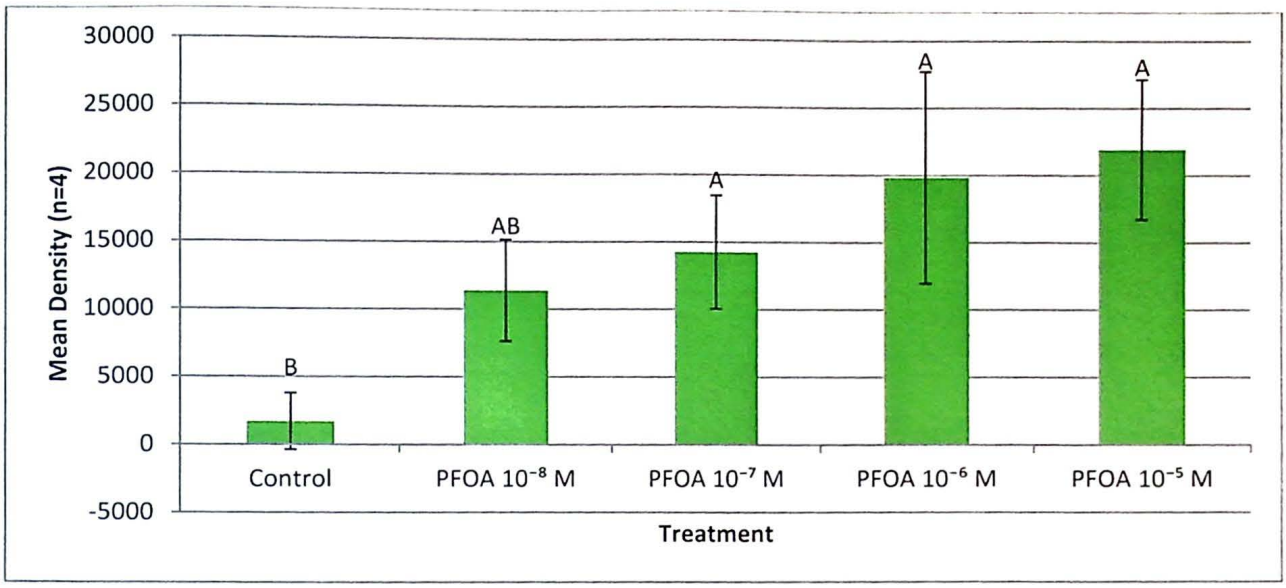

Figure 8. The effect of PFOA on proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. PFOA 10^{-8} M was not significantly different than the control but all other concentrations of PFOA were different. This suggests that at least three concentrations of PFOA induced a proliferative effect on the cells.

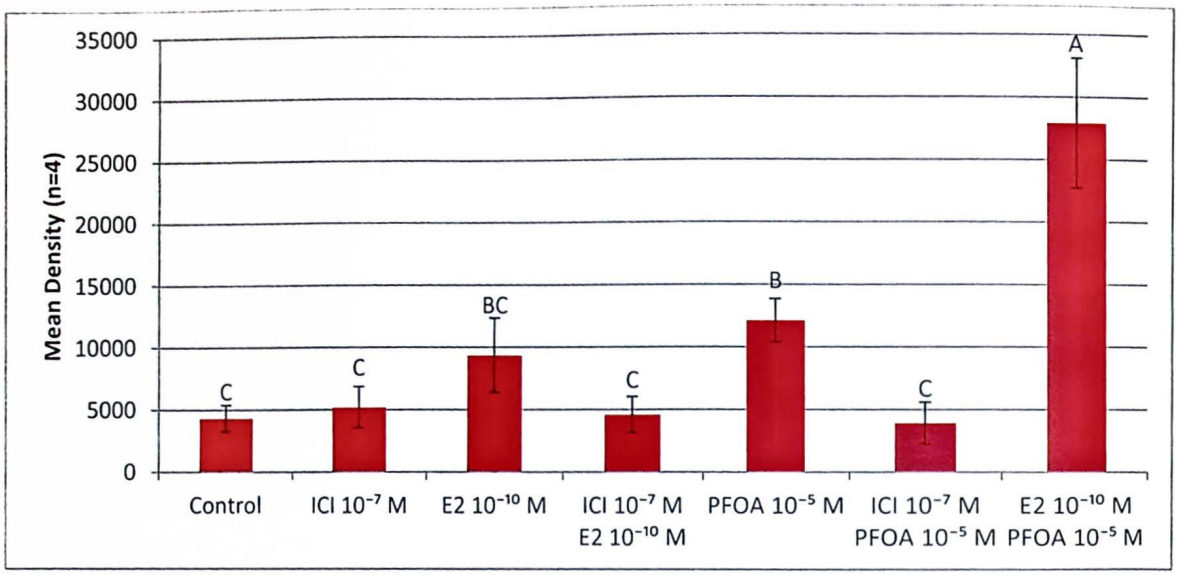

Figure 9. The effect of PFOA and ICI on proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M treatment was not significantly different than the control, PFOA or any combination of the inhibitors. However, the E2 10^{-10} M + PFOA 10^{-5} M treatment was significantly different than the E2, control, PFOA and all combinations of inhibitors. The PFOA 10^{-5} M treatment was significantly different than all inhibitor combinations. This suggests that the ICI inhibited the proliferative effect of PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect greater than E2 alone.

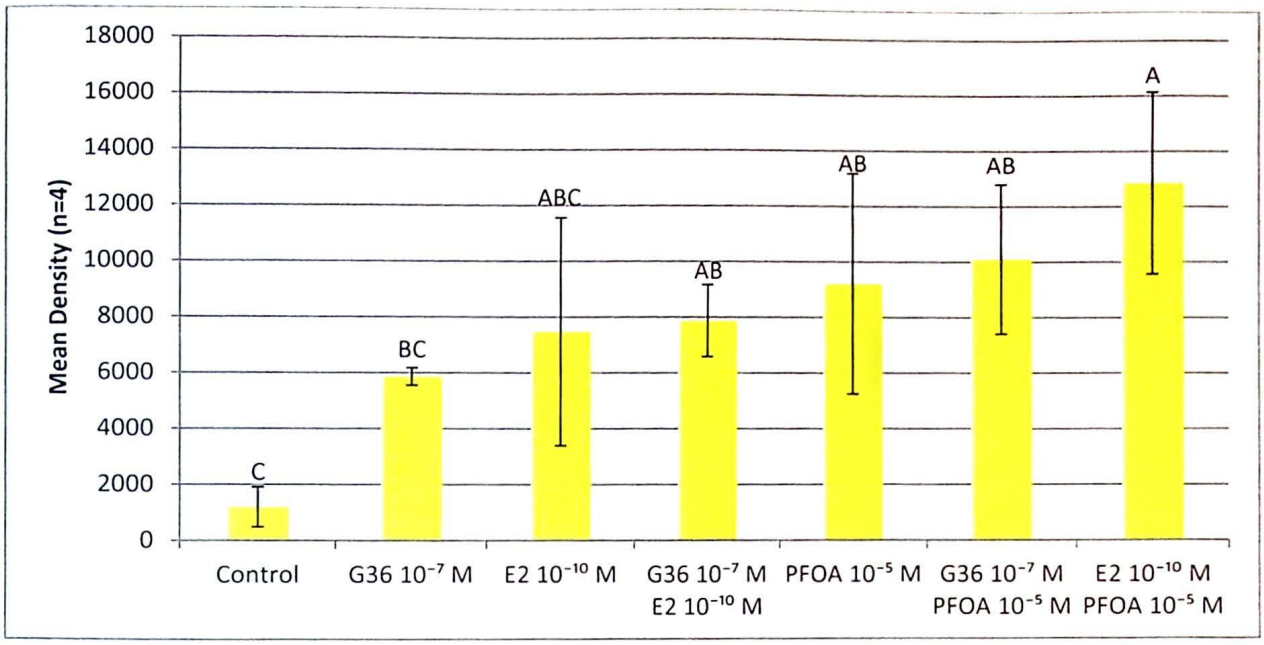

Figure 10. The effect of PFOA and G36 on proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. PFOA 10^{-5} M was not significantly different than the G36 10^{-7}+ PFOA 10^{-5} M, E2 10^{-10} + PFOA 10^{-5}M, or E2 treatments. However, G36 10^{-7}M was higher, albeit not significantly higher. PFOA 10^{-5}M was significantly different than the control. This suggests that G36 did not effectively inhibit the proliferative effect of the PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

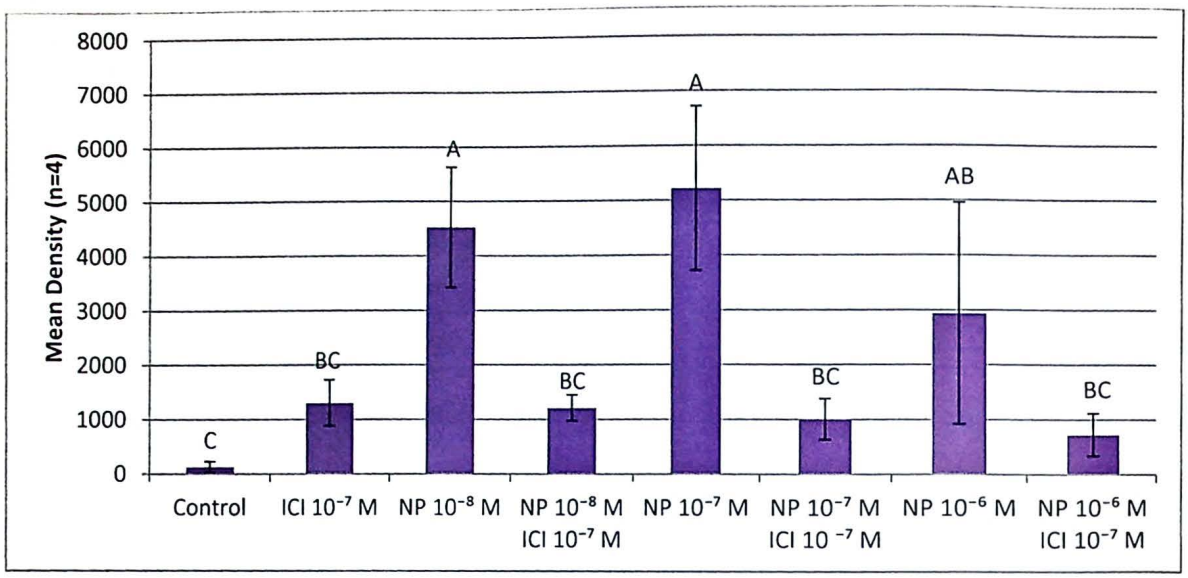

Figure 11. The effect of NP and ICI proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. Treatments of NP at the 10^{-7} M and 10^{-8} M concentrations were statistically different than the corresponding treatment with ICI suggesting ICI blocked the effects of NP at those concentrations. However, the control, NP 10^{-6} M + ICI 10^{-7} M and NP 10^{-6} M were not statistically different. This suggests that ICI 10^{-7} M did not effectively block the effects of NP on the cells at that concentration.

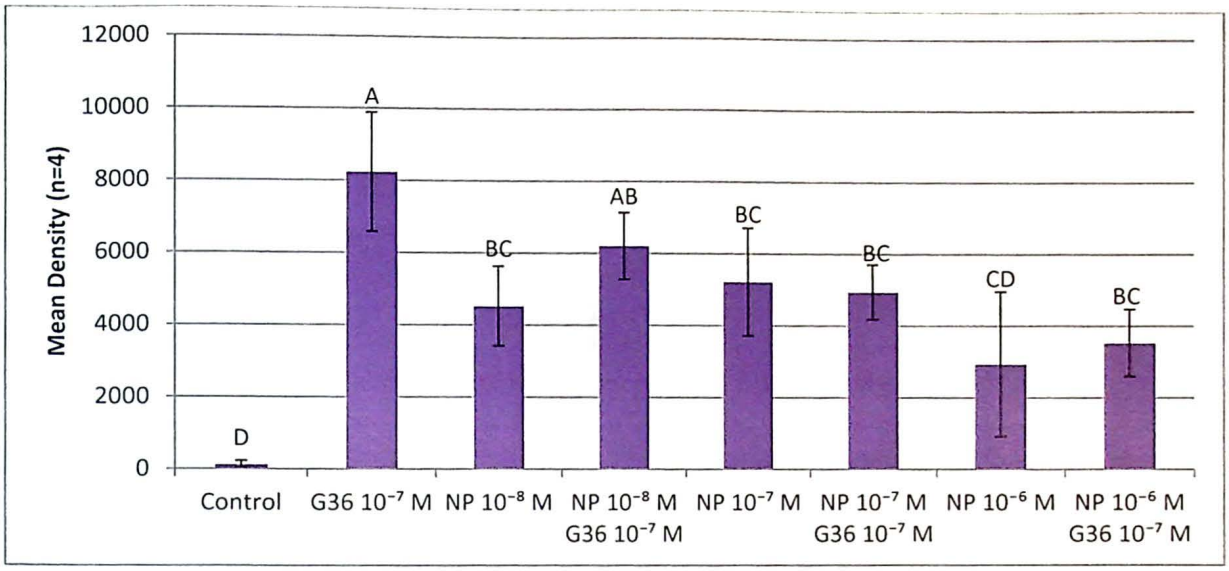

Figure 12. The effect of NP and G36 on proliferation of MCF-7 cells shown as mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. None of the concentrations of NP + G36 were statistically different than its corresponding concentration of NP. However, all treatments were statistically different than the control. This indicates that G36 had little or no effect on blocking the effects of NP.

4.2 Calcium ratioing

Alone, E2, PFOA, and NP failed to induce a detectable Ca^{++} flux in MCF-7 cells. However, when coupled with ATP, which triggers a transitory Ca^{++} flux in MCF-7 cells, these estrogenic compounds appear to modulate the recovery phase of ATP induced Ca^{++} release. A frame of cells were recorded for 20 minutes on each treatment/plate and the results are displayed below in terms of mean change in 340/380 ratio over a period of time (20 minutes).

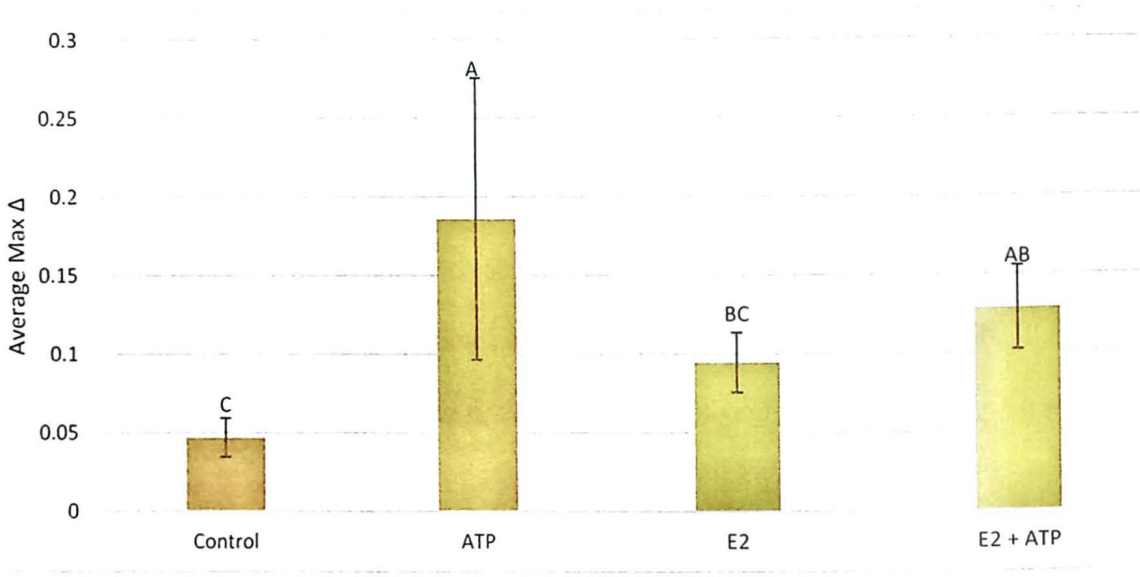

Figure 13. The mean maximum change in 340/380nm λ in at least 5 or more ROI's. Treatments not connected by the same letter are statistically significantly different. ATP and E2 + ATP were significantly different than the control and E2 alone but not from each other suggesting that the two highest responding treatments did cause a Ca^{++} flux. E2 was not significantly different than the control suggesting that E2 did not cause a significant flux of Ca^{++}.

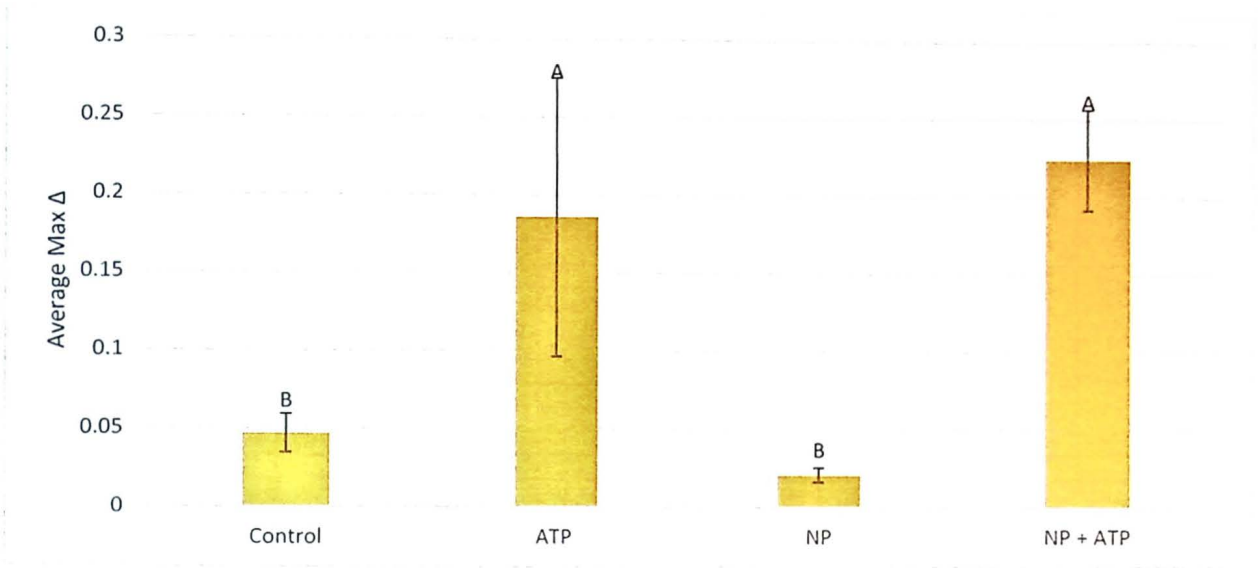

Figure 14. The mean maximum change in 340/380nm λ in at least 5 or more ROI's. Treatments not connected by the same letter are statistically significantly different. ATP and NP + ATP were significantly different than the control and NP alone but not from each other suggesting that the two highest responding treatments did cause a Ca^{++} flux. NP was not significantly different than the control suggesting that NP did not cause a significant flux of Ca^{++}.

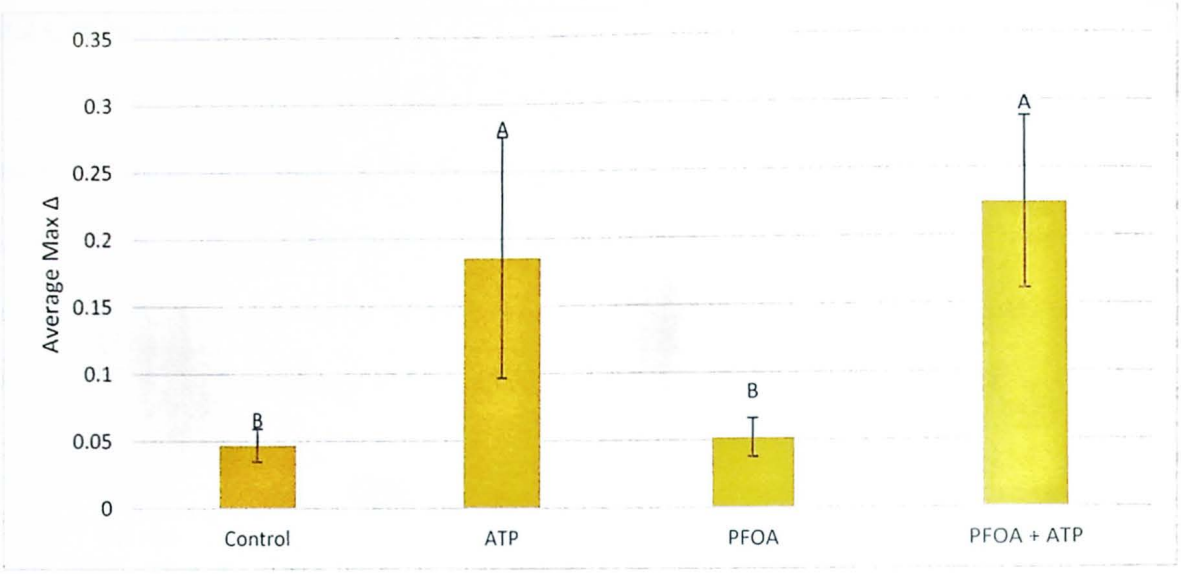

Figure 15. The mean maximum change in 340/380nm λ in at least 5 or more ROI's. Treatments not connected by the same letter are statistically significantly different. ATP and PFOA + ATP were significantly different than the control and PFOA alone but not from each other suggesting that the two highest responding treatments did cause a Ca^{++} flux. PFOA was not significantly different than the control suggesting that E2 did not cause a significant flux of Ca^{++}.

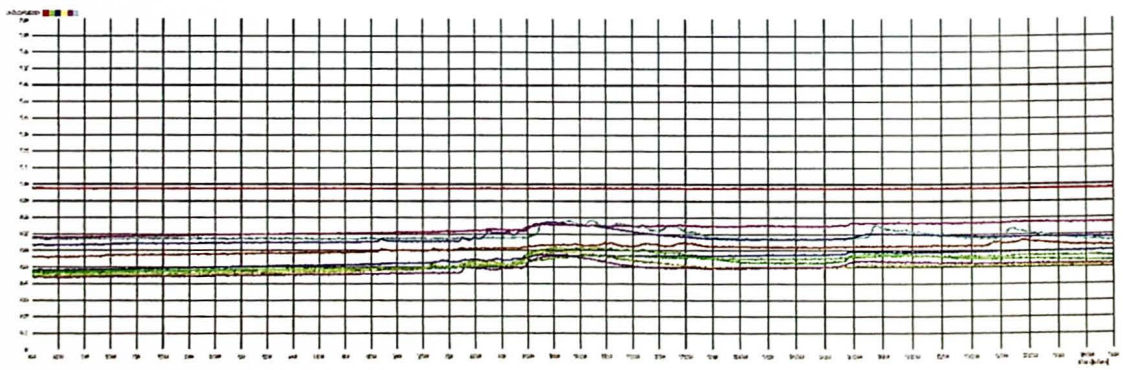

Figure 16. The release and eventual recovery of basal Ca^{++} levels in MCF-7 cells when treated with ATP 10μM.

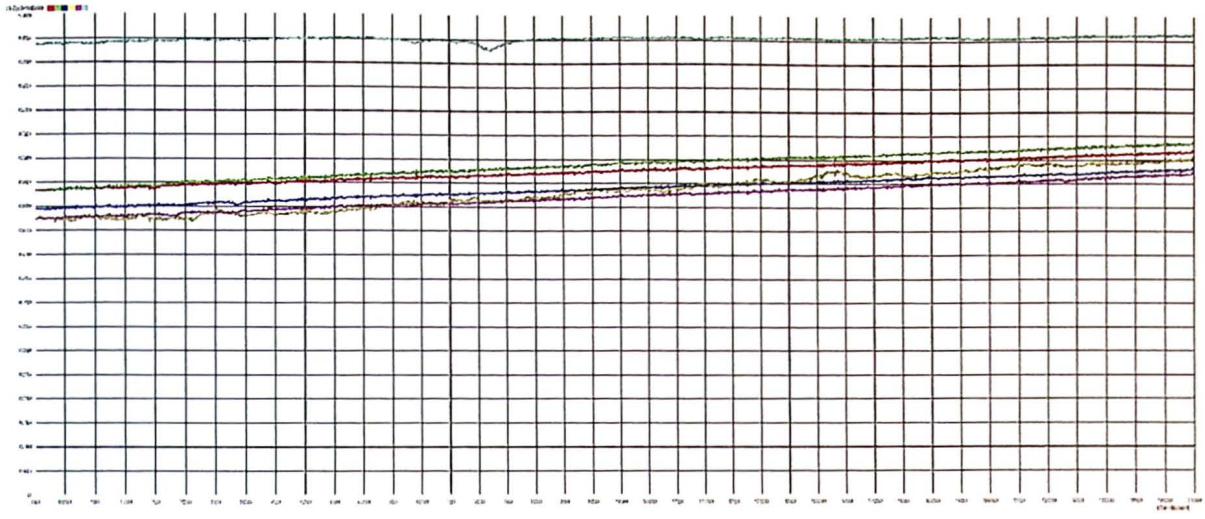

Figure 16. MCF-7 cell Ca^{++} ratioing after treatment with E2.

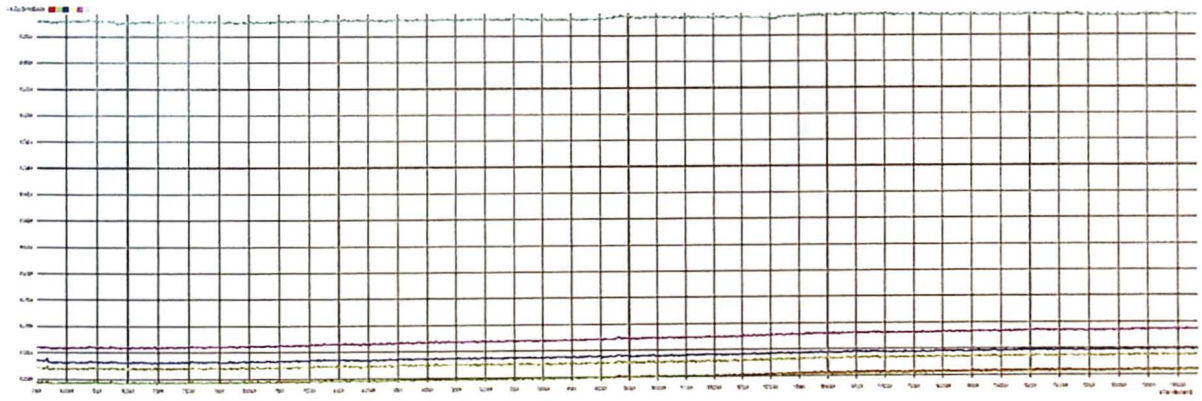

Figure 17. MCF-7 cell Ca^{++} ratioing after treatment with NP.

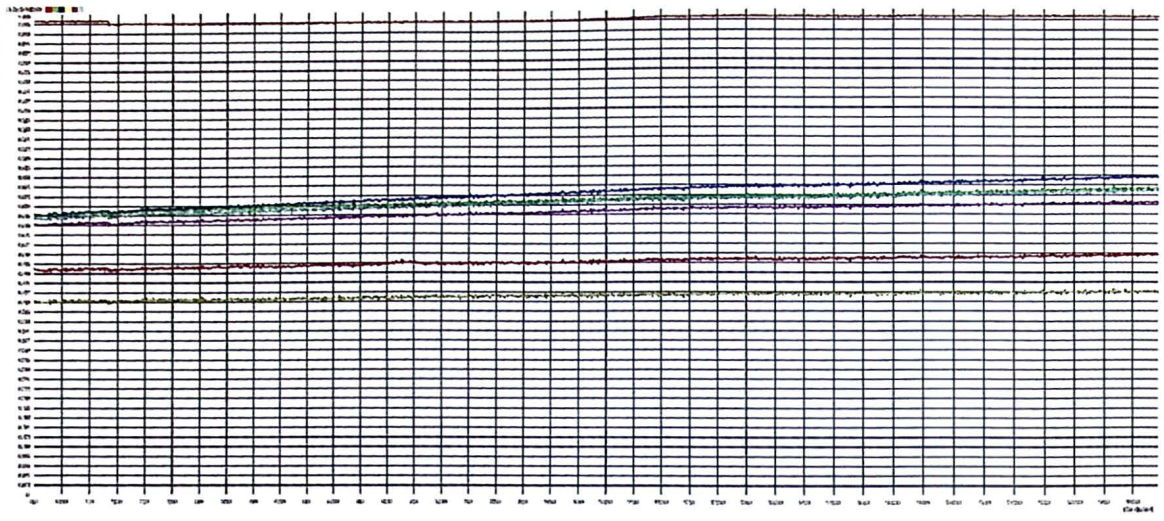

Figure 18. MCF-7 cell Ca^{++} ratioing after treatment with PFOA.

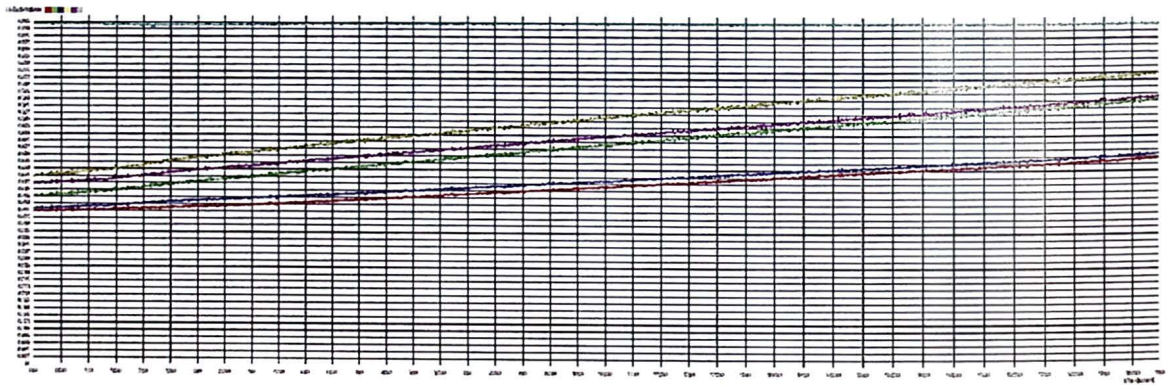

Figure 19. The steady release of Ca^{++} in MCF-7 cells when treated with PFOA coupled with ATP 10μM.

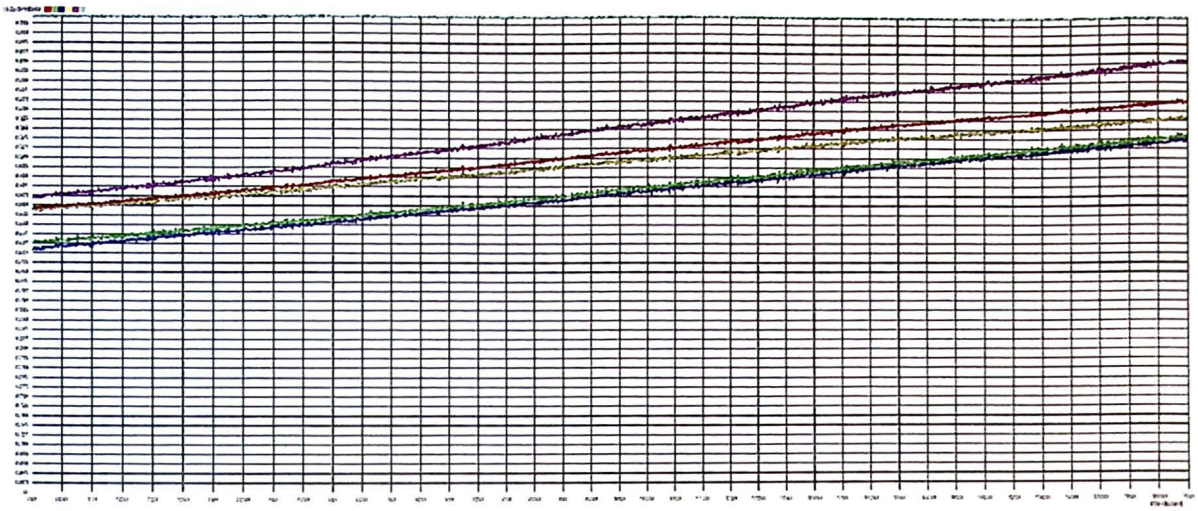

Figure 20. The steady release of Ca++ in MCF-7 cells when treated with NP coupled with ATP 10μM.

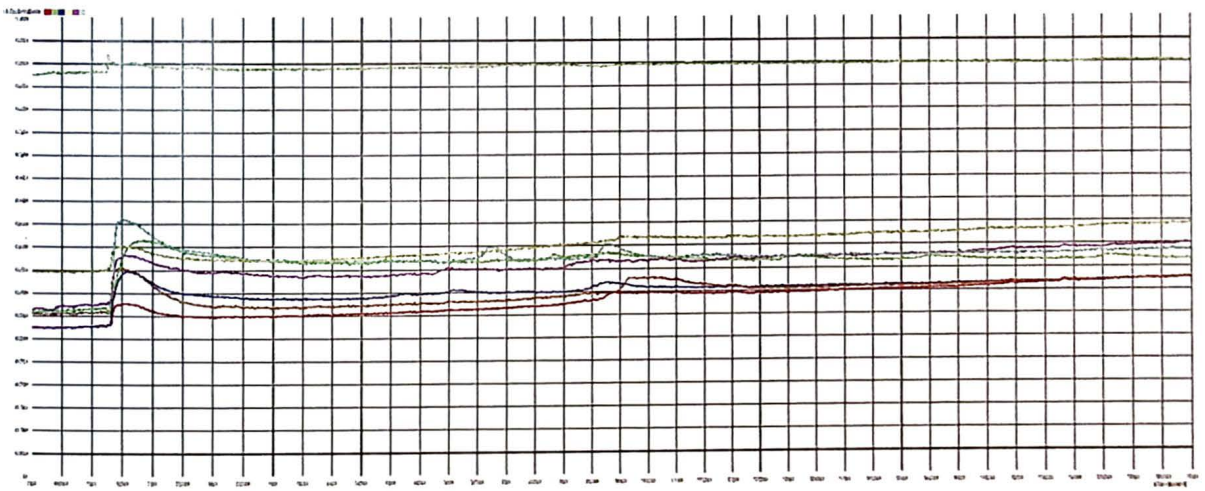

Figure 21. The steady release of Ca^{++} in MCF-7 cells when treated with E2 coupled with ATP 10μM.

CHAPTER V DISCUSSION

The objective of this research project was to screen PFOA and NP, found in everyday household goods, for estrogenic properties and determine to which estrogen receptor they bind. Nonylphenol and perfluorooctanioc acid were screened with the epithelial adenocarcinoma breast cancer MCF-7 cells. Compounds with estrogenic properties were then evaluated for calcium releasing properties through calcium ratioing. The results of this project demonstrate that both NP and PFOA possess estrogenic properties in at least one concentration in the E-Screen assay and E2, NP and PFOA, when coupled with ATP cause calcium flux.

The MCF-7 cell assay can be used as a tier one screening assay for estrogenic and anti-estrogenic compounds through the quantification of resazurin fluorescence. The MCF-7 cells are highly responsive to E2 (T. Wiese et al., 1992). Estrogen receptors are ligand activated transcriptions factors. When E2 binds to the ER, it causes a structural change in the protein which then allows it to bind to the control elements on the target genes (N. Heldring et al, 2007). Compounds that imitate endogenous estradiol will act as agonists on the receptors and cause an increase in proliferation (K. Korach et al, 1988).

Estradiol did show a proliferative effect in MCF-7 cells, most often the E2 10^{-10} M and E2 10^{-9} M concentrations, while higher concentrations did not appear to have as strong of an effect resulting in a non-statistically significant difference from the control. The results are supported by A. Soto & C. Sonnenschein (1985). The estradiol binds to the ERα which activates transcription factors which, in turn, increase cell division (S. Hayashi et al, 2003).

Perfluorooctanoic acid did show strong estrogenic properties in MCF-7 cells in nearly every passage. PFOA is a known endocrine disruptor and operates through the ER (G. Du., et al

2013). These results indicate that PFOA binds to ERα and imitates endogenous estradiol. The results are supported by E. Hood (2008) and Wei et al (2007). PFOA was effectively inhibited by the ER inhibitor ICI but not by the GPER inhibitor G36. Based on these results, in contrast to what was previously hypothesized, PFOA operates, at least in part, through the ERα and induces increased proliferation. Additionally, PFOA did not show anti-estrogenic properties; on the contrary, PFOA displayed a synergistic effect with estradiol to increase proliferation at an even faster rate on more than one occasion. This possibly could be due to PFOA activating transcription factors on the PPARα in addition to the activating the ERα causing the increased proliferation (G. Kennedy et al, 2004, C. Lau et al., 2007). Another plausible hypothesis, as stated before, PPARα activation is responsible for the up-regulation of estradiol receptors in certain instances (K. Suchanek et al, 2002, C. Crisafulli et al, 2009). As a result, the cells exposed to PFOA in addition to E2 saw an up-regulation of ERs due to the PFOA activating the PPARα and E2 binding to the ER thus causing the synergistic-like reaction as seen in nearly all passages.

Nonylphenol also showed strong estrogenic properties in the MCF-7 cells in nearly every passage. The last passage NP was used in various concentrations when combined with the inhibitors ICI and G36 since the concentration used in the previous passages were not statistically different than the control. G36 failed to inhibit NP proliferation while ICI effectively inhibited proliferation. After analysis of these results, it was determined that NP binds to the ERα and not the GPER and increases proliferation through that route and not the GPER pathway. Additionally, NP did not consistently show anti-estrogenic properties.

The MCF-7 cells did not show a significant Ca^{++} flux when treated with estradiol, NP and PFOA alone. However, when coupled with ATP, these compounds induced a statistically

significant Ca^{++} flux. Interestingly enough, only cells treated with E2 combined with ATP recovered from the calcium flux back to basal or near basal levels by the end of the 20-minute experiment as shown in Figure 22.

Cells treated with NP and PFOA coupled with ATP saw a statistically significant calcium flux. Conversely, unlike E2, the cells did not see a recovery of Ca^{++} levels but a steady Ca^{++} flux shown in Figures 20 and 21. This leads us to believe NP and PFOA operate through a different mechanism than ATP to lock open the Ca^{++} channels, possibly acting as a ligand activated Ca^{++} channel (D. Clapham, 2007). An alternative hypothesis is that NP and PFOA desensitize the negative feedback system embedded in the purinergic receptor signaling pathway through blocking the arrestin molecule or preventing the phosphorylation via the GRK. Upon further investigation of this notion, B. Roufogalis et al (1999) determined that NP inhibits plasma membrane bound Ca^{++} ATPase. The inhibition of this protein would not allow a feedback to the endoplasmic reticulum and thus would cause a continual release of calcium. However, there is no literature shedding light on the operational mechanisms on PFOA. Consequently, it can be deemed as a plausible explanation for the continual release of calcium in the presence of ATP, however, further research is needed to confirm this.

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Images Reference

http://resources.ama.uk.com/glowm_www/graphics/figures/v5/0380/07.gif

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APPENDICES

Appendix I

Control	E2 10^{-10} M	E2 10^{-8} M	ICI 10^{-7} M	ICI 10^{-7} M + E2 10^{-10} M	PFOA 10^{-8} M	PFOA 10^{-6} M	NP 10^{-8} M	NP 10^{-6} M	ICI 10^{-7} M + PFOA 10^{-5} M	G36 10^{-7} M + PFOA 10^{-5} M	E2 10^{-10} M + PFOA 10^{-5} M
Control	E2 10^{-10} M	E2 10^{-} M	ICI 10^{-7} M	ICI 10^{-7} M + E2 10^{-10} M	PFOA 10^{-8} M	PFOA 10^{-6} M	NP 10^{-8} M	NP 10^{-6} M	ICI 10^{-7} M + PFOA 10^{-5} M	G36 10^{-7} M + PFOA 10^{-5} M	E2 10^{-10} M + PFOA 10^{-5} M
Control	E2 10^{-10} M	E2 10^{-8} M	ICI 10^{-7} M	ICI 10^{-7} M + E2 10^{-10} M	PFOA 10^{-8} M	PFOA 10^{-6} M	NP 10^{-8} M	NP 10^{-6} M	ICI 10^{-7} M + PFOA 10^{-5} M	G36 10^{-7} M + PFOA 10^{-5} M	E2 10^{-10} M + PFOA 10^{-5} M
Control	E2 10^{-10} M	E2 10^{-8} M	ICI 10^{-7} M	ICI 10^{-7} M + E2 10^{-10} M	PFOA 10^{-8} M	PFOA 10^{-6} M	NP 10^{-8} M	NP 10^{-6} M	ICI 10^{-7} M + PFOA 10^{-5} M	G36 10^{-7} M + PFOA 10^{-5} M	E2 10^{-10} M + PFOA 10^{-5} M
Blank	E2 10^{-9} M	E2 10^{-7} M	G36 10^{-7} M	G36 10^{-7} M + E2 10^{-10} M	PFOA 10^{-7} M	PFOA 10^{-5} M	NP 10^{-7} M	NP 10^{-5} M	ICI 10^{-7} M + NP 10^{-5} M	G36 10^{-7} M + NP 10^{-5} M	E2 10^{-10} M + NP 10^{-5} M
Blank	E2 10^{-9} M	E2 10^{-7} M	G36 10^{-7} M	G36 10^{-7} M + E2 10^{-10} M	PFOA 10^{-7} M	PFOA 10^{-5} M	NP 10^{-7} M	NP 10^{-5} M	ICI 10^{-7} M + NP 10^{-5} M	G36 10^{-7} M + NP 10^{-5} M	E2 10^{-10} M + NP 10^{-5} M
Blank	E2 10^{-9} M	E2 10^{-7} M	G36 10^{-7} M	G36 10^{-7} M + E2 10^{-10} M	PFOA 10^{-7} M	PFOA 10^{-5} M	NP 10^{-7} M	NP 10^{-5} M	ICI 10^{-7} M + NP 10^{-5} M	G36 10^{-7} M + NP 10^{-5} M	E2 10^{-10} M + NP 10^{-5} M
Blank	E2 10^{-9} M	E2 10^{-7} M	G36 10^{-7} M	G36 10^{-7} M + E2 10^{-10} M	PFOA 10^{-7} M	PFOA 10^{-5} M	NP 10^{-7} M	NP 10^{-5} M	ICI 10^{-7} M + NP 10^{-5} M	G36 10^{-7} M + NP 10^{-5} M	E2 10^{-10} M + NP 10^{-5} M

Appendix II

Passage 1

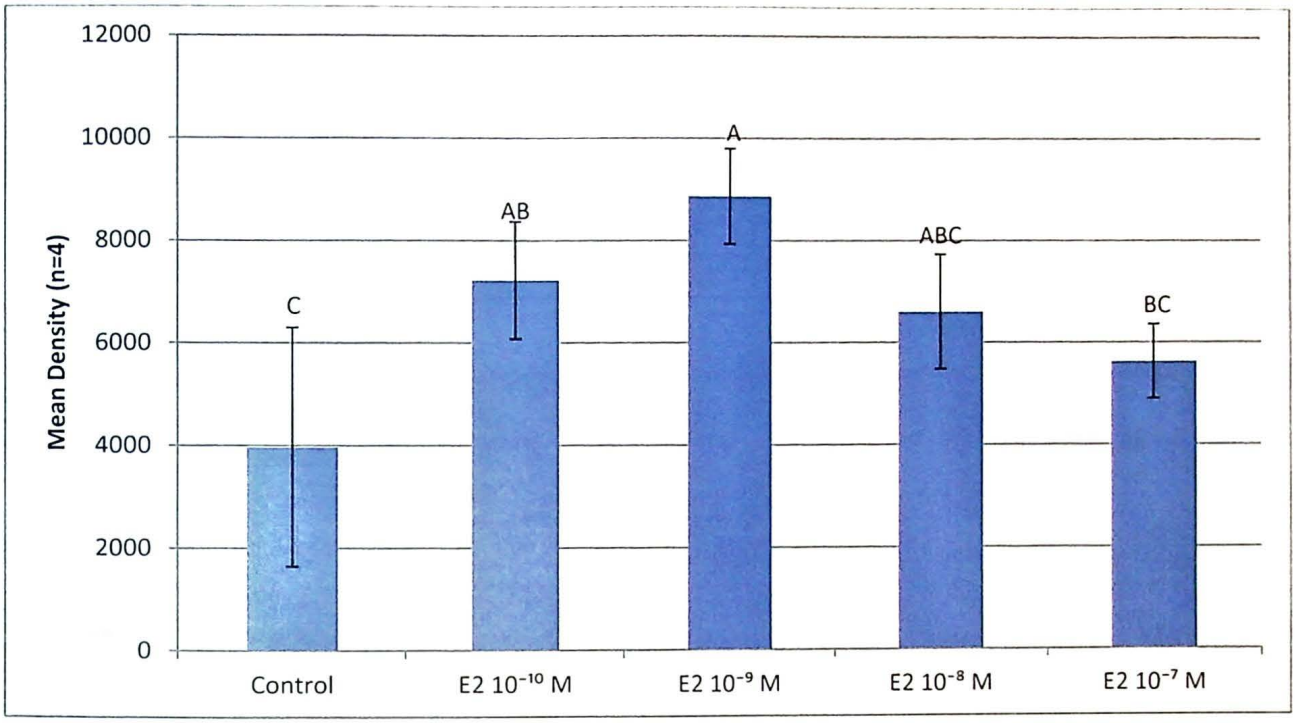

Figure 22. The effect of E2 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The $E2^{-10}$ M and $E2^{-9}$ M treatments were statistically different than the control, contrary to the $E2^{-8}$ M and $E2^{-7}$ M treatments which were not. This suggests that at least 2 concentrations of E2 had a proliferative effect on the cells.

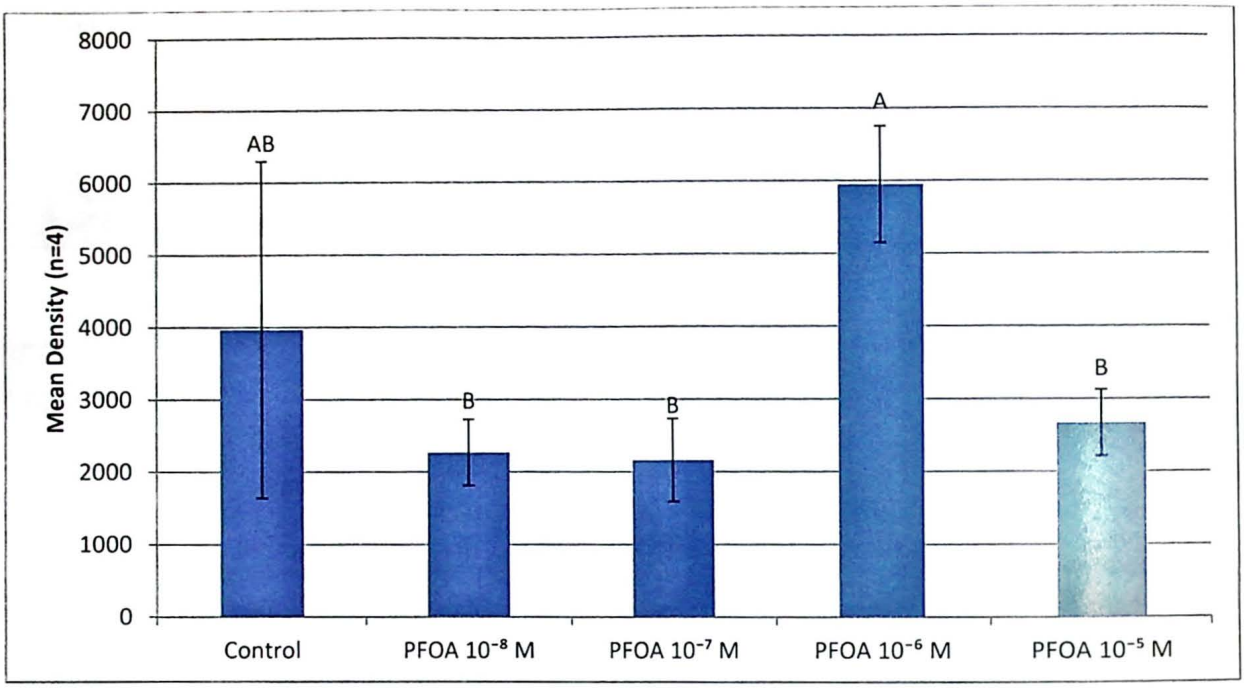

Figure 23. The effect of PFOA on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. None of the concentrations of PFOA were significantly different than the control. However, PFOA 10^{-6} M was significantly different than the other concentrations of PFOA. This suggests that in this passage of PFOA did not stimulate proliferation of the cells.

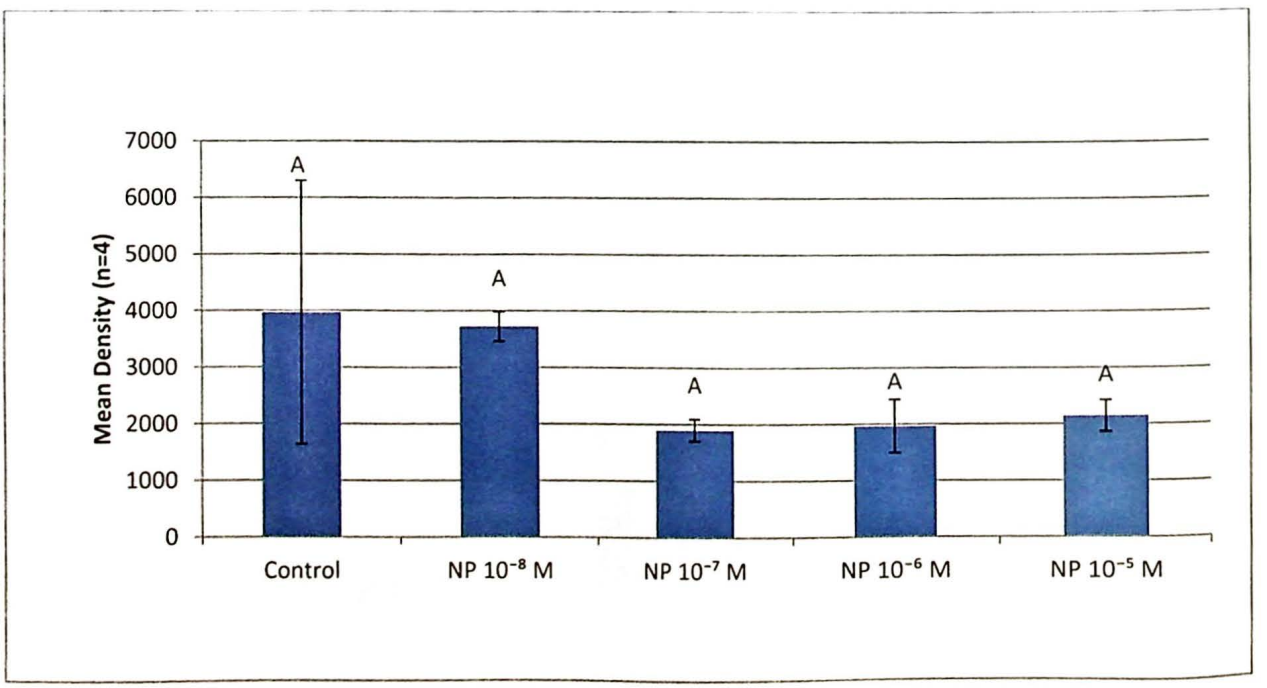

Figure 24. The effect of NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. None of the concentrations of NP were significantly different than the control. This suggests that in this passage of NP treatment did not stimulate proliferation of the cells.

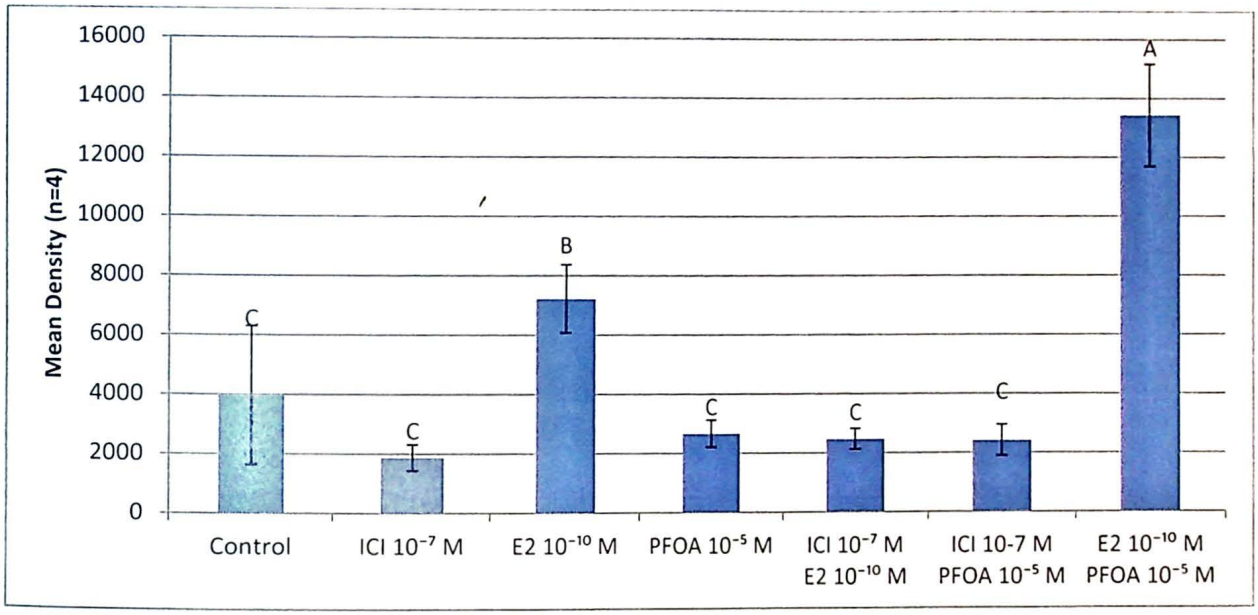

Figure 25. The effect of PFOA and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. $E2^{-10}$ M resulted in a significant difference than the control, PFOA, and all combinations of inhibitors. $E2^{-10}$ M was also significantly different than the $E2^{-10}$ M + PFOA 10^{-5} M treatment. The $E2^{-10}$ M + PFOA 10^{-5} M treatment was significantly different from all other treatments. This suggests that the ICI effectively inhibited the proliferative effect of PFOA, however the particular concentration of PFOA did not induce a proliferative effect, so that assumption cannot be made. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

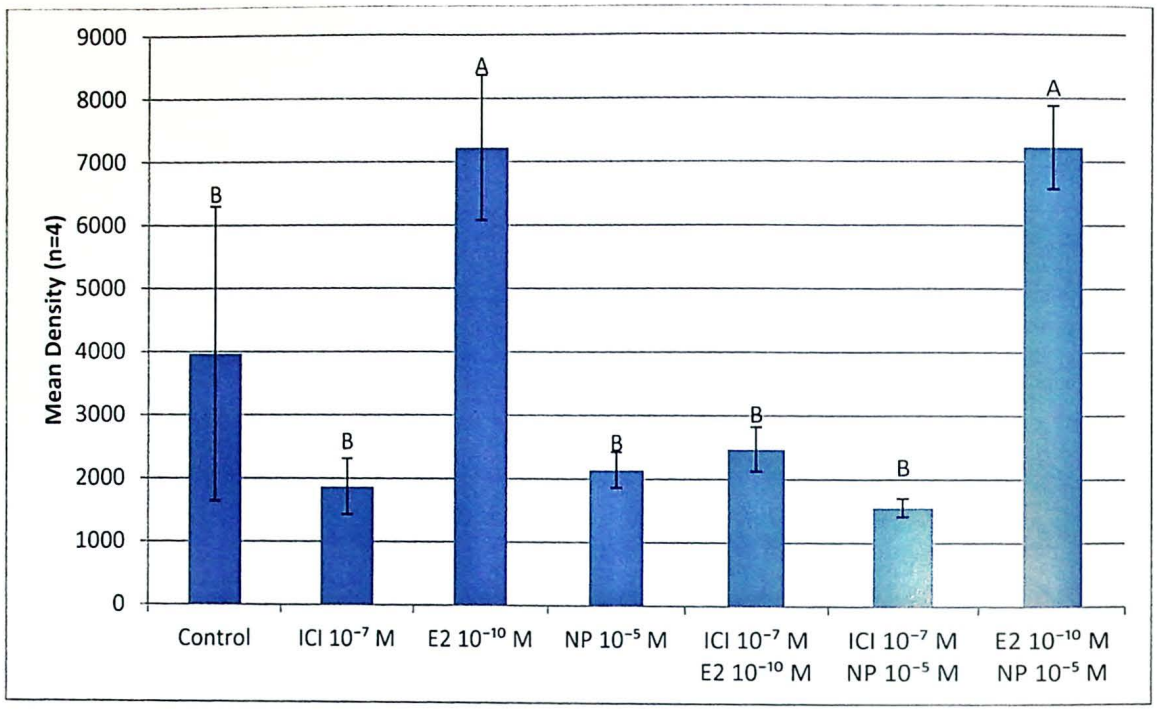

Figure 26. The effect of NP and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. $E2^{-10}$ M resulted in a significant difference than the control, NP and all combinations of inhibitors. The $E2^{-10}$ M + NP 10^{-5} M treatment was significantly different from all other treatments except $E2^{-10}$ M. This suggests that the ICI effectively inhibited the proliferative effect of NP, however the NP did not induce a proliferative effect, so that assumption cannot be made. Additionally, the data suggests the combination of E2 and NP caused a significant proliferative effect.

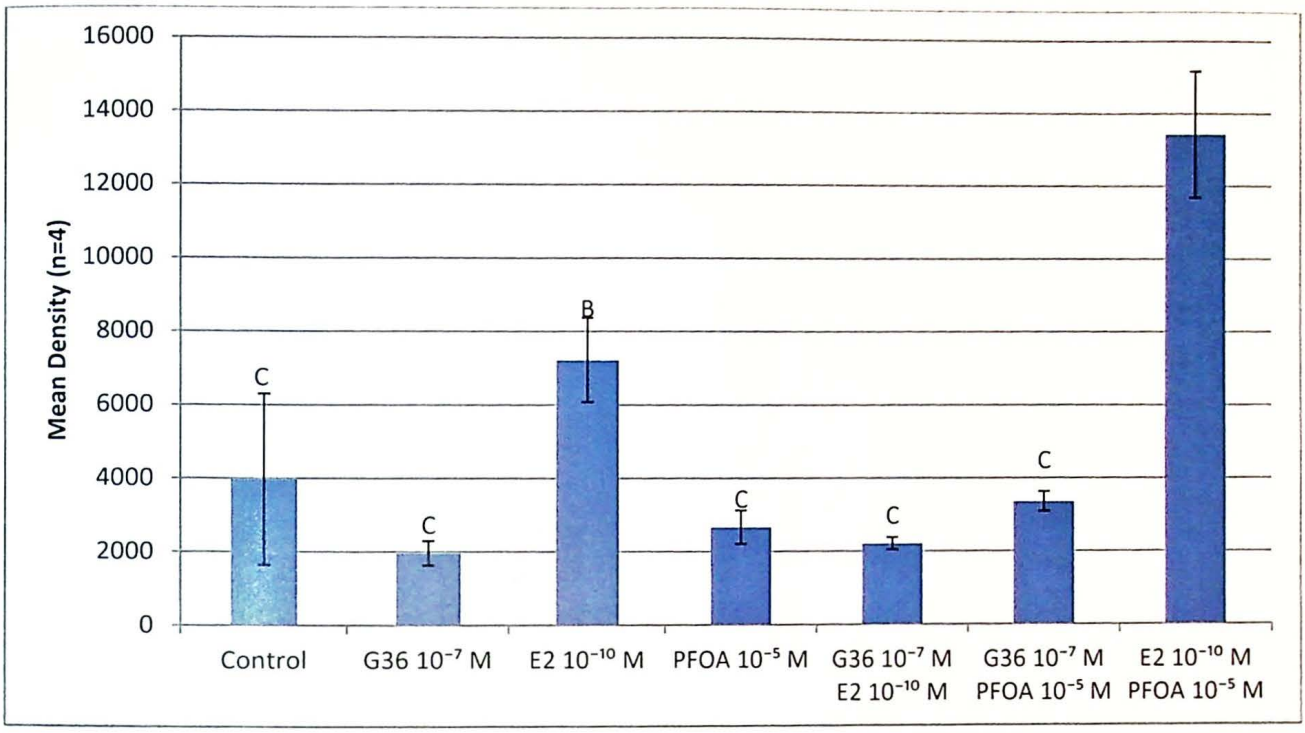

Figure 27. The effect of PFOA and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. $E2^{-10}$ M resulted in a significant difference than the control, PFOA and all combinations of inhibitors. $E2^{-10}$ M + PFOA 10^{-5} M showed a significant difference than the $E2^{-10}$ M, control and all combinations of inhibitors. This suggests that the G36 effectively inhibited the proliferative effect of PFOA, however the particular concentration of PFOA did not induce a proliferative effect, so that assumption cannot be made. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect greater than E2 alone.

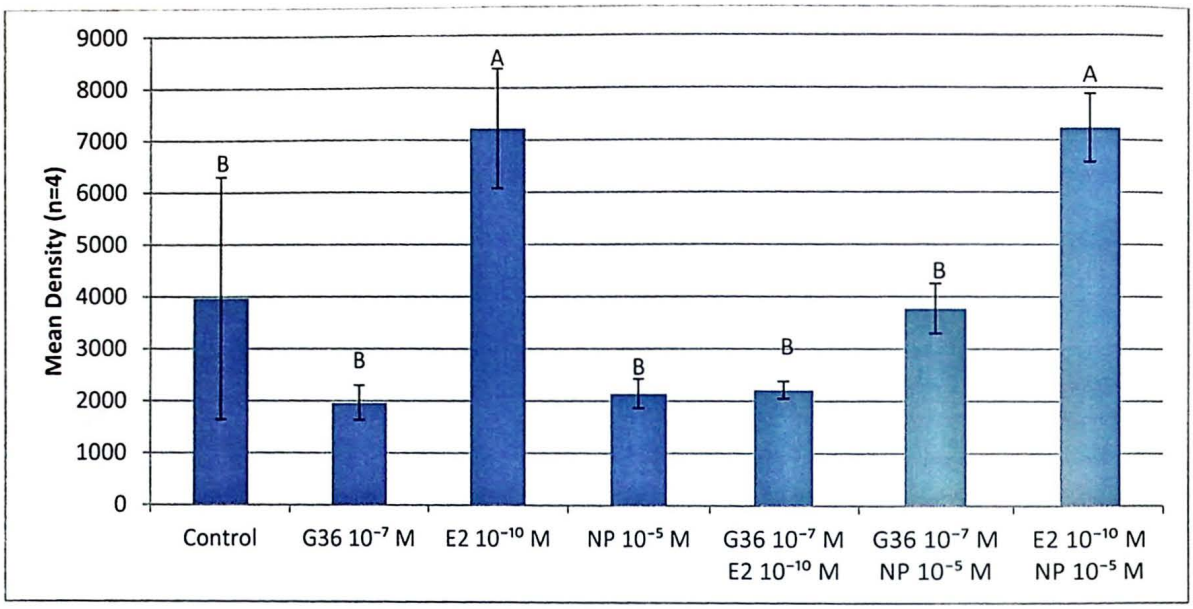

Figure 28. The effect of NP and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on first passage of the experiment. Treatments not connected by the same letter are statistically significantly different. $E2^{-10}$ M resulted in a significant difference than the control, NP and all combinations of inhibitors. $E2^{-10}$ M + NP 10^{-5} M showed a significant difference than the control and all combinations of inhibitors but not the $E2^{-10}$ M treatment. This suggests that the G36 did not inhibit the proliferative activity of NP but because that particular concentration of NP did not induce a proliferative effect, that assumption cannot be made. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

Passage 2

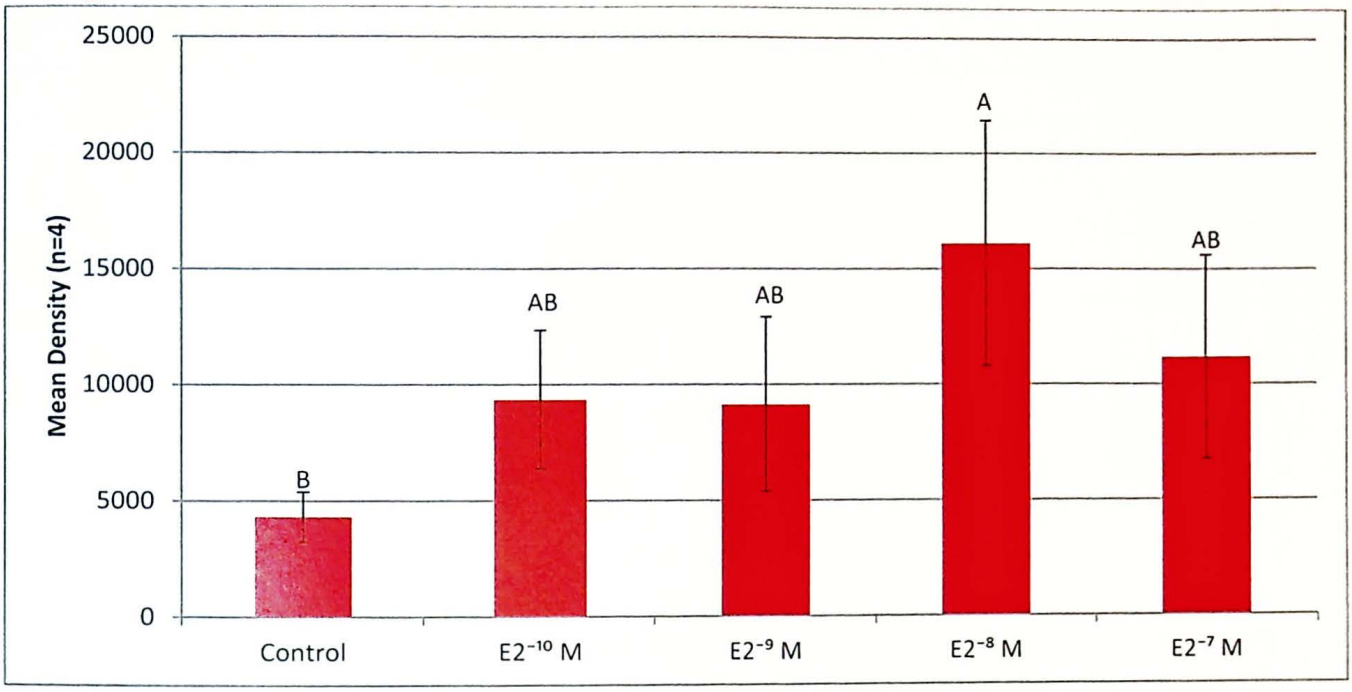

Figure 29. The effect of E2 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on second passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The $E2^{-8}$ M treatment showed a significant difference than the control while all the other E2 treatments did not. This suggests that at least one concentration of E2 induced a proliferative effect on the cells.

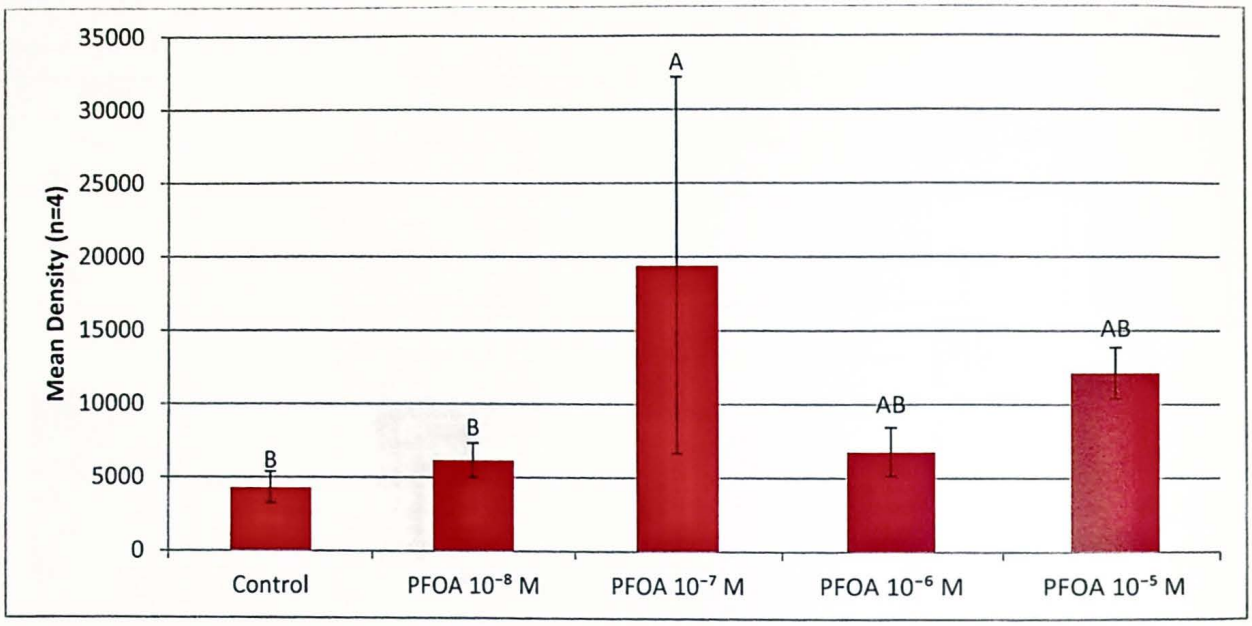

Figure 30. The effect of PFOA on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on second passage of the experiment. Treatments not connected by the same letter are statistically significantly different. PFOA 10^{-7} M treatment was significantly different than the control and PFOA 10^{-8} M treatment but not the other. This suggests that at least one concentration of PFOA induced a proliferative effect on the cells.

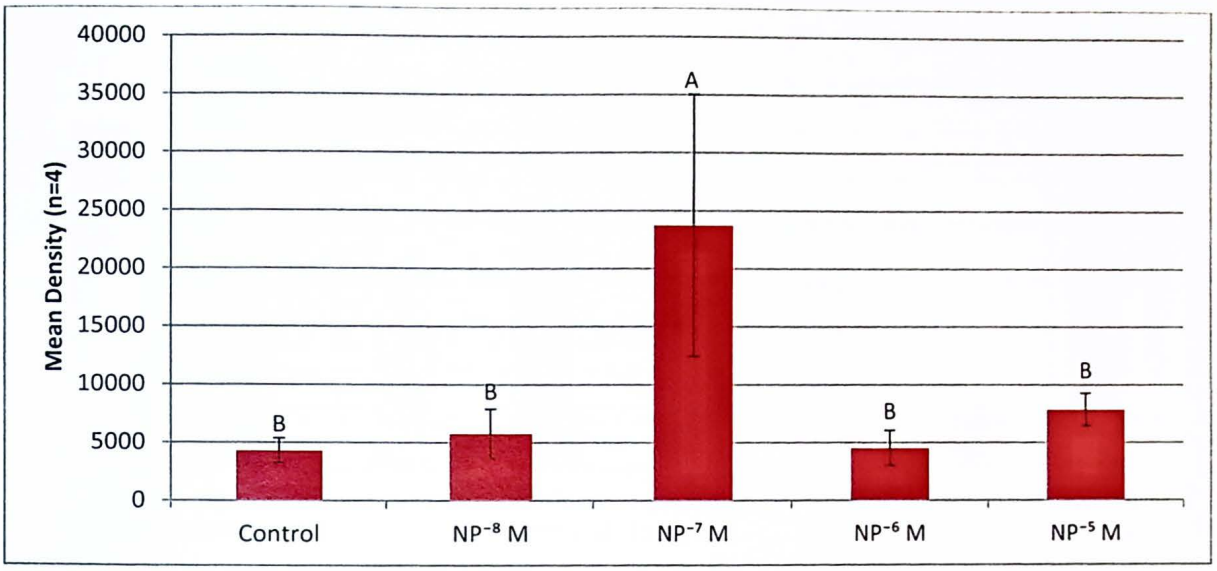

Figure 31. The effect of NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. NP 10^{-7} M treatment showed a significant difference between the control and all other concentrations of NP. This suggests that at least one concentration of NP induced a proliferative effect on the cells.

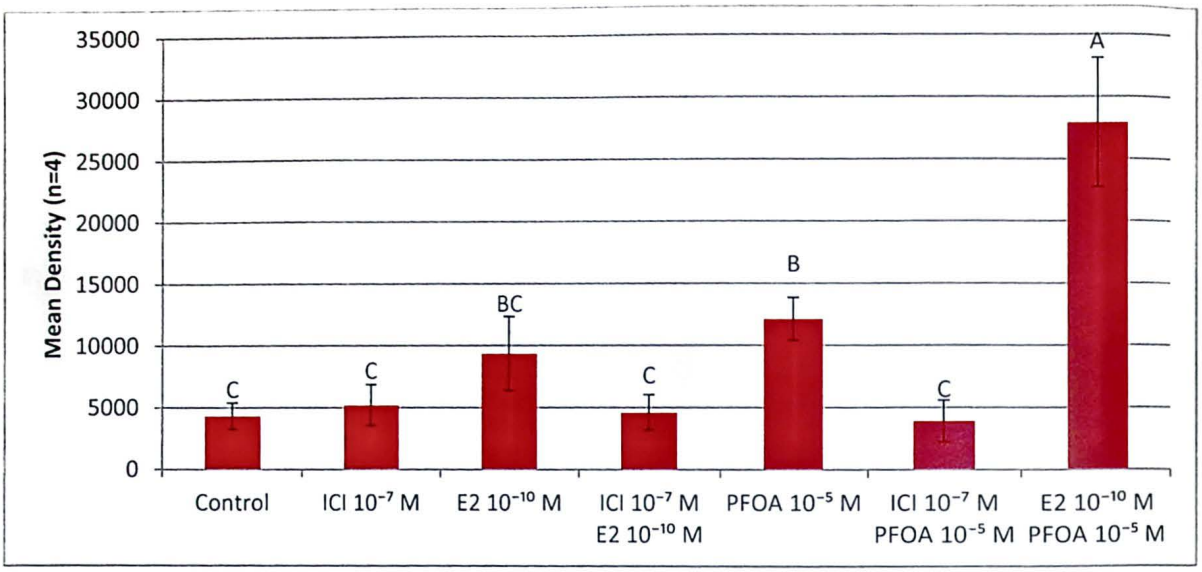

Figure 32. The effect of PFOA and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on second passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M treatment was not significantly different than the control, PFOA or any combination of the inhibitors. However, the E2 10^{-10} M + PFOA 10^{-5} M treatment was significantly different than the E2, control, PFOA and all combinations of inhibitors. The PFOA 10^{-5} M treatment was significantly different than all inhibitor combinations. This suggests that the ICI inhibited the proliferative effect of PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect greater than E2 alone.

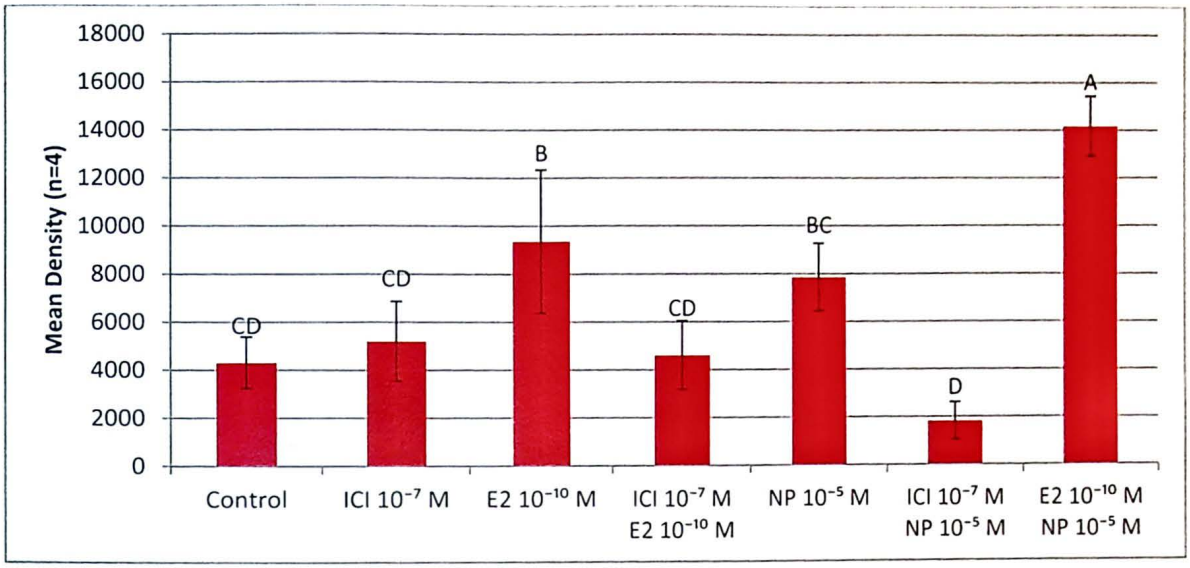

Figure 33. The effect of NP and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on second passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M treatment was significantly different than the control and all inhibitor combinations. The E2 10^{-10} M treatment was not significantly different than the NP treatment but different than the E2 10^{-10} M + NP 10^{-5} M. The NP 10^{-5} M treatment however, was not significantly different than the control, ICI 10^{-7} M, and E2 10^{-7} + ICI 10^{-7}. All inhibitor combinations were not significantly different than the control. This suggests that the ICI effectively inhibited the proliferative effect of NP, however the NP did not induce a proliferative effect, so that assumption cannot be made. Additionally, the data suggests the combination of E2 and NP caused a significant proliferative effect greater than E2 alone.

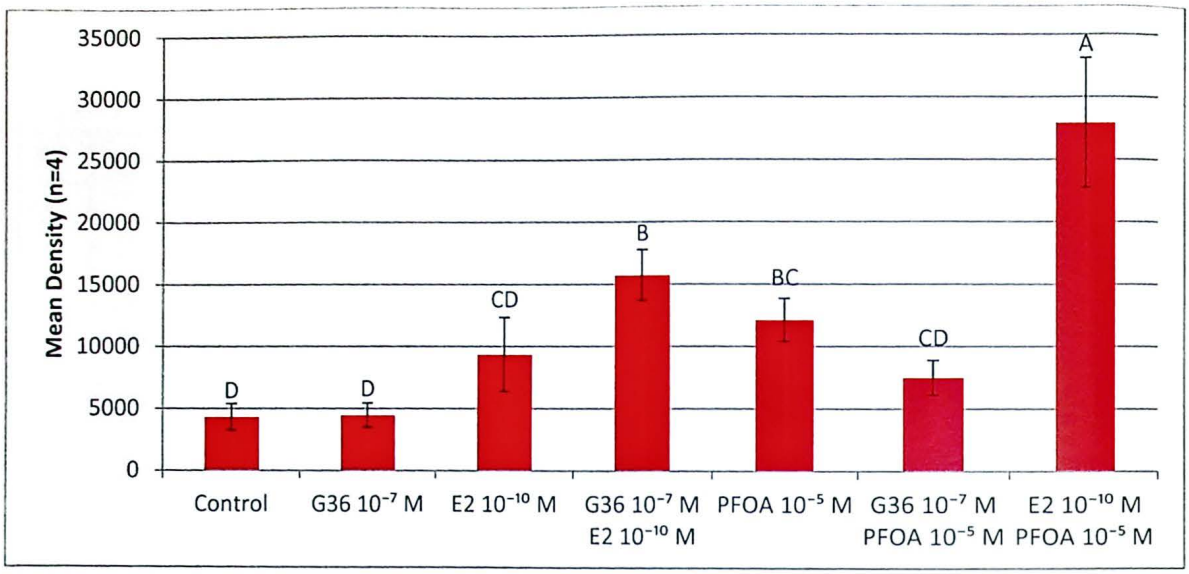

Figure 34. The effect of PFOA and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on second passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M treatment was not significantly different than the control, PFOA, G36 10^{-7} M and G36 10^{-7} M + PFOA 10^{-5} M. The E2 10^{-10} M + PFOA 10^{-5} M treatment was significantly different than the control, G36 10^{-7} M + PFOA 10^{-5} M, PFOA 10^{-5} M, and all combinations of inhibitors. PFOA 10^{-5} M treatment was significantly different than the control yet not significantly different than the G36 10^{-7} M + PFOA 10^{-5} M or G36 10^{-7} M + E2 10^{-10} M treatment. This suggests that the G36 did not effectively inhibit the proliferative effect of PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect greater than E2 alone and the combination of G36 and E2 had a significant proliferative effect greater than E2 alone.

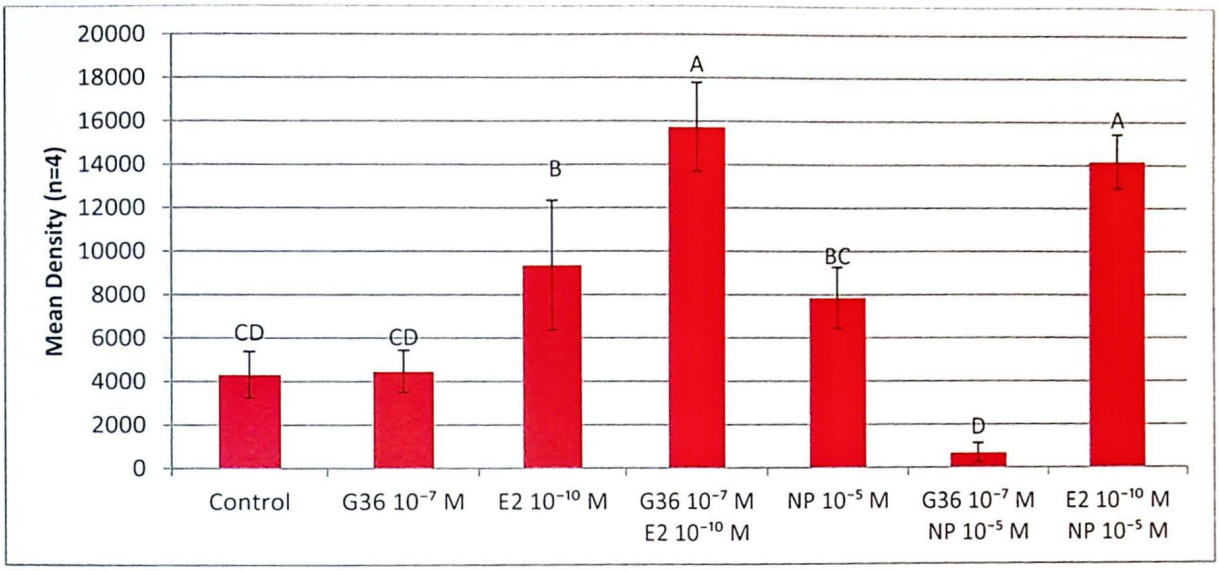

Figure 35. The effect of NP and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on second passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M treatment was significantly different than the control but not NP 10^{-5} M. NP 10^{-5} M was significantly different than the G36 10^{-7} M + NP 10^{-5} M treatment however, not the control or G36 10^{-7} M. This suggests that the G36 effectively inhibited the NP but not the E2. However, a combination of E2 and G36 resulted in a higher proliferative effect than with E2 alone. Additionally, the data suggests the combination of E2 and NP caused a significant proliferative effect greater than E2 alone

Passage 3

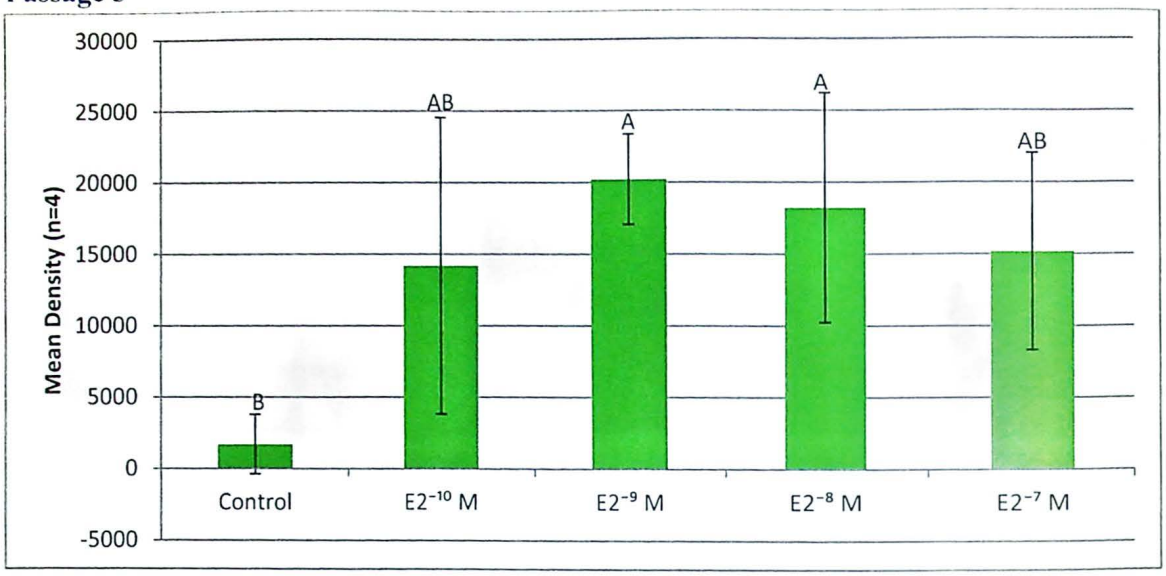

Figure 36. The effect of E2 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on third passage of the experiment. Treatments not connected by the same letter are statistically significantly different. E2 10^{-8} M and E2 10^{-9} M treatments were significantly different than the control but not the other E2 concentrations. This suggests that at least two concentrations of E2 induced a proliferative effect on the cells.

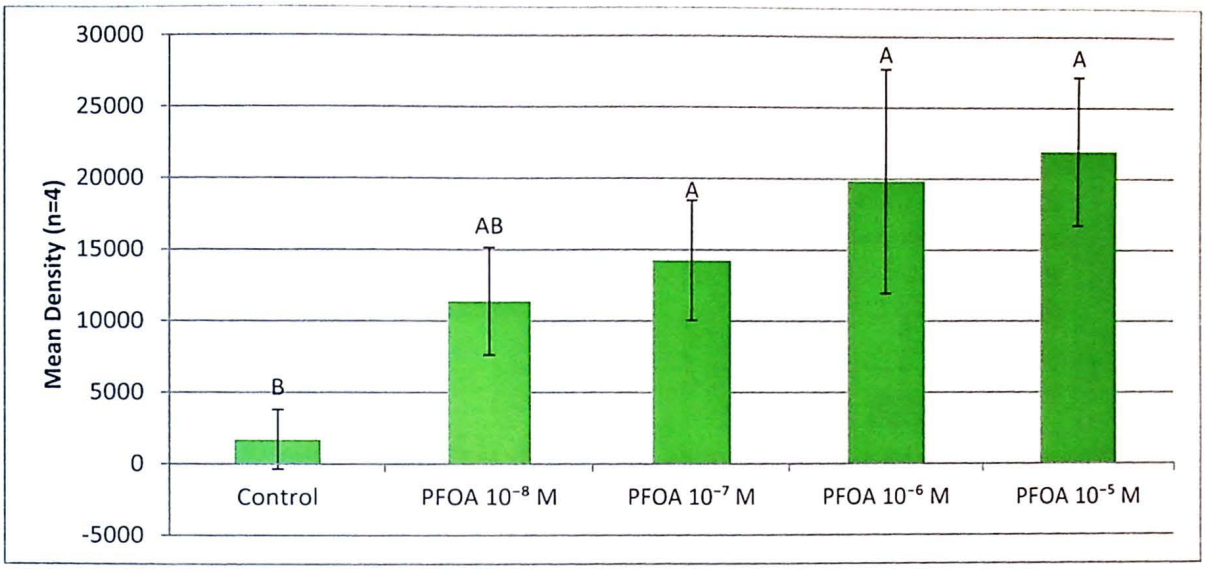

Figure 37. The effect of PFOA on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on third passage of the experiment. Treatments not connected by the same letter are statistically significantly different. PFOA 10^{-8} M was not significantly different than the control but all other concentrations of PFOA were different. This suggests that at least three concentrations of PFOA induced a proliferative effect on the cells.

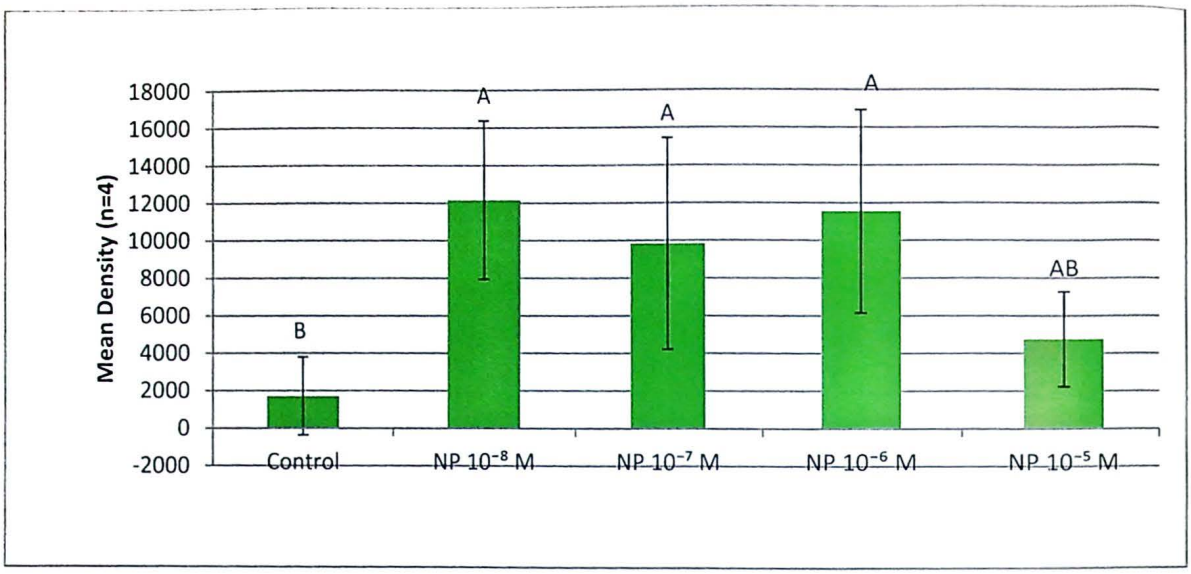

Figure 38. The effect of NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells. Treatments not connected by the same letter are statistically significantly different. All concentrations of NP were significantly different than the control except NP 10^{-5} M. This suggests that at least three concentrations of NP induced a proliferative effect on the cells.

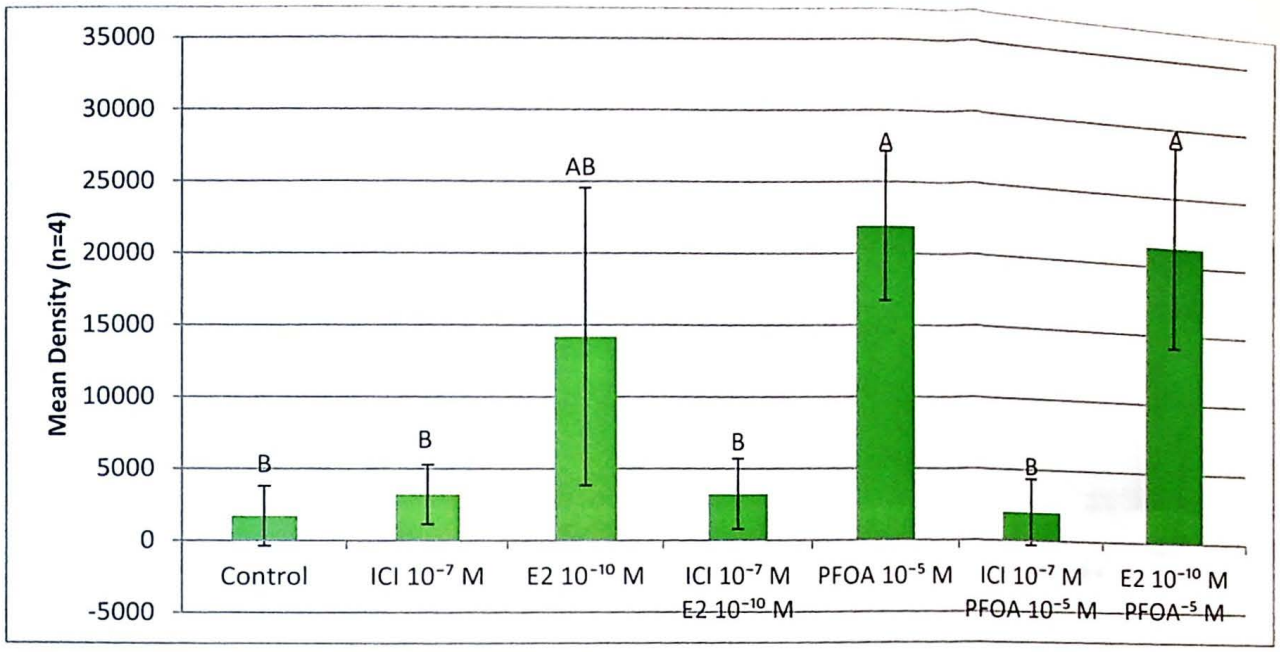

Figure 39. The effect of PFOA and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on third passage of the experiment. Treatments not connected by the same letter are statistically significantly different. E2 10^{-10} M treatment was not significantly different than the control or any combination of the inhibitors. The PFOA 10^{-5} M treatment was significantly different than the control and all combinations of inhibitors but not different than the E2 10^{-10} M. The E2 10^{-10} M + PFOA 10^{-5} M was not significantly different from each other. This suggests that ICI effectively inhibited the proliferative effect of PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

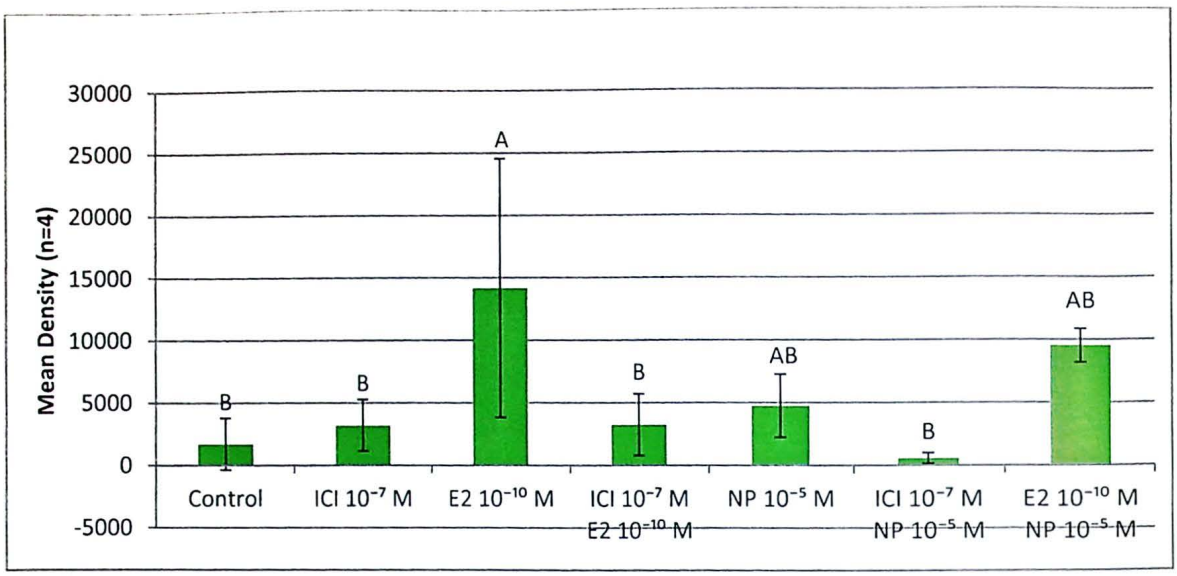

Figure 40. The effect of NP and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on third passage of the experiment. Treatments not connected by the same letter are statistically significantly different. E2 10^{-10} M treatment was significantly different than the control and all combinations of inhibitors. The NP^{-5} M treatment was not different than the E2 10^{-10} M or any of the inhibitor combination. The E2 10^{-10} M + NP 10^{-5} M treatment was not significantly different than the control, any combination of inhibitor, E2 10^{-10} M, or the NP 10^{-5} M treatment. This suggests that the ICI effectively inhibited the proliferative effect of NP, however the NP at that concentration did not induce a proliferative effect, so that assumption cannot be made. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

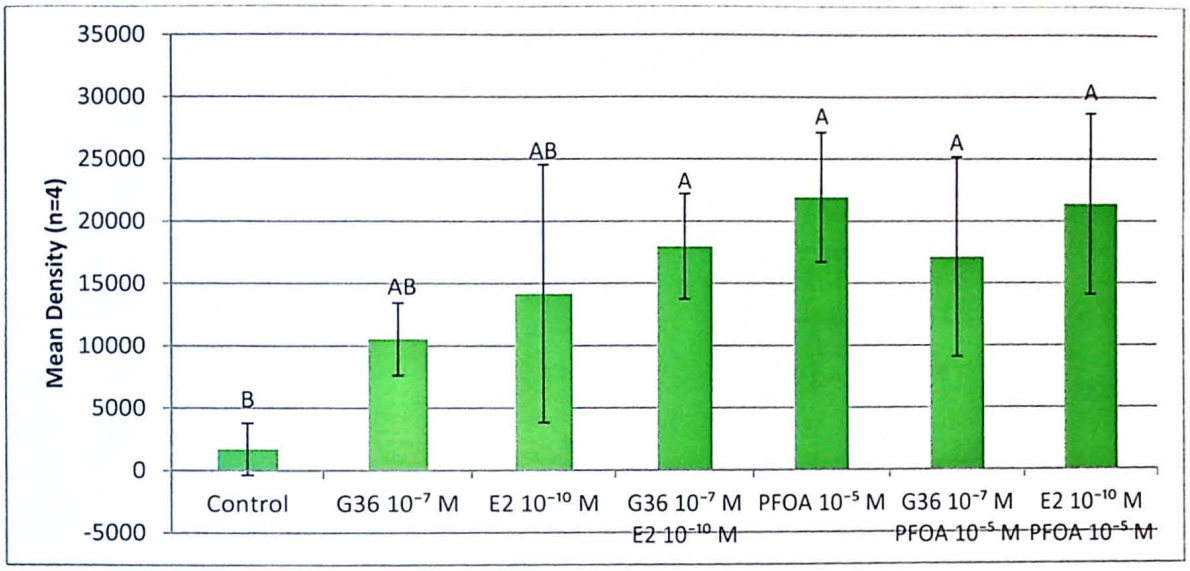

Figure 41. The effect of PFOA and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on third passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The E2 10^{-10} M treatment was significantly different than the control. The PFOA 10^{-5} M treatment was significantly different than the control but not the E2 10^{-10} M, any of the inhibitor combinations or E2 10^{-10} M + PFOA 10^{-5} M treatments. The E2 10^{-10} M + PFOA 10^{-5} M treatment was different than the control but none of the other treatments. This suggests that the G36 did not effectively inhibit the proliferative effect of PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

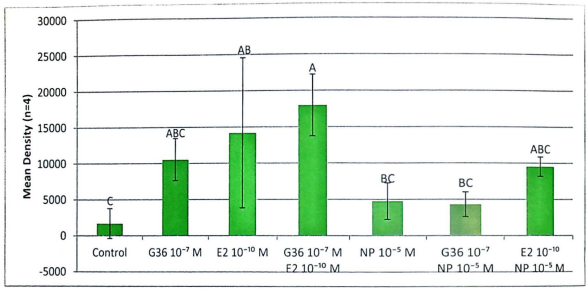

Figure 42. The effect of NP and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on third passage of the experiment. Treatments not connected by the same letter are statistically significantly different. E2 10^{-10} M was significantly different than the control but not any combination of inhibitor or NP 10^{-5} M. NP 10^{-5} M treatment was significantly different than the G36 10^{-7} M + E2 10^{-10} M treatment but NP 10^{-5} M yielded a lower mean density. NP 10^{-5} M was not significantly different than the control, E2 10^{-10} M or the other inhibitor combinations, with the exception of G36 10^{-7} M + E2 10^{-10} M. The E2 10^{-10} M + NP 10^{-5} M treatment was not significantly different than any other treatment. This suggests that the G36 effectively inhibited the proliferative effect of NP, however the NP at that concentration did not induce a proliferative effect, so that assumption cannot be made. However, the data suggests that G36 did not inhibit the proliferative effect of E2.

Passage 4

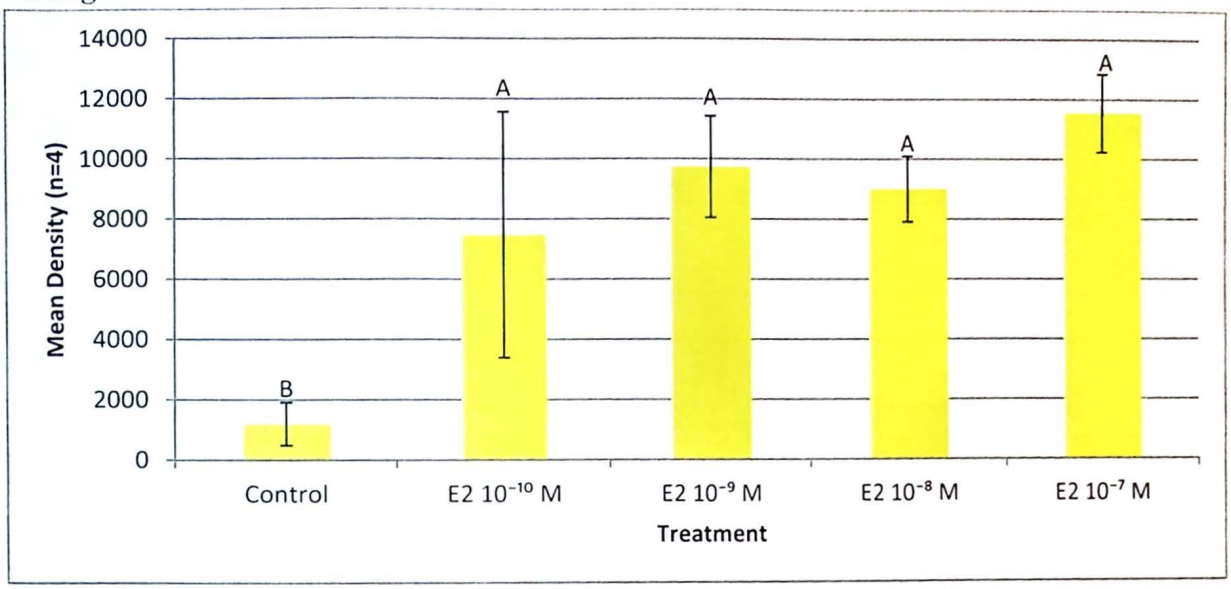

Figure 43. The effect of E2 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. All concentrations of E2 were significantly different than the control but not from each other. This suggests that all 4 concentration of E2 induced a proliferative effect on the cells.

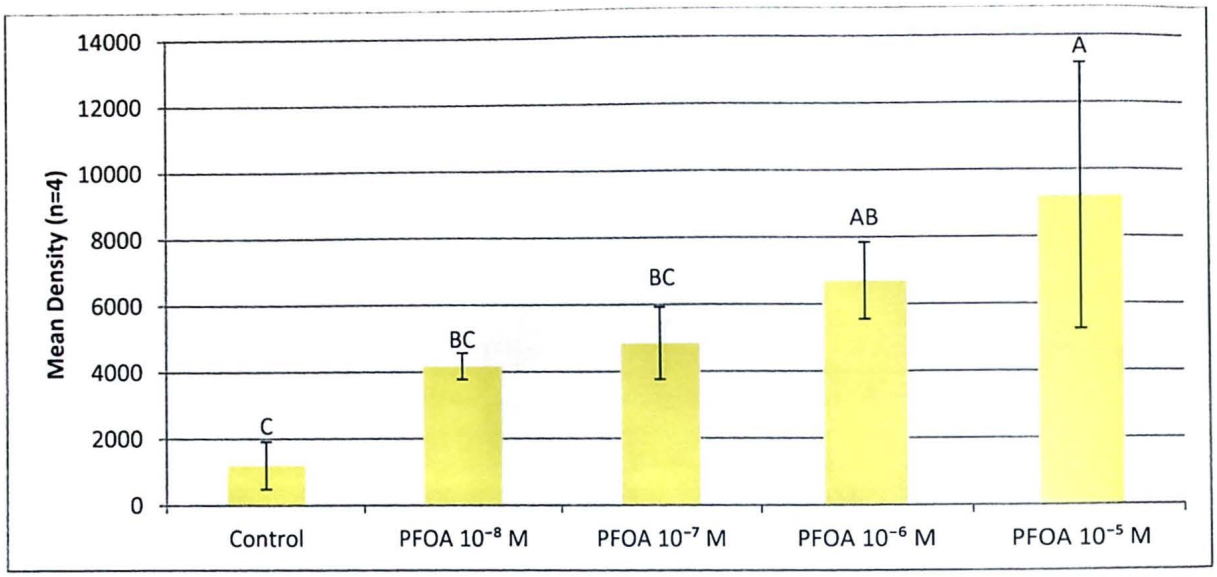

Figure 44. The effect of PFOA on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. PFOA at the 10 10^{-5} and 10 10^{-6}M were significantly different than the control but only the 10 10^{-5}M concentration was significantly different than the two lowest concentrations of PFOA. This suggest that at least 2 concentrations of PFOA induced a proliferative effect on the cells.

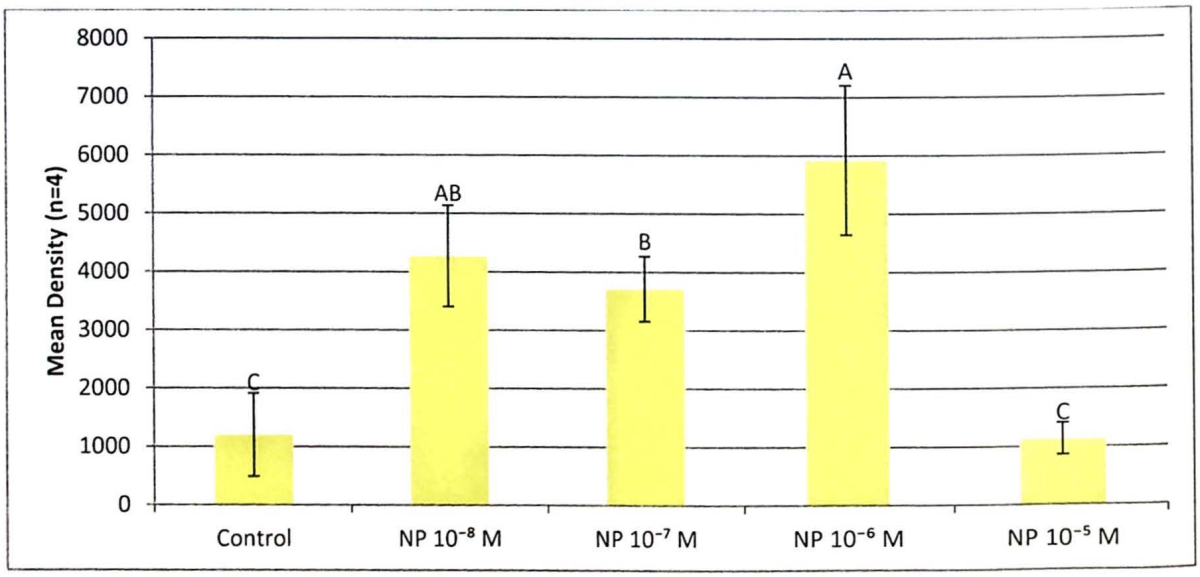

Figure 45. The effect of NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. All concentrations of NP were significantly different than the control except the 10^{-5}M. This suggests that at least 3 concentrations of NP induced a proliferative effect on the cells.

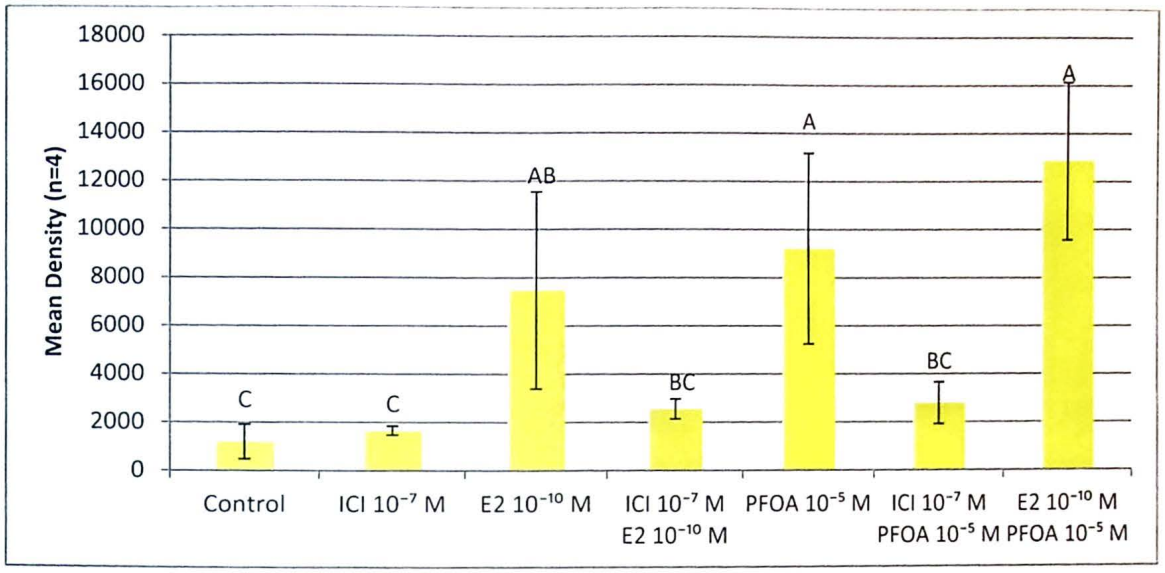

Figure 46. The effect of PFOA and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. PFOA 10^{-5}M showed a significant difference than the control, ICI, ICI + PFOA and ICI + E2. However, the PFOA 10^{-5}M was not significantly different than the E2 and E2 + PFOA treatment. This suggests that the ICI effectively inhibited the PFOA proliferative effect. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

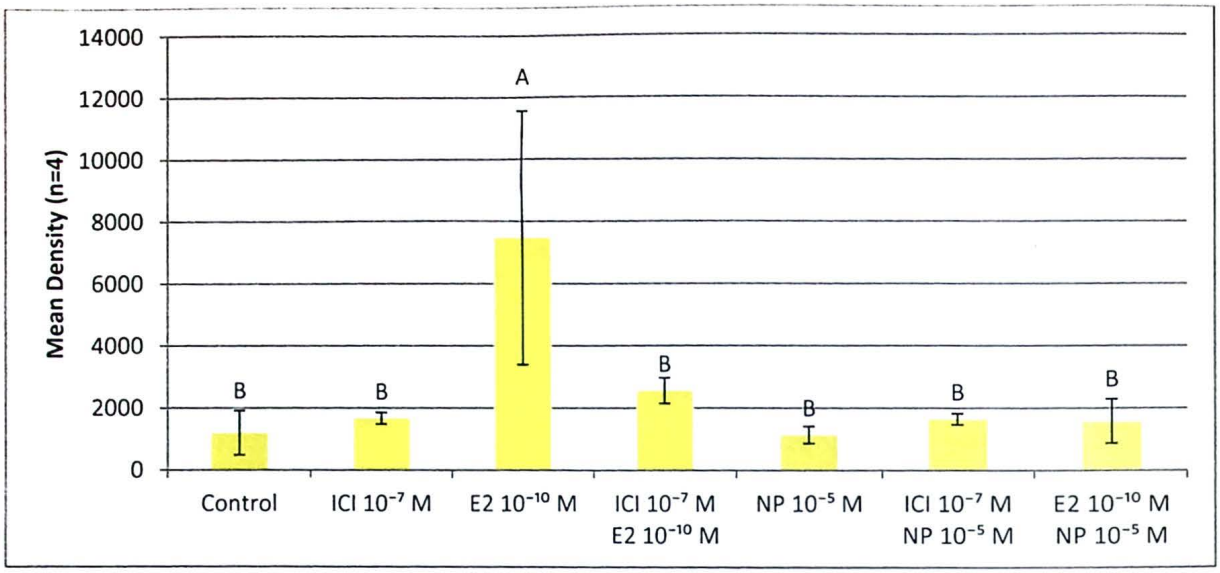

Figure 47. The effect of NP and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The NP 10^{-5} M treatment was not significantly different than any of the treatments except E2 10^{-10} M. This suggests that the ICI effectively inhibited the proliferative effect of NP, however the NP at that concentration did not induce a proliferative effect, so that assumption cannot be made.

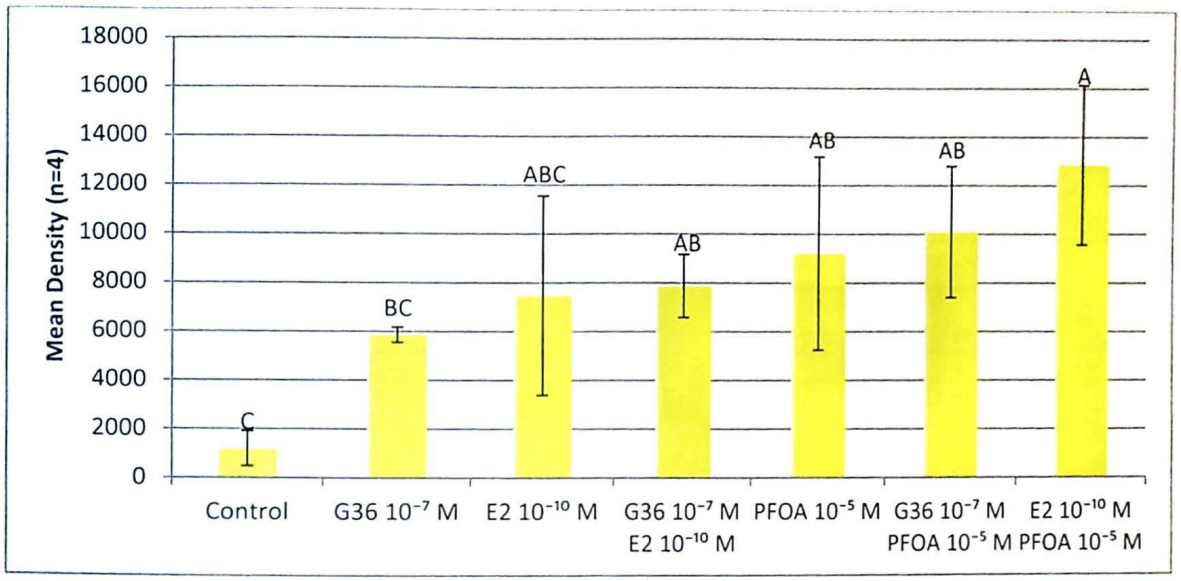

Figure 48. The effect of PFOA and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. PFOA 10^{-5} M was not significantly different than the G36 10^{-7}+ PFOA 10^{-5} M, E2 10^{-10} + PFOA 10^{-5}M, or E2 treatments. However, G36 10^{-7}M was higher, albeit not significantly higher. PFOA 10^{-5}M was significantly different than the control. This suggests that G36 did not effectively inhibit the proliferative effect of the PFOA. Additionally, the data suggests the combination of E2 and PFOA caused a significant proliferative effect.

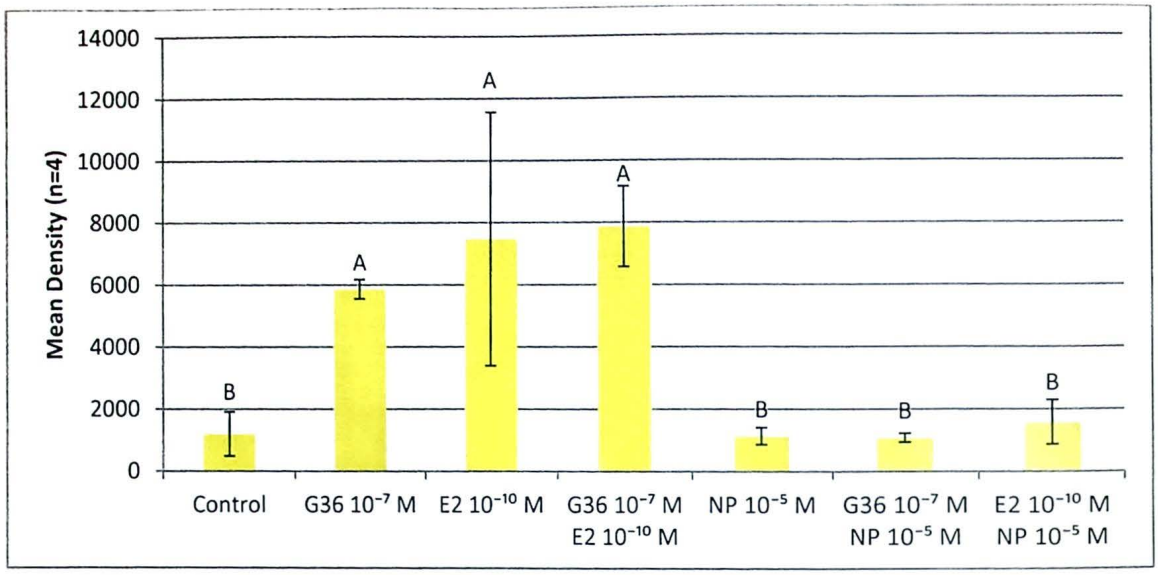

Figure 49. The effect of NP and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fourth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. The 10^{-5}M concentration of NP was not significantly different than the G36 + NP, NP + E2 or the control. The G36, E2, and G36 + E2 were not significantly different from each other but different than the rest of the treatments. This suggests that the G36 effectively inhibited the proliferative effect of NP, however the NP at that concentration did not induce a proliferative effect, so that assumption cannot be made. However, G36 alone and G36 combined with E2 induced a significant proliferative effect. Additionally, the combination of E2 and NP appeared to inhibit the proliferative effect of E2.

Passage 5

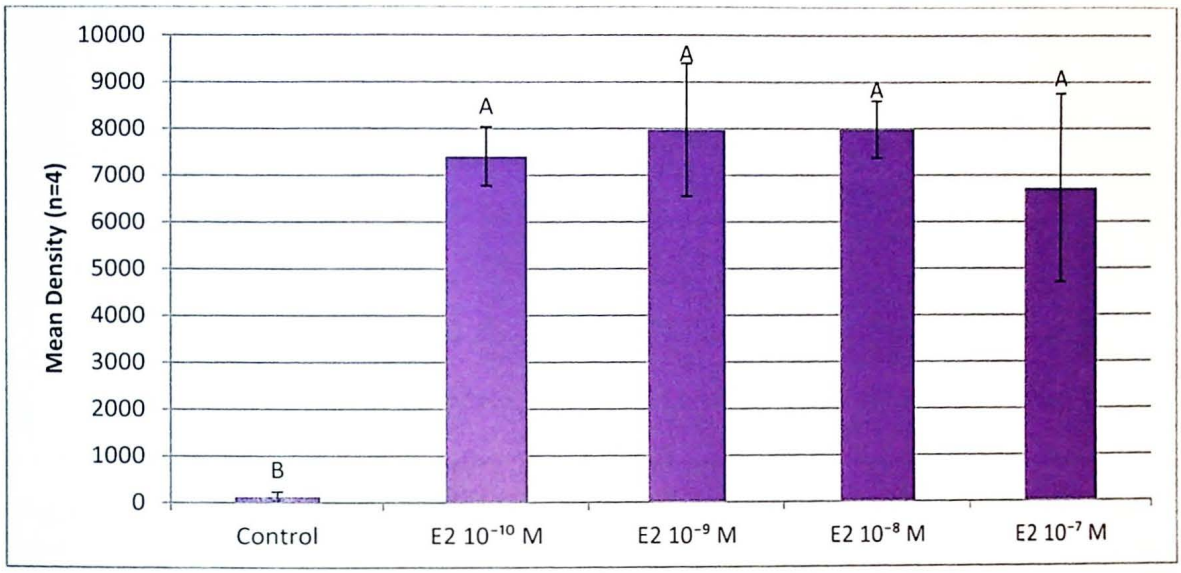

Figure 50. The effect of E2 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. All concentrations of E2 were significantly different than the control but not from each other suggesting E2 has a proliferative effect on the cells. This suggests that all concentrations of E2 caused a proliferative effect in the cells.

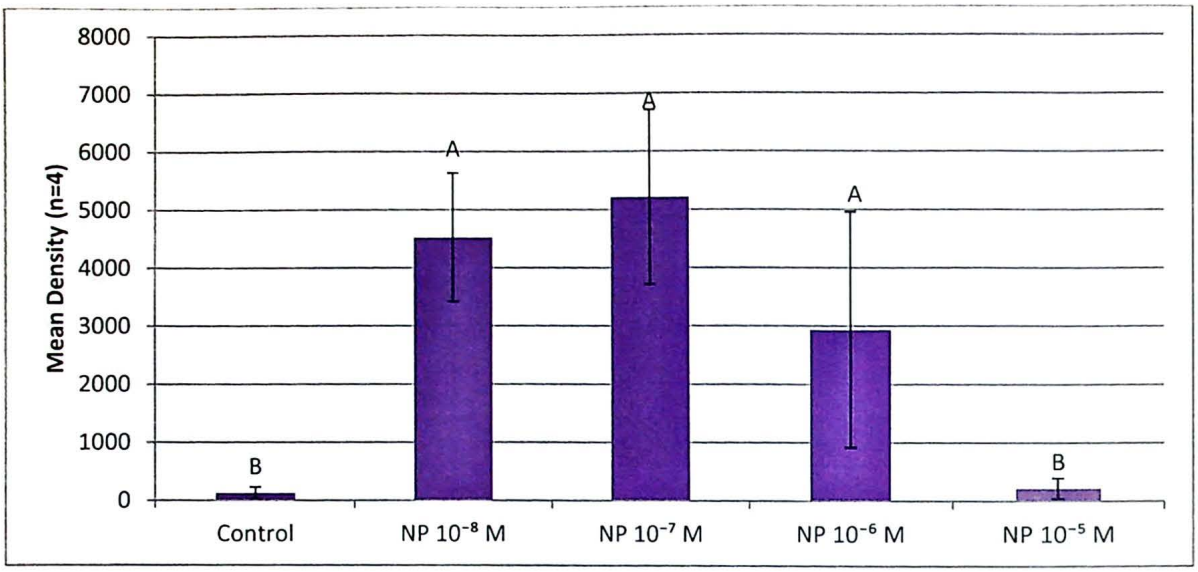

Figure 51. The effect of NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. All concentrations of NP were significantly different than control except the NP 10^{-5} M suggesting that NP has a proliferative effect on the cells. This suggests that at least 3 concentrations of NP caused a proliferative effect in the cells.

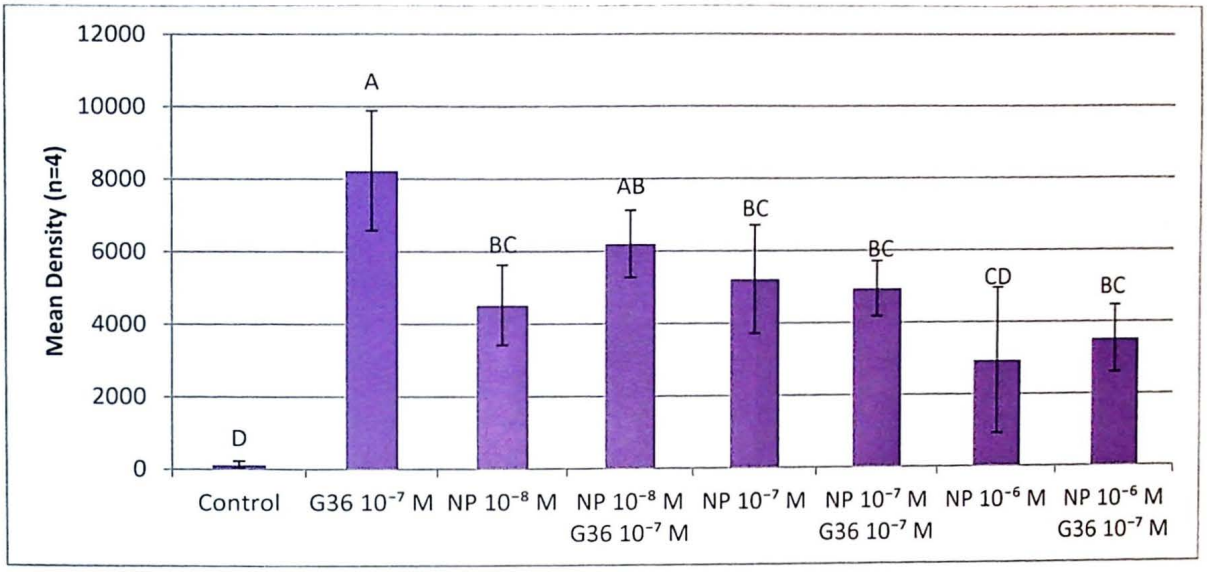

Figure 52. The effect of NP and G36 on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. None of the concentrations of NP + G36 were statistically different than its corresponding concentration of NP. However, all treatments were statistically different than the control. This indicates that G36 had little or no effect on blocking the effects of NP.

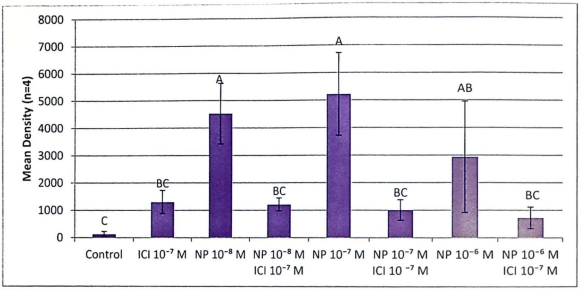

Figure 53. The effect of NP and ICI on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. Treatments of NP at the 10^{-7} M and 10^{-8} M concentrations were statistically different than the corresponding treatment with ICI suggesting ICI blocked the effects of NP at those concentrations. However, the control, NP 10^{-6} M + ICI 10^{-7} M and NP 10^{-6} M were not statistically different. This suggests that ICI 10^{-7} M did not effectively block the effects of NP on the cells at that concentration.

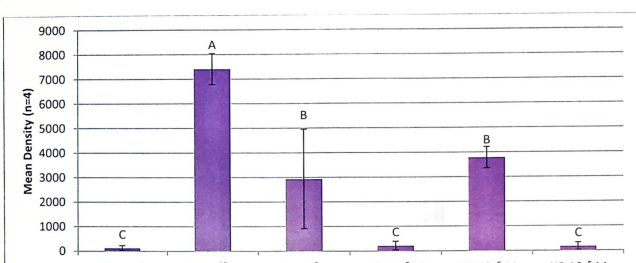

Figure 54. The effect of E2 and NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. NP 10^{-6} M + E2 10^{-10} M was statistically different than the E2 10^{-10} M treatment suggesting the NP 10^{-6} M treatment acted, at least in part, as an inhibitor to E2.

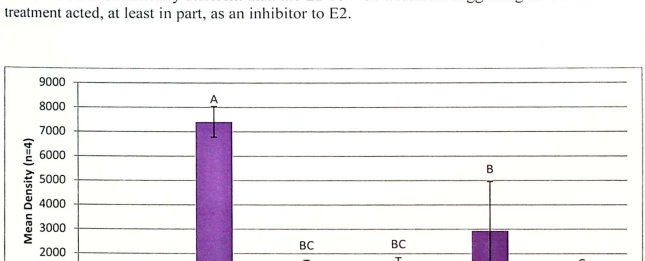

Figure 55. The effect of E2, ICI and NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. ICI 10^{-7} M + E2 10^{-10} M was statistically different than the E2 10^{-10} M treatment. NP 10^{-6} M statistically different than the NP 10^{-6} M + ICI 10^{-7} M treatment. This suggests that ICI works through the same mechanism to block E2 as it does for NP.

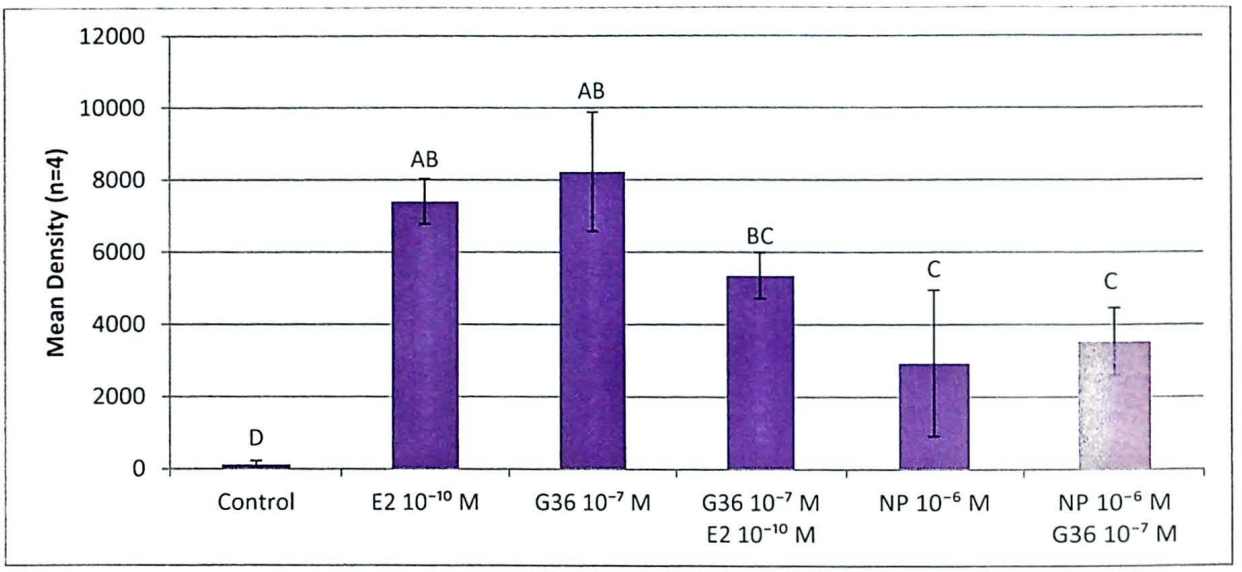

Figure 56. The effect of E2, G36 and NP on proliferation of MCF-7 cells. This graph shows the mean density and standard deviations of four replicate wells on fifth passage of the experiment. Treatments not connected by the same letter are statistically significantly different. E2 10^{-10} M and E2 10^{-10} M + G36 10^{-7} M were not statistically different from each other. NP 10^{-6} M and NP 10^{-6} M + G36 10^{-7} M were not statistically different from each other. This suggests that G36 does not block the operation of NP or E2 and that E2 and NP operate through the same mechanisms.