Use of fish cell lines to compare the cytotoxicity of Tetrabromobisphenol A with its degradation products and with an alternative brominated flame retardant

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2011

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Tetrabromobisphenol A, (TBBPA or Br4BPA), is a widely used brominated flame retardant (BFR). Although TBBPA and its breakdown products been found in river sediments, the environmental impact of their contamination is largely unknown. One breakdown product of TBBPA is bisphenol A (BPA), which has been studied intensively for its toxicology because it is used in the manufacturing of plastics and leaches from food containers, water bottles and pipes. Other breakdown products of TBBPA include tribromobisphenol A (Br3BPA), dibromobisphenol A (Br2BPA), and monobromobisphenol A (BrBPA) but little is known about their toxicology. Since TBBPA is toxic, there is a need to search for an alternative BFR, with one being tetrabromobisphenol A bis(2,3-dibromopropylether) or TBBPA-DBPE. However, almost nothing is known about the toxicology of this compound. Hence, two rainbow trout cell lines, RTL-W1 from liver and RTgill-W1 from gill, were used to evaluate the cellular toxicity of TBBPA, BPA, BrBPA, Br2BPA, Br3BPA and TBBPA-DBPE.

The cells were exposed to these compounds for 24 h in the basal medium, L-15, to study their cytotoxicity and in L-15 with fetal bovine serum (FBS) to evaluate their capacity to induce 7-ethoxyresorufin o-deethylase (EROD) activity. Viability was measured with three fluorometric indicator dyes: Alamar Blue (AB) for metabolism, 5-carboxyfluorescein diacetate acetoxymethyl (CFDA AM) for cell membrane integrity, and Neutral Red (NR) for lysosomal activity. The concentrations causing a 50 % reduction in viability (EC50) as measured with these three dyes were used to compare the relative cytotoxicity of these chemicals. For both cell lines and with all viability endpoints, TBBPA was the most cytotoxic, with EC50s ranging from 2.33 to 3.11 μg/ml. BPA, BrBPA, Br2BPA, and Br3BPA also caused dose-dependent declines in cell viability but showed no consistent order of potency. None of the six compounds induced EROD activity, which suggests that they do not activate the aryl hydrocarbon receptor (AhR). Regardless of the endpoint or cell line, TBBPA-DBPE was not cytotoxic. This suggests

that, from a toxicological perspective, this compound may be a suitable replacement for TBBPA as a BFR.

BPA stood out from the other compounds in two regards. BPA caused a dose-dependent decline in cell viability for cultures in L-15 with FBS, whereas for the other compounds, little or no change in viability was seen in cultures with FBS. BPA elicited a decline in the ability of cells to reduce AB almost immediately upon its addition to cultures in a simple buffer, whereas as for other compounds a decline took time to develop. These results suggest that BPA exerts its cytotoxicity by a different mechanism different from the other compounds.

Acknowledgements

I would like to acknowledge the following people for their involvement in my research:

Tee, Catherine[1]; Sherry, Jim [2]; Bols, Niels C[1]

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Also, I would like to thank the other members from the Bols' lab for their kind support.

Dedication

I dedicate this thesis to my family for their love and support. And, to my supervisor, Dr Niels Bols, for the opportunities and encouragements he had given me.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AB	Alamar Blue
AhR	Aryl Hydrocarbon receptor
ATSDR	Agency for Toxic Substances and Disease Registry
BFRs	Brominated flame retardants
BrBPA	Monobromobisphenol A or 3-monobisphenol A
Br_2BPA	Dibromobisphenol A or 3,3'-dibromobisphenol A
Br_3BPA	Tribromobisphenol A or 3,3',5-tribromobisphenol A
Br_4BPA	Tetrabromobisphenol A or 3,3', 5,5' tetrabromobisphenol A
decaBDE	Decabromo diphenyl ether
EC_{50}	Half maximal effective concentration
ER	Estrogen receptor
EROD assay	Ethoxyresorufin-O-deethylase assay
EU	European Union
FBS	Fetal Bovine Serum
GC-MSD	Gas chromatography–mass spectrometry
HBCD	Hexabromocyclododecane
HPLC	High performance liquid chromatography
K_{ow}	Octanol-Water Partition Coefficient
NMR	Nuclear magnetic resonance
NR	Neutral Red dye
octaBDE	Octabromo diphenyl ether
PBBs	Polybrominated Biphenyls

PBDEs	Polybrominated diphenyl ether
pentaBDE	Pentabromo diphenyl ether
RFU	Raw fluorescent unit
RFU	Relative fluorescent unit
RTgill-W1	Rainbow trout gill cell line
RTL-W1	Rainbow trout liver cell line
SDS	Sodium dodecyl sulphate
T_3	Triiodothyronine
T_4	Thyroxine
TBBPA	Tetrabromobisphenol A or 3,3,5,5' tetrabromobisphenol A
TBBPA-DBPE	Tetrabromobisphenol A bis(2,3-dibromopropylether)
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TTR	Transthyretin
US EPA	US Environmental Protection Agency
WHO	World Health Organization

Chapter 1 Introduction

1.1 Brominated Flame retardants

In the US, it is estimated that there are over a million fires per year, responsible for killing thousands and injuring more than 17,000 people. This has resulted in more than $20 billion USD in property damages (US National Fire Protection Association, 2009). Every year, fires kill more than 100,000 people worldwide at a cost of 1% of the world's GDP (European Flame Retardants Association, 2010). For the past 25 years, the incidence of fires had already dramatically decreased due to improvement of standards in fire safety, such as mandatory use of flame retardants in industrial and household products (Birnbaum & Staskal, 2004). For example, countries within the European Union are required to have all manufactured and imported household products such as interior decorations, furniture, consumer equipments and car interiors pass fire standard tests which include the evaluation of ignition time (European Flame Retardants Association, 2010).

Flame retardants are used in the manufacture of household products, and most widely used ones are brominated flame retardants (BFRs). BFRs are used in electronics, plastics, and building materials (de Wit, 2002; Birnbaum & Staskal, 2004). They can be additives or as components in the backbone of plastic products that have a high risk of ignition, such as electric cables and circuit boards. As a result, BFRs are present in almost all electronics. According to Greenpeace International (2005), the lifespan of computers and mobile phones averaged around 2 years in first-world countries. With a predicted use of 716 million new computers in 2010 in developing countries like China and India, electronic waste or "e-waste" is now the fastest

growing component of the municipal solid waste. Mobile phones, computers, televisions, audio equipments, etc. are upgraded, hence disposed, more frequently than ever before (Greenpeace International, 2005). The US Environmental Protection Agency (EPA) has estimated that there is more than 1.8 million tons of e-waste in US landfills (US EPA, 2005). The health of nearby communities and its organisms maybe negatively affected because the chemicals in e-waste, such as BFRs which can leach into the soil and water bodies. The most common BFRs include polybrominated biphenyls (PBBs), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA). Their structures are below:

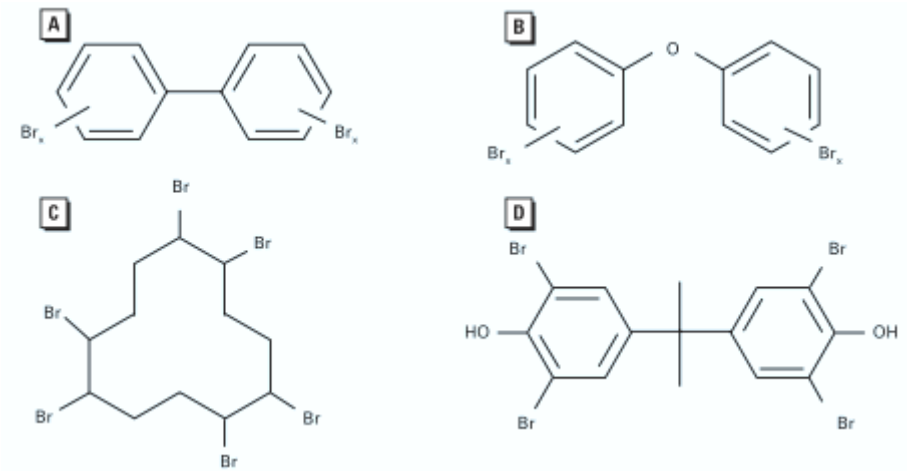


Figure 1.1:Structures of (A) PBBs, (B)PBDEs, (C) HBCD, and (D) TBBPA (Birnbaum & Staskal, 2004).

In the early 1970s, several thousand pounds of "Firemaster FF-1", a commercial PBB mixture was accidentally added to a livestock feed that was distributed to farms in Michigan, US. This eventually led to more than $100 million loss in livestock and poultry (Carter, 1976). Most

significantly, there were long term health impacts for the farm families who were exposed because PBBs were later found to be mutagenic and nephrotoxic (National Library of Medicine HSDB Database, 2009). In some studies, long term exposure induced neoplastic nodules in the liver and in some cases hepatocellular carcinoma in animals (Silberhorn *et al*, 1990). Since the toxicity of PBBs was known, it became highly regulated by countries around the world. Despite such a horrific incident, little toxicity information is still known about the long term use of other BFRs.

PBDEs or polybrominated diphenyl ethers is a widely used BFR in the US today, with more than 24,500 metric tons per region used as estimated in 2001(Birnbaum & Staskal, 2004). PBDEs are diphenyl ethers with varying number of bromine atoms (1-10) bound to the diphenyl rings. There are around 209 possible congeners (de Wit, 2002). The commercialized PBDEs are not a single congener but a mixture. Three mixtures of PBDEs which are pentaBDE, octaBDE and decaBDE are the main ones used today. These compounds are highly lipophilic (K_{ow} between 4.28 and 9.9), hence very likely to bioaccumulate in fat tissues (de Wit, 2002). The lower the bromination of the PBDE product, the more likely it is to bioaccumulate because it is more mobile than higher brominated ones. The higher brominated PBDEs may be less mobile because of their molecular weight. Therefore, lower brominated mixtures are more toxic than the higher brominated PBDEs. Indeed, pentaBDE was found to be more toxic than octaBDE and decaBDE in various studies using invertebrates, and decaBDE is found to be essentially non-toxic (Birnbaum & Staskal, 2004). The largest concern for PBDEs is neurotoxicity observed in organisms such as mice. When newborn mice were exposed to pentaBDE (commercial name "BDE 99") during the critical window for brain growth, motor behavior was found to be

impaired permanently (Viberg *et al*, 2002). Due to raising concerns, the EU has banned the use of pentaBDEs in products produced or imported since 2004. Therefore, the most used PBDEs around the world are decaBDEs (US EPA National Center for Environmental Assessment & Lorber, 2010). The US National Toxicology Program has conducted a two year study of decaBDEs using mice to monitor its long term effects (National Toxicology Program, 2011). So far, it had the most extensive data in both acute and chronic studies.

Various studies have found the effects of PBDEs to include interference in T_4 hormone level (Chevrier *et al*, 2010). Whether PBDEs affect estrogenic activities have yet to be further investigated as its effects were sometimes found in *in vitro* but not in *in vivo* (Birnbaum & Staskal, 2004). Some PBDEs are found to be an activator of aryl hydrocarbon (dioxin) receptor (Zhou *et al*, 2002). For instance, Zhou et al. (2002) found DE-71, another commercial pentaBDE mixture, to induce EROD activity which is a classic indicator of activation of Ah receptor. In humans, given the increasing production of PBDEs, health concerns for the chemical also escalate. Many studies are now being conducted to explore its potential toxicity to newborns through breast milk (Hooper & McDonald, 2000; Kalantzi *et al*, 2004).

Another BFR which is widely used in European countries is hexabromocyclododecane (HBCD). PBDEs such as pentaBDEs are banned in the EU. Hence, BFRs such as HBCD and TBBPAs were used to replace them. Studies have showed that HBCD are highly likely to bioaccumulate in organisms with a bioaccumulation factor of 18,100 in Fathead Minnows (*Pimephales promelas)* (Veith *et al*, 1979). It is also very persistent in the environment in sediments (Sellström *et al*, 1998). Studies with aquatic organisms found toxicity near levels of solubility (Organisation for Economic Co-operation and Development, 2003). Sometimes, results

of studies in HBCD are conflicting (Birnbaum & Staskal, 2004). This indicates a need for continued research.

Among all of the BFRs above, tetrabromobisphenol A (TBBPA) remains the most highly used BFR in the world. The amount of TBBPA used was close to that of PBDEs in the US but it was far more popular than other BFRs in Asian countries. In fact, 82.64% of the TBBPA produced worldwide was being used in Asia (Bromine Science and Environmental Forum, 2004). This is linked to a large electronic industry in China, Korea and Japan (Bromine Science and Environmental Forum, 2004).

BFRs are decomposed in the environment through many ways. This include by UV radiation from the sun and in different aerobic and anaerobic conditions (de Wit, 2002). The BFRs will then breakdown into metabolites and other products by microbes (Lobos *et al*, 1992; Ravit *et al*, 2005; Zalko *et al*, 2006; Sakai *et al*, 2007). The breakdown products may or may not be harmful. Only the effects of TBBPA and its potential breakdown products BrBPA, Br_2BPA, Br_3BPA and BPA will be discussed in this thesis.

1.2 Tetrabromobisphenol A

Tetrabromobisphenol A (TBBPA or Br_4BPA) is used to make epoxy resin in circuit boards in electronics. It is the highest produced brominated flame retardant, with more than 120,000 tons produced annually around the world (Ronisz, 2004; Bromine Science and Environmental Forum, 2004).

Brominated bisphenols are a group of chemicals with bromines attached to a bisphenol A (BPA) group. They are made through bromination of BPA. TBBPA is made of 4 bromine atoms

attached to a BPA group. TBBPA is highly lipophilic (K_{ow} = 4.5) and is not very water soluble (de Wit, 2002; Birnbaum & Staskal, 2004). The toxicity of TBBPA depends on its use. If it is use as a component of the plastic material, it is less likely to be toxic as it is chemically bonded. In contrast, if TBBPA is used as an additive, it may leach into the environment more readily. TBBPA has been found in sediments in Sweden and in sewage sludge in Canada, Sweden, US and also in Japan (Watanabe *et al*, 1983; Zalko *et al*, 2006).

Currently, TBBPA is proposed by the European Union to be "very toxic" to aquatic organisms (Institute for Health and Consumer Protection, 2006). TBBPA is known to decrease reproductive success in zebrafish at environmentally relevant concentrations (Kuiper *et al*, 2006). The compound is also found to be an endocrine disruptor in experimental systems both *in vitro* and *in vivo* (Legler, 2008). Its effects to aquatic organism are a concern based on its heavy use. Therefore, having a non-toxic alternative would be a beneficial option for both human and the environment in the future.

The toxicity of TBBPA and its intermediates are of interest because of their significant presence in the environment due to the huge amount of e-waste. The breakdown of TBBPA can be accomplished by microorganisms under anaerobic conditions in sediments (Ronen & Abeliovich, 2000; Arbeli & Ronen, 2003; Ravit *et al*, 2005) (Figure 1.2). Some of the intermediates recently identified by methods such as high-performance liquid chromatography are tribromobisphenol A (Br_3BPA), dibromobisphenol A (Br_2BPA), monobisphenol A (BrBPA) (Arbeli & Ronen, 2003). One of the end products of TBBPA degradation by microbes is BPA (Ronen & Abeliovich, 2000). BPA can then undergo aerobic mineralization by the gram-negative strain of *Sphingomonas* (Sakai *et al*, 2007). Some studies have also suggested that

TBBPA is broken down by UV radiation under the sunlight through debromination into Br_3BPA and other compounds (de Wit, 2002). TBBPA was found to be excreted through feces and bile in rats (Birnbaum & Staskal, 2004). Inside the body, TBBPA is found to be metabolized oxidatively and by the conjugative enzyme-dependent pathways (Zalko *et al*, 2006). It is deduced that TBBPA is broken down by enzymes through cleavages leading to different metabolites. *In vitro*, these metabolites are processed by cytochrome P450 in the liver and the end products, detected by nuclear magnetic resonance (NMR), are hydroxylated parts of TBBPA (Zalko *et al*, 2006). No reports have yet appeared as to how the intermediates BrBPA, Br_2BPA, Br_3BPA from environmental degradation are processed in animals.

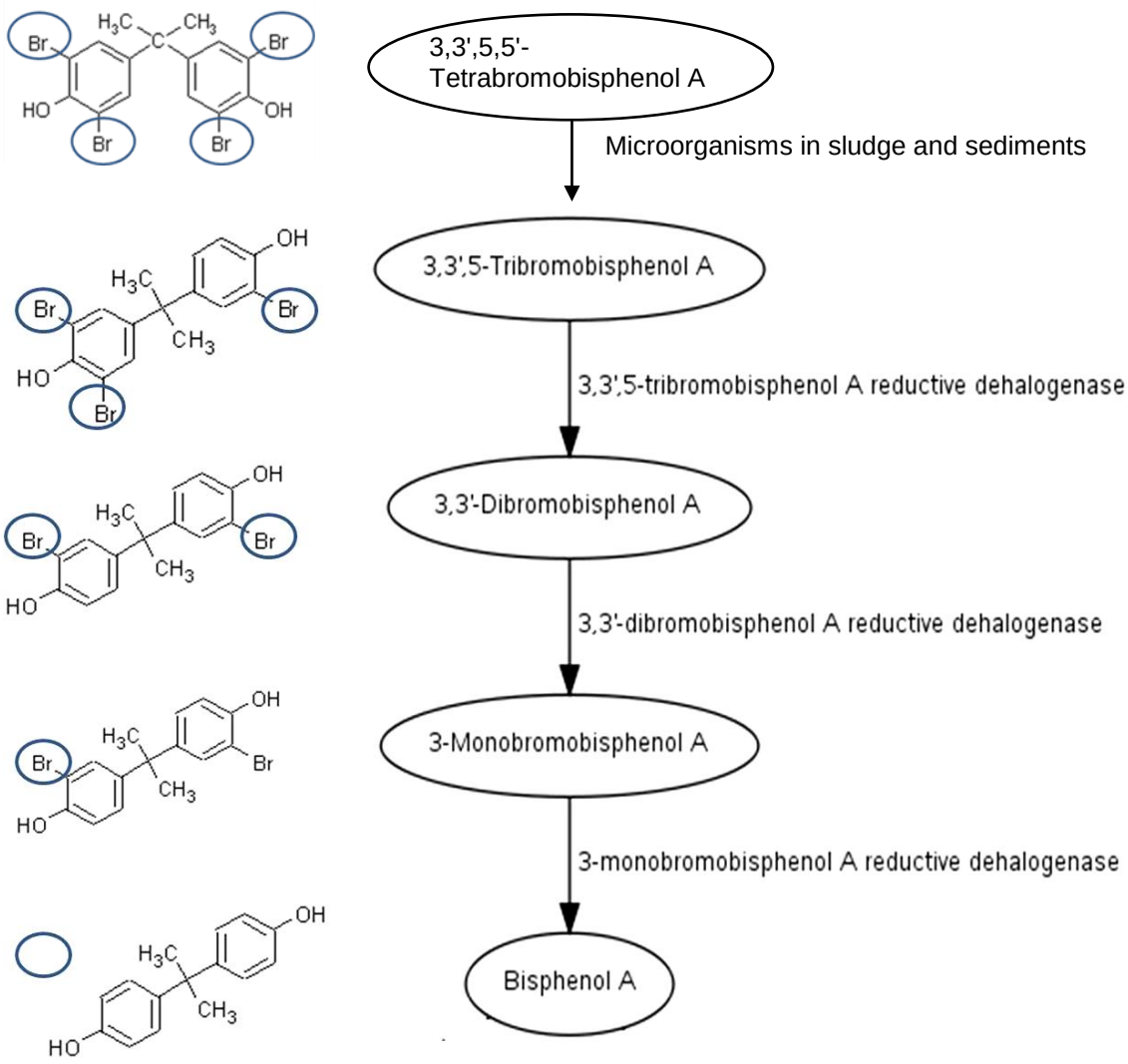


Figure 1.2: Degradation of Tetrabromobisphenol A, adapted from Arbeli & Ronen (2003).

1.3 The physiochemistry of TBBPA and its possible metabolites BrBPA, Br_2BPA, Br_3BPA

TBBPA is structurally based on its parent compound which is also its potential breakdown product at the end, bisphenol A (BPA). BPA was found to be weakly estrogenic (Vandenberg *et al*, 2007). TBBPA was found to have little estrogenic effect, however, the more it debrominates, the more its structure and activity may resemble BPA. Furthermore, TBBPA was found to inhibit triiodothyronine (T_3) and also transthyretin (TTR), a fluid transporter of thyroxine (T_4).

Thyroid hormones are in charge of metabolism and growth. T_4 in the blood gets converted to T_3 in target tissue where it signals different growth pathways such as those in charge of essential development of the brain (Silverthorn, 2004). Because both T_3 and T_4 are lipophilic, the transport of these compounds which affect their availability in the body is controlled by a carrier such as TTR (Silverthorn, 2004). TBBPA was found to be ten times more likely than T_4 to bind to TTR (de Wit, 2002). Because of that, free T_4 hormone levels in blood may be higher than usual and less of it will be transported to the right target tissue to be converted into T_3. The decreased level of T_3 may impact growth and other functions of the organism (Silverthorn, 2004) (Figure 1.3). Furthermore, TTR also play a role in delivering T_4 hormone to the developing fetus through placental blood, the lack of T_4 in the fetus may lead to impaired brain development (Birnbaum & Staskal, 2004). The intricate balance of T_3/ T_4

hormone in the body also affects different organs and functions such as mood and behavior of the animal.

The influence of TBBPA to human is still under investigation. However, its effects may be magnified in smaller organisms such as mice. Currently, more research has yet to be done about the endocrine disrupting abilities of BrBPA, Br_2BPA and Br_3BPA as nothing is known about these chemicals.

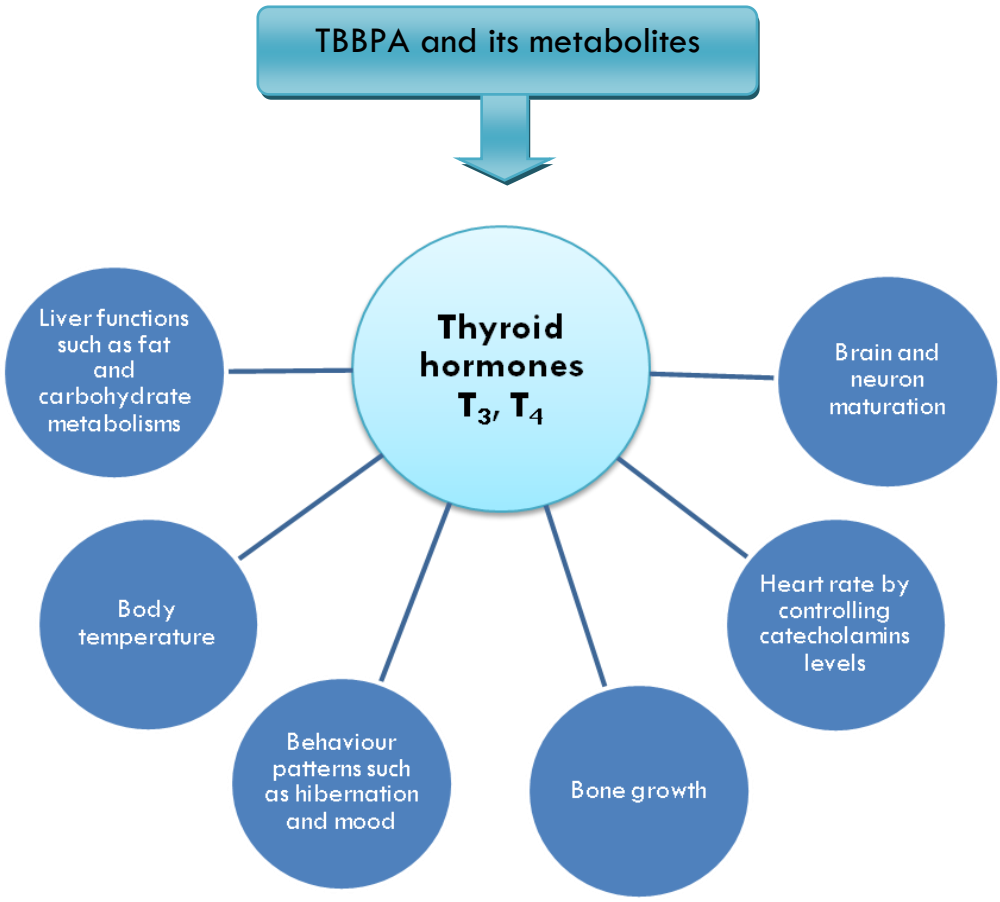


Figure 1.3: Possible physiological influences of TBBPA.

1.4 Toxicological significance of bisphenol A

The potential risk of bisphenol A (BPA) has long been a concern. For the past 50 years, BPA has been an industrially important chemical in the manufacture of plastics. It is used at up to 6 billion pounds per year worldwide (Vandenberg *et al*, 2008). Apart from its use as a primary material in plastics, it's also used to make other chemicals such as flame retardants and polyesters (Tsai, 2006). BPA is not produced in nature and is released to the environment by industrial plants and plastics disposed in landfills (Yamamoto *et al*, 2001). Humans come into contact with BPA through BPA leaching from plastic bottles and food containers. It has been identified as an environmental hormone which can act as an endocrine disruptor (Tsai, 2006; Vandenberg *et al*, 2007; Bonefeld-Jørgensen *et al*, 2007; Watson *et al*, 2007). An endocrine disruptor can mimic the body's own hormones and bind to corresponding receptors, leading to adverse health effects. Mammals in their early development stages will be the most sensitive to endocrine disruptors. Safety levels were determined for humans, but those levels are being questioned or reviewed as a result of new scientific studies (Vandenberg *et al*, 2007). Therefore, BPA became a public concern. Governments around the world started to investigate the possibility of limiting this chemical and its release from industrial processes. The European Commission and European Food Safety Authority did not ban this chemical, stating that the public was exposed to levels that are "well below levels considered harmful" (Tsai, 2006). United States and Canada also expressed concerns over BPA. Canada first banned the use of BPA in baby bottles in April, 2008 but not in other plastic materials (CBC, 2008). However, in an historic move in October, 2010, Canada declared BPA to be toxic and is the first country in

doing so despite controversies among other countries citing there are enough research evidence to prove that it is harmful to the health of both humans and the environment (Reuters, 2010).

1.5 Exposure to BPA in the environment

BPA exists in the environment through many sources. It is leached to the environment from plastics and industrial plants (Tsai, 2006). When expelled to the environment, there is a low to moderate potential for it to partition in water (log K_{ow}=3.3) (Tsai, 2006). Microorganisms also take part in biodegrading BPA. BPA is not expected to persist very long in the environment due to biodegradation and bioaccumulation, but low levels of it existed in water bodies around the world. Most monitoring shows that BPA in water bodies is around 1 µg/L (Tsai, 2006). Other sources of BPA include dust from indoors and outdoors and direct contact from plastics (Tsai, 2006). Residents in offices or homes in urban areas are ubiquitously exposed to plastics, and thus, BPA. However, exposure to consumer products is expected to be 1 µg/kg body weight/day which is much lower than the safe dose of 50 µg/kg/day recommended by the United States Environmental Protection Agency (US EPA) (Tsai, 2006).

Apart from water exposure, the most common route of exposure to humans is considered to be intake of food and fluids in contact with BPA. During digestion, BPA forms glucuronidate metabolites and is excreted rapidly from the body (Vandenberg *et al*, 2007). However, the consistent exposure to BPA is a concern as many animal studies demonstrated endocrine disruptive effects such as a decrease in sperm production and impaired neurological development (Yamada *et al*, 2002; Vandenberg *et al*, 2008).

1.6 What are the effects of BPA in low dose?

Since exposure to hormones during developmental processes may have an permanent organizational effect on the individual, exposure to endocrine disruptors may indeed influence development (Richert *et al*, 2000; Yamada *et al*, 2002; Rayner *et al*, 2005; Vandenberg *et al*, 2008). For instance, the critical window for organogenesis in mammals occurs in the first trimester. Female mice which were exposed to the pesticide Atrazine (a possible endocrine disruptor) during gestation period produced significantly smaller pups because of impaired mammary gland development (Rayner *et al*, 2005). Therefore, it is possible that exposure to BPA during the first trimester of pregnancy and in puberty of an organism may have adverse effects as it is also an endocrine disruptor (Vandenberg *et al*, 2008).

Many studies indicated BPA may have a low dose effect. For example, Somm et al. (2009) demonstrated that early adipogenesis in female rats born to mothers exposed to low levels of BPA (0.1 mg BPA/kg BW per day) during gestation was altered. The rats were significantly heavier than those not exposed to BPA (Somm *et al*, 2009).

A detectable level of BPA was found in the serum of pregnant women and in their fetus' umbilical cord blood and plasma indicating that the compound can cross the maternal-fetal placental barrier (Yamada *et al*, 2002; Vandenberg *et al*, 2007). BPA has also been detected in human urine from populations around the world (Vandenberg *et al*, 2008). Hence, there is clearly a need for further investigations.

1.7 Physiochemical properties of BPA

The –OH groups of BPA can undergo a variety of chemical reactions under acidic or basic conditions, such as esterification and etherification (Figure 1.4). It may yield compounds like phenol, 4-isopropyl phenol, and semiquinones (Tsai, 2006). BPA's structure is similar to estradiol with two hydroxyl groups and a hydrocarbon backbone (benzene rings) (Figure 1.4 & Figure 1.5). It fits in the binding site of estrogenic receptors (ER) in the body and may induce an effect (Figure 1.6). However, it's considered as weakly estrogenic because it has 10,000-fold weaker affinity for ER as compared to estradiol (Vandenberg *et al*, 2007). Binding of BPA to different ER may alter their ability to recruit co-activators for DNA transcription in tissue-specific responses (Figure 1.6) (Vandenberg *et al*, 2008).

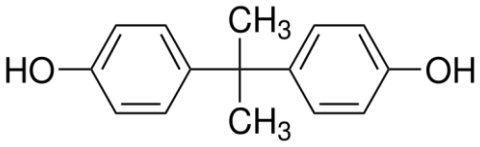


Figure 1.4: Structure of BPA.

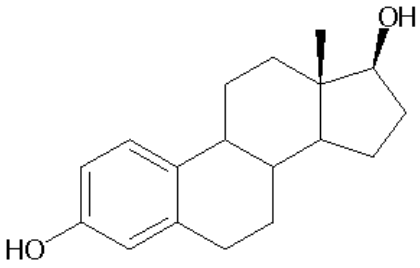


Figure 1.5: Structure of estradiol (US EPA, 2011).

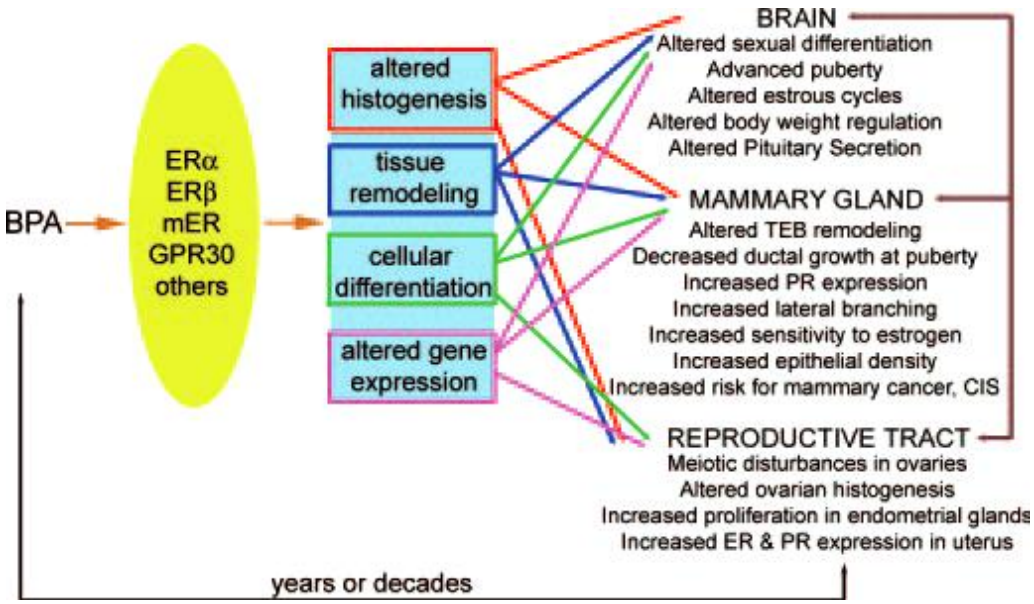


Figure 1.6: Possible mode of action of BPA in mammals (Vandenberg *et al*, 2008).

In vitro studies have shown that BPA may utilize signaling pathways downstream of receptor activation; hence its affect was not limited by its low affinity for ER. For example, low levels of BPA produced a calcium influx which increased levels of prolactin in cells (Watson *et al*, 2007). *In vivo* studies have observed increase in vitellogenin production in male fishes exposed to high levels of BPA in effluent which lead to male fish to produce sperm and ova concurrently (Hill, 2010).

1.8 Tetrabromobisphenol A bis(2,3-dibromopropyl ether) or TBBPA-DBPE

TBBPA (Figure 1.8) has been found to be toxic and this has led to a search for alternatives with tetrabromobisphenol A bis (2,3-dibromopropyl ether) , or TBBPA-DBPE, being one (Figure 1.7). TBBPA-DBPE is manufactured by Albemarle Corporation, the Great Lakes Chemical Corporation, and several chemical corporations in China (EPA, 2007; Cai, 2008). It was first introduced to replace the flame retardant decabromodiphenyl oxide (DBDPO) which was banned due to its negative environmental impacts in the late 90s. Since then, it has been produced up to10 million pounds annually in the US (US EPA, 2006). TBBPA-DBPE released into the environment is expected to accumulate in sediments and sewage sludge as it is highly hydrophobic (Haneke, 2002). Concerns have been raised that TBBPA-DBPE could possibly be broken down into dibromo-1-propanol (DBP) which was proven carcinogenic to B6C3F 1 mice and F344/N rats (Heneke, 2002). Later, it was found that the probability of forming DBP is low (Knudsen *et al*, 2007). In a study of absorption, distribution, metabolism and excretion of this chemical using F344 rats, TBBPA-DBPE was found to be metabolised in the body to glucuronides conjugates in the liver similar to the mechanism of BPA, it is then excreted via the bile or feces (Knudsen *et al*, 2007). Acute toxicity was also found to be low (LD_{50} = 20g/kg in rats and > 20g/kg in mice). The compound was found to have no T_4 competing potency in contrast with other brominated bisphenols (Hamers *et al*, 2006). Almost nothing is known about TBBPA-DBPE's effects to aquatic organisms.

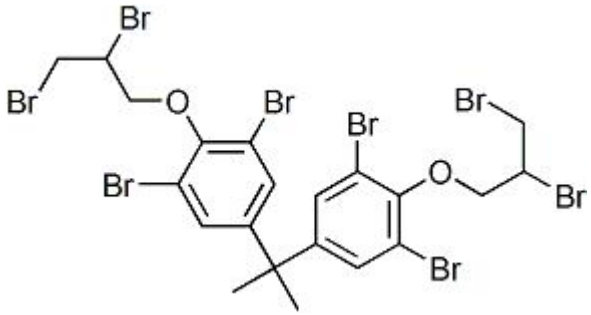


Figure 1.7 Tetrabromobisphenol A bis (2, 3-dibromopropyl ether)

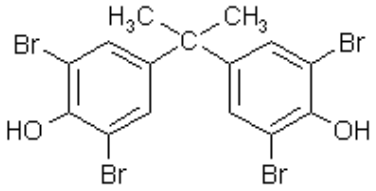


Figure 1.8: Tetrabromobisphenol A (TBBPA)

1.9 *In vitro* study of contaminants using fish cell-lines

Toxicity studies using fish cell lines offer numerous benefits. It can save time, money and labor. Established cell lines such as Rainbow trout *(Oncorhynchus mykiss)* liver and gill cells (RTL-W1 and RTgill-W1) takes between 1-3 weeks to reach confluency (to grow into a mono-layer) in a 75 cm^2 flask ready for experimentation. In contrast, it may take months for live fishes to mature and even more time to dissect them and harvest organs for experiments. Thus, the *in vitro* approach is an ideal system to complement *in vivo* during primary screenings by decreasing the amount of animals sacrificed. Rainbow trout is a model organism in contaminant studies because of their broad distribution in lakes and streams of North America and their use in aquaculture around the world (Environment Canada, 2010). Its organs, such as the liver and the gill, are selected for experimentation based on their physiological significance. The liver is

responsible for detoxifying compounds in the environment and secretes many enzymes such as cytochrome P450 1A (CYP1A) that oxidizes lipophilic compounds into more hydrophilic metabolites. Monitoring levels of such enzymes in the liver provides information about how the animal deals with chemical insults. The gills of a fish are the first gateway for contaminants to enter the body. Impaired functions of the gill may lead to failing physiological functions in the body. Using cell lines of the above organs, or the *in vitro* method, provides an understanding of what is going on at the cellular level.

One approach that has been used to study TBBPA and BPA toxicity had also been the *in vitro* one. TBBPA is found to act as an endocrine disruptor and interferes with thyroid hormone homeostasis by competing with thyroxin T_4 hormone *in vitro* (Canesi *et al*, 2005). Studies by Mariussen and Frode (2003) showed that Tetrabromobisphenol A (TBBPA) inhibit plasma membrane uptake of the neurotransmitters dopamine, glutamate and γ-amino-*n*-butyric acid (GABA) and other vesicles in rat brain synaptosomes. As a result, the accumulated neurotransmitters, such as dopamine, in the plasma membrane are oxidized producing reactive oxygen species. As for BPA, it is observed to bind specifically to human estrogen-related receptor-γ in HeLa cell lines (Okada *et al*, 2008), thus may possibly influence development.

1.10 Cytotoxicity Endpoints

Various endpoints would measure various components of cellular health. The endpoints selected for this study would be a combination of fluorescent dyes measuring viability: Alamar Blue (AB) for metabolism, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA AM) for cell membrane integrity and Neutral Red (NR) for lysosomal membrane activity. Together, these assays will provide information about the components which are vital to the survival of the cell. The endpoints are measured 24 hours after exposure to the target chemical. Afterward, the cells studied are disposed.

In additional, the above fluorescent dye assays were improved to measure acute response of the cells to the compounds almost concurrently in the first 1-3 hours of exposure.

Ethoxyresorufin-O-deethylase (EROD) assay is another endpoint used to measure activation of the aryl hydrocarbon receptors (AhR). The ligands of AhR are dioxin-like compounds. These include polycyclic aromatic hydrocarbons (PAHs). Some of the PAHs identified by Agency for Toxic Substances and Disease Registry (ATSDR) include benzo(e)pyrene, benzo(a)anthracene and pyrene (ATSDR, 2009). These substances are produced through burning fuels such as coal, wood, petroleum, petroleum products, or oil. Some of the PAHs are found to be carcinogenic (ATSDR, 2009). Other substances which act through the AhR may have similar toxic outcomes as PAHs. EROD assay used in this study will indirectly measure the activation of AhR if there is an elevated production of CYP1A (Figure 1.9).

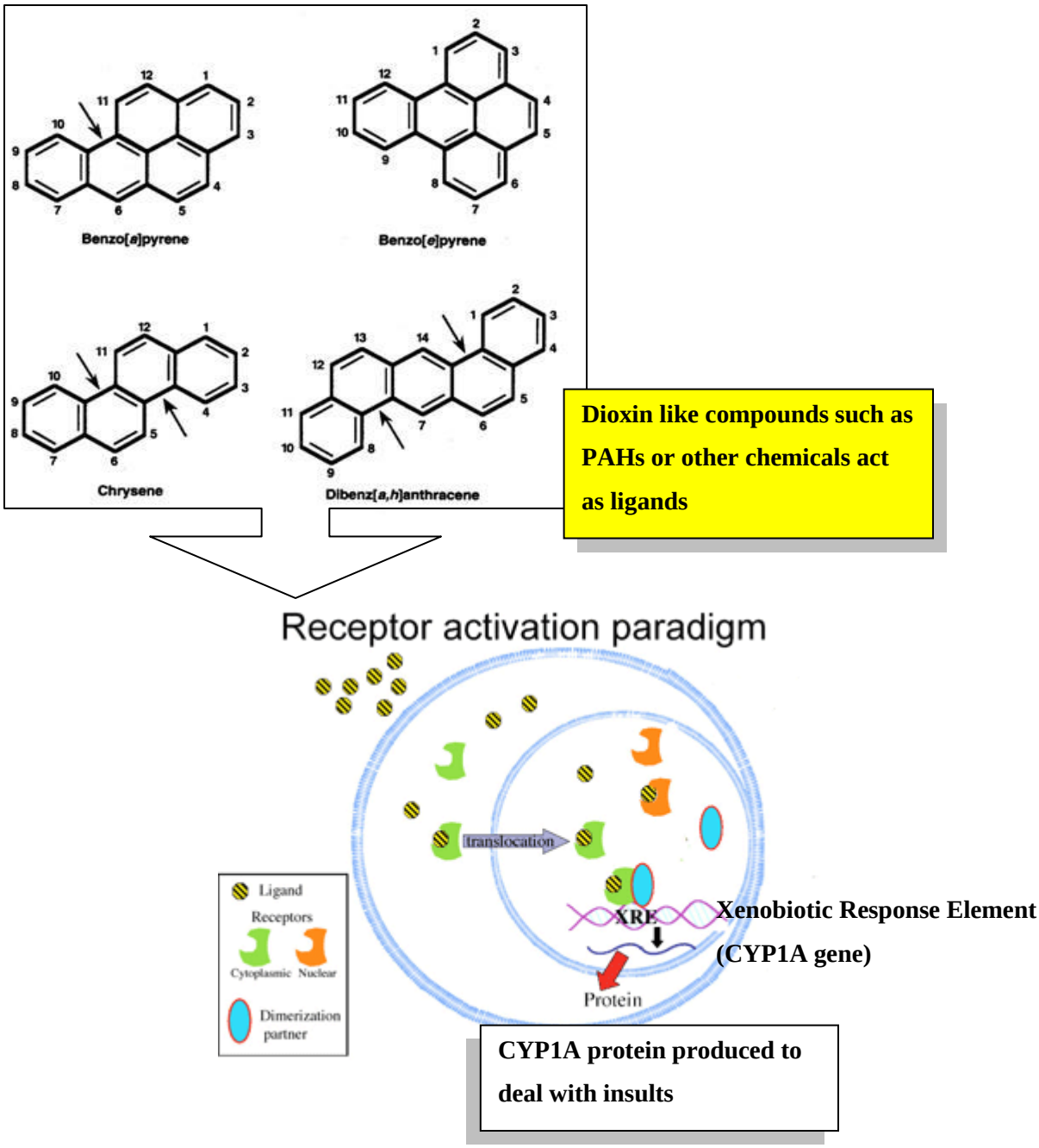


Figure 1.9: AhR receptor activation paradigm (ATSDR, 2009; Hahn, 2005).

1.11 Objectives of study

To investigate the possible cytotoxic effects of BPA, Tetrabromobisphenol A (TBBPA), its breakdown products Tribromobisphenol A (Br_3BPA), Dibromobisphenol A (Br_2BPA), monobisphenol A (BrBPA), as well as a potential alternative TBBPA-DBPE, using the fish cell lines RTL-W1and RTgill-W1.

Chapter 2 Materials and Methods

2.1 Fish cell cultures

RTL-W1 and RTgill-W1 are, respectively, liver and gill cell lines from Rainbow trout (Lee *et al*, 1993; Bols *et al*, 1994). All of the cell lines were grown in 75cm^2 tissue culture-treated flasks at room temperature in Leibovitz's L-15 culture medium (Sigma-Aldrich, Ltd., Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1 % penicillin-streptomycin solution (10 000 units/mL penicillin, 10 mg/mL streptomycin, Sigma-Aldrich). Routine sub-cultivations were made from confluent flasks with Tryple E solution (Invitrogen, CA, USA).

2.2 Exposure of Fish cells to BFRs

BPA, BrBPA, Br_2BPA, Br_3BPA, TBBPA and TBBPA-DBPE were all provided in powder form by Environment Canada. Stock solutions with concentration of 100 mg/mL were made by dissolving 100 mg of the chemical in powder into 1 mL dimethyl sulfoxide (DMSO). The purity of each of the stock compound in powder form is >99% pure based on GC-MSD and NMR analysis, as specified by Environment Canada. For Br_2BPA, the structure is 3,3'-dibromobisphenol A with no 3,5-dibromobisphenol A in preparation (please see Figure 1.2 for structure).

Exposures for cytotoxicity assays were done in 96-well plates with a density of 37500 cells/well in 200 µL of L-15, without FBS. Trials with 10% FBS were also done to explore the

differences in toxicity. For each of the compounds tested for cytotoxicity, there were 8 replicates (Figure 2.1). The compounds were dosed directly with a Hamilton syringe. This involved adding a small volume of 1µL of the stock solution directly to the wells. The final concentrations of each toxicant in the well were 1µg/ml to 50µg/ml.

Exposures for the detection of EROD activity were done in 48-well plates in a density of 75 000 cells/well in 500 µL of L-15, supplemented with 5% FBS and 1% penicillin-streptomycin solution. A much wider range from 0.078125µg/ml to 5µg/ml was used for the detection of EROD activity. In the EROD assays, for each of the chemicals tested, there were 6 replicates for each concentration. All compounds were directly dosed.

All of the above experiments were done in room temperature and incubated for 24 hours.

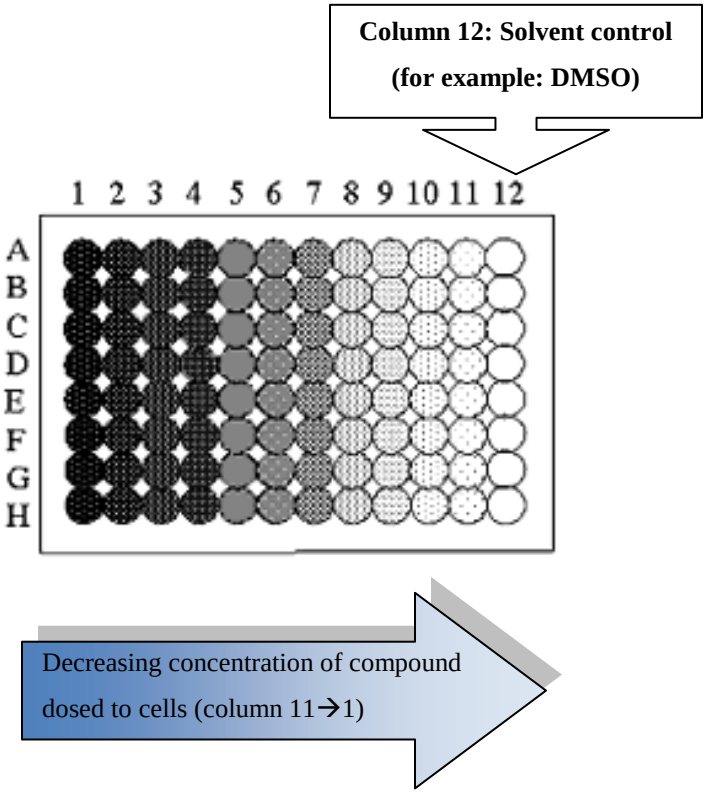


Figure 2.1: An example of dosing using a 96-well plate.

2.3 Cytotoxicity Assays

Various cytotoxicity tests were done to explore the toxicity of the above compounds. Cell viability was measured by three fluorometric assays, allowing the detection of three endpoints. These were Alamar Blue (AB) for metabolism, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA AM) for cell membrane integrity and Neutral Red (NR) for lysosomal membrane integrity (Figure 2.2).

100µL of mixture containing 526µL of Alamar Blue dye, 10.4 µL of CFDA AM in 10mL of L-15/ex was added to each well. The cells were incubated in the solution for 1 hour in the dark and afterward read with CytoFluor 4000 (PerSeptive Biosystem, Burlington, ON, Canada) at excitation and emission wavelengths of 530 nm and 590 nm for AB, and 485 nm and 530 nm for CFDA AM respectively. After the readings were taken, the Alamar Blue dye mixture was discarded and 100µL of mixture containing 180 µL of Neutral Red dye in 11.8 mL of phosphate buffer solution (PBS, Sigma-Aldrich) was added to each well. The cells were incubated in the dark for one hour. The mixture was discarded again and the plate was washed with a fixative solution containing 0.5% (v/v) formaldehyde and 1 % $CaCl_2$. Afterward, each well was filled with 100 µL of extractive solution containing 1% (v/v) acetic acid and 50% (v/v) ethanol. The plate was shaken at high speed of about 600 rpm for 10 minutes. The absorbance of Neutral Red was measured at the excitation and emission wavelengths of 530 nm and 645 nm, respectively.

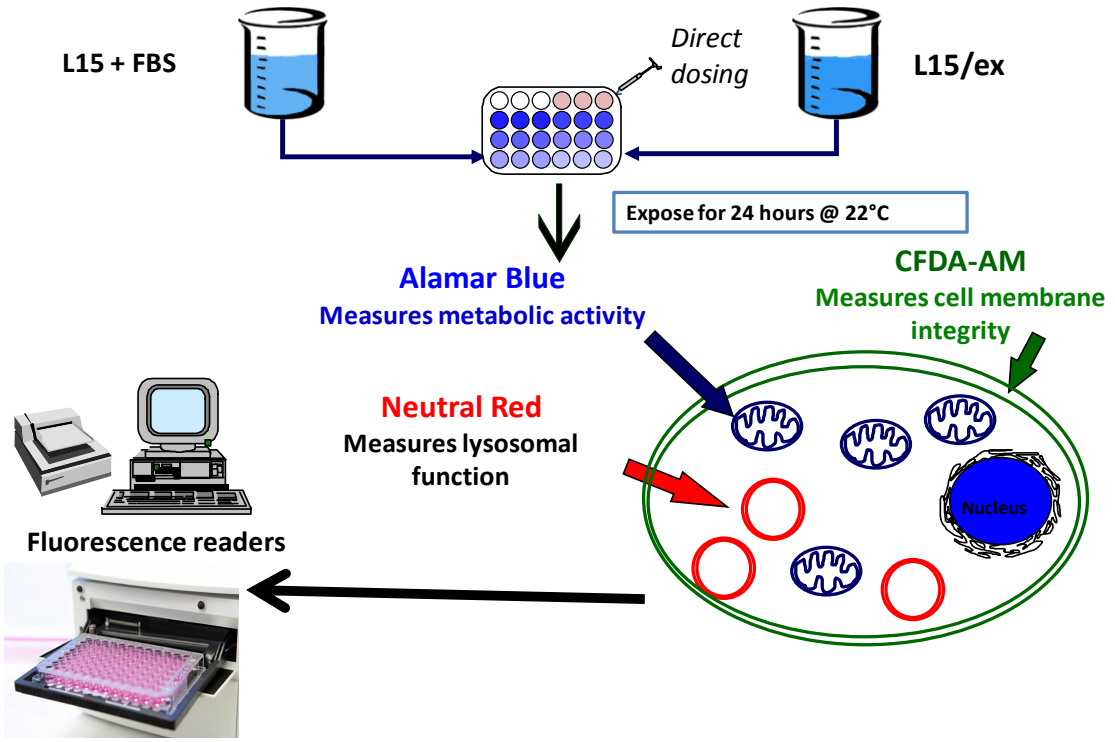


Figure 2.2: Run down procedure for 24 h cytotoxicity endpoints

2.4 Analysis of Dose-Response Curves from the Cytotoxicity Assays

Dose response curves were constructed with the GraphPad Prism 4 (GraphPad Software, Inc., CA, USA) non-linear curve fitting module using fluorescence readings expressed as the percentage of control. The values for the EC_{50} (half maximal effective concentration) for each of the endpoints were obtained from the graph. The data from each experiment were examined with one-way analysis of variance (ANOVA) and with Tukey-Kramer Multiple Comparison Test using GraphPad InStat (GraphPad Software, Inc., CA, USA). If the *p* value was less than 0.05, the toxicant was concluded to be a significant stressor.

2.5 Concurrent cytotoxicity assay for metabolism and cell membrane integrity

A concurrent cytotoxicity assay was developed to investigate the acute response of the fish cells to the compounds tested. The readings of Alamar blue and CFDA AM were measured every 15 min from cells dosed with the same range of concentrations of the traditional 24 h assay (Section 2.3). To do this, Alamar Blue and CFDA AM mixtures with varying concentrations of the compounds dissolved in DMSO are added to the plate of cells right away and is measured as time = 0 min. The control wells consisted of cells dosed with only AB / CFDA AM mixture and DMSO. The plate is then monitored by a fluorescent plate reader every 15 minutes up to 1 hour. The plate reader used are CytoFluor 4000 (PerSeptive Biosystem, Burlington, ON, Canada) and VICTOR 3V 1420 (Perkin Elmer, Woodbridge, ON, Canada). The fluorescent readings collected were expressed as % of control with time. This experiment was done using a 24-well plate without the addition of FBS.

2.6 Detecting EROD induction

All of the above toxicants were tested along with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for their ability to induce EROD. One approach to quantify EROD activity is to estimate the potency relative to TCDD, a potent activator of AhR. Cells on the plate were dosed directly with the toxicants from a range of 0 to 5 µg/ml, and one row with TCDD in the range of 1.5 pM to 97.6 pM. The plate is then incubated for 24 hour and washed with PBS afterwards. 250 µL of a reaction solution consisting of 0.8 µL of 7-Ethoxyresorufin (7ER) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-aldrich) was added to each well. The DMEM used here does not contain phenol red. The fluorescent product, resorufin, was detected by the

CytoFluor 2350 multi-well plate reader (PerSeptive Biosystem, Burlington, ON, Canada) in excitation and emission wavelengths of 530 nm and 590 nm, respectively. As soon as the reaction mixture was added, a reading was taken as time 0. The plate was then shaken at 100 rpm and readings were taken every 15 minutes for 1 hour. The substrate 7ER will be oxidized to resorufin if CYP1A is present in the cell (Figure 2.3). The amount of protein was detected by a solution of fluorescamine as described by Lorenzen and Kennedy (1993). A linear resorufin standard curve was generated periodically with the plate reader using known concentrations. Fluorescent units from each experiment are converted to picomoles of resorufin using the below expression:

$$\text{pmol of resorufin} = \frac{Fluorecent\,units\,of\,time\,(x) - \,fluorescent\,units\,of\,time\,(0)}{slope\,of\,the\,resorufin\,standard\,curve}$$

If CYP1A is present in the cell:

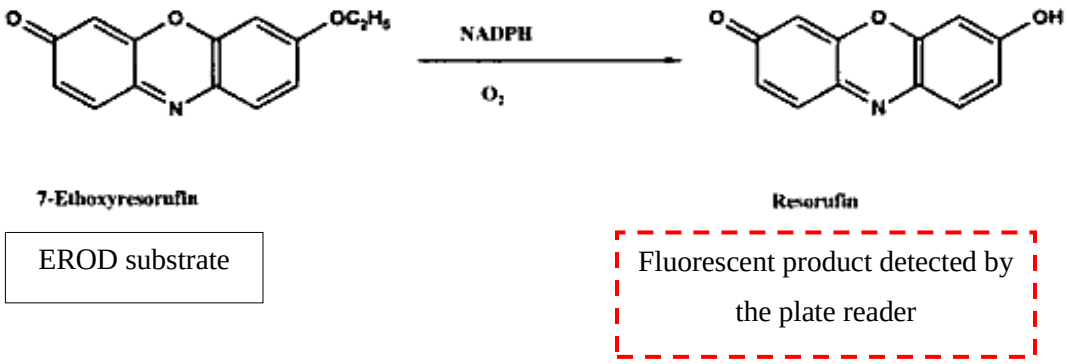


Figure 2.3: Mechanism of EROD assay (Mothersill & Austin, 2003).

2.7 Analysis of Dose-response curves for EROD

EROD activity was first calculated from the data and expressed as pmol resorufin/mg of protein/min. Dose response curves were constructed with GraphPad Prism 4 (GraphPad Software, Inc., CA, USA) using the values of EROD activity. The data from each experiment were examined with one-way ANOVA using GraphPad InStat (GraphPad Software, Inc., CA, USA). If the *p* value was less than 0.05, the toxicant was concluded to be an EROD inducer.

Chapter 3 Results

3.1 Capacity of BPA and brominated flame retardants (BFRs) to induce EROD

Exposure from approximately 2 to 100 pM TCDD for 24 h strongly induced EROD activity in RTL-W1 cultures in L-15/FBS but exposures under the same conditions to TBBPA-DBPE, TBBPA (Br_4BPA), Br_3BPA, Br_2BPA, BrBPA, or BPA at up to 10 µg/ml had little or no effect on EROD activity (Figure 3.1). One possible explanation for the failure to see an increase in EROD activity could be the carry-over of these compounds from the induction period into the EROD assay and having them interfere with the catalytic activity of CPY1A, which is responsible for EROD activity. To test this, microwell cultures of RTL-W1 in 48-well plates were exposed to 97.6 pM TCDD for 24 h. The induction medium was removed and EROD activity was measured in microwells in which BPA had or had not been added. EROD activity was not diminished by the presence of BPA in the assay. Overall, these results suggested that the tested BFRs were unable to induce EROD activity in RTL-W1. Therefore their potential to be cytotoxic was evaluated at higher concentrations and in the absence of serum (-FBS).

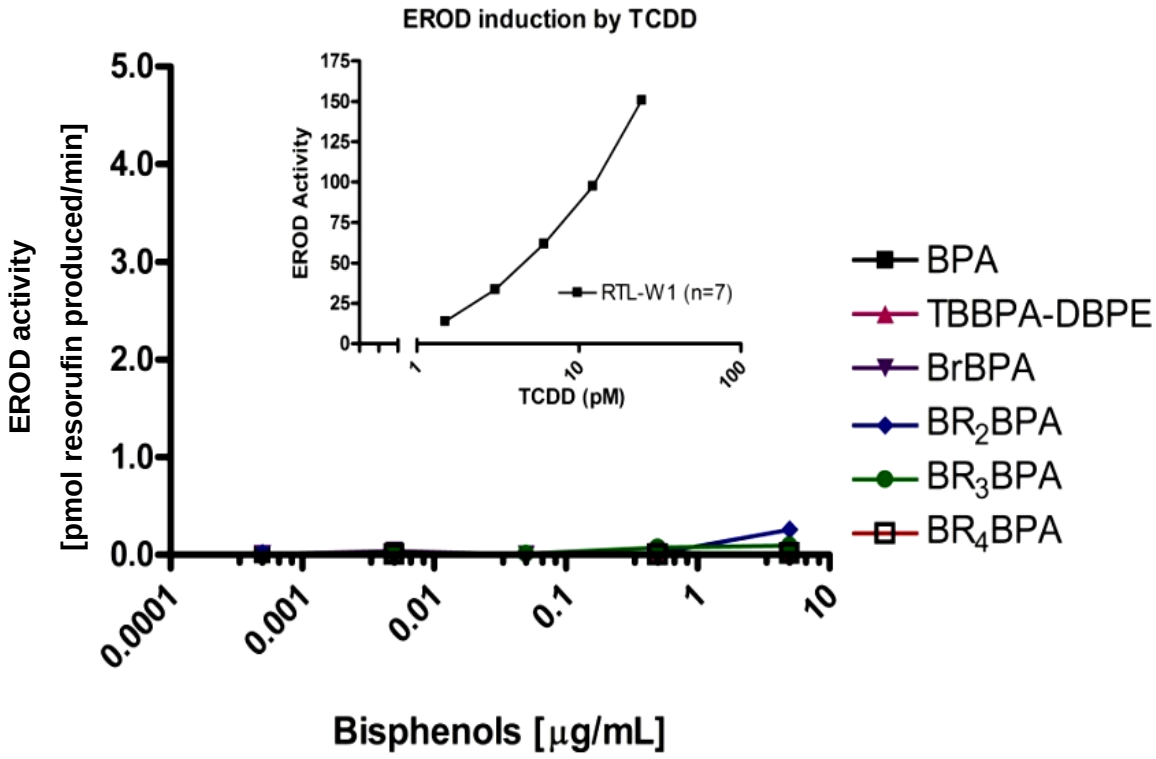


Figure 3.1: EROD activity as compared to the positive control TCDD in RTL-W1.

3.2 Cytotoxicity of tetrabromobisphenol A bis(2,3-dibromopropylether) (TBBPA-DBPE)

TBBPA-DBPE was not cytotoxic. Exposure of RTgill-W1 and RTL-W1 in L-15 to this alternative flame retardant at up to 50 μg/ml (52.97 μM) caused no change in cell morphology, as judged by phase contrast microscopy (Figure 3.5). When cultures were exposed to TBBPA-DBPE at concentrations from 0 to 50 μg/ml for 24 h and evaluated with the fluorescent indicator dyes AB, CFDA AM and NR, little or no change in readings, expressed as raw fluorescent units (RFUs), were observed relative to the control cultures (Figure 3.3). By contrast, increasing concentrations of the other BFR compounds did bring about a progressive decline in readings as described in the next section.

3.3 Cytotoxicity of Brominated BPAs and BPA

TBBPA, Br_3BPA, Br_2BPA, BrBPA and BPA were cytotoxic when exposures were done in L-15. For TBBPA, concentrations up to approximately 2 μg/ml (3.68 μM) in L-15 caused little change in the appearance of cultures, but at higher concentrations, the cultures were observed to have cells with altered shapes. This was also true for Br_3BPA, Br_2BPA, BrBPA and BPA below 3 μg/mL (10.75 μM, 12.95 μM, 16.28 μM & 21.9 μM, respectively). As the concentrations increased above 6 μg/ml, readings with the indicator dyes progressively declined (Figure 3.2) allowing EC_{50}s to be calculated for each endpoint in each cell line (Table 3.1). The EC_{50}s ranged from a low of 2.33 μg/ml to a high of 15.14 μg/ml.

The most cytotoxic compound was TBBPA. For TBBPA in both cell lines, the EC_{50}s for at least one cell viability assay was different (Tukey-Kramer multiple comparison test, $p < 0.05$) from the EC_{50}s for the same assay with each of the other four compounds (Tables 3.2 and 3.3). For example with RTL-W1, TBBPA was significantly different from Br_2BPA only for the CFDA AM assay, from BrBPA and Br_3BPA for both the Alamar Blue and Neutral Red assays, and from BPA for all three assays (Table 3.2). With RTgill-W1, TBBPA was significantly different from the other compounds in all assays (Table 3.3).

For Br_3BPA, Br_2BPA, BrBPA and BPA, the EC_{50}s varied with the cell line and the cell viability assay and so the cytotoxic ranking of these compounds cannot be easily stated and they might be regarded generally as equally cytotoxic. In both cell lines and with all three assays, BPA did not differ from Br_2BPA, and BrBPA did not differ from Br_3BPA (Tables 3.2 and 3.3).

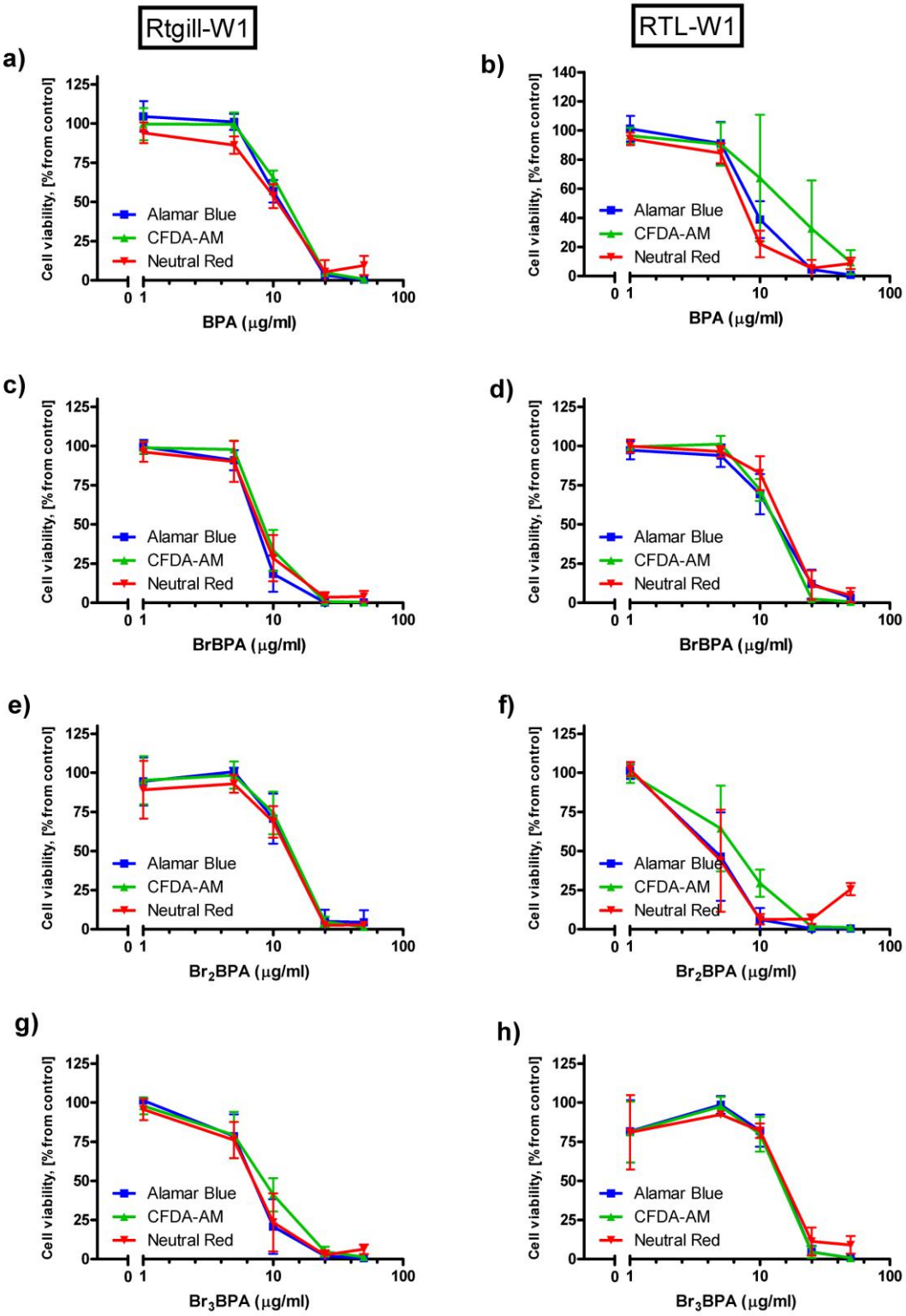


Figure 3.2: Effects of different bisphenol compounds on RTgill-W1 (Right side: a, c, e, g) versus effects on RTL-W1 (Left side: b, d, f, h) (n= 4-6).

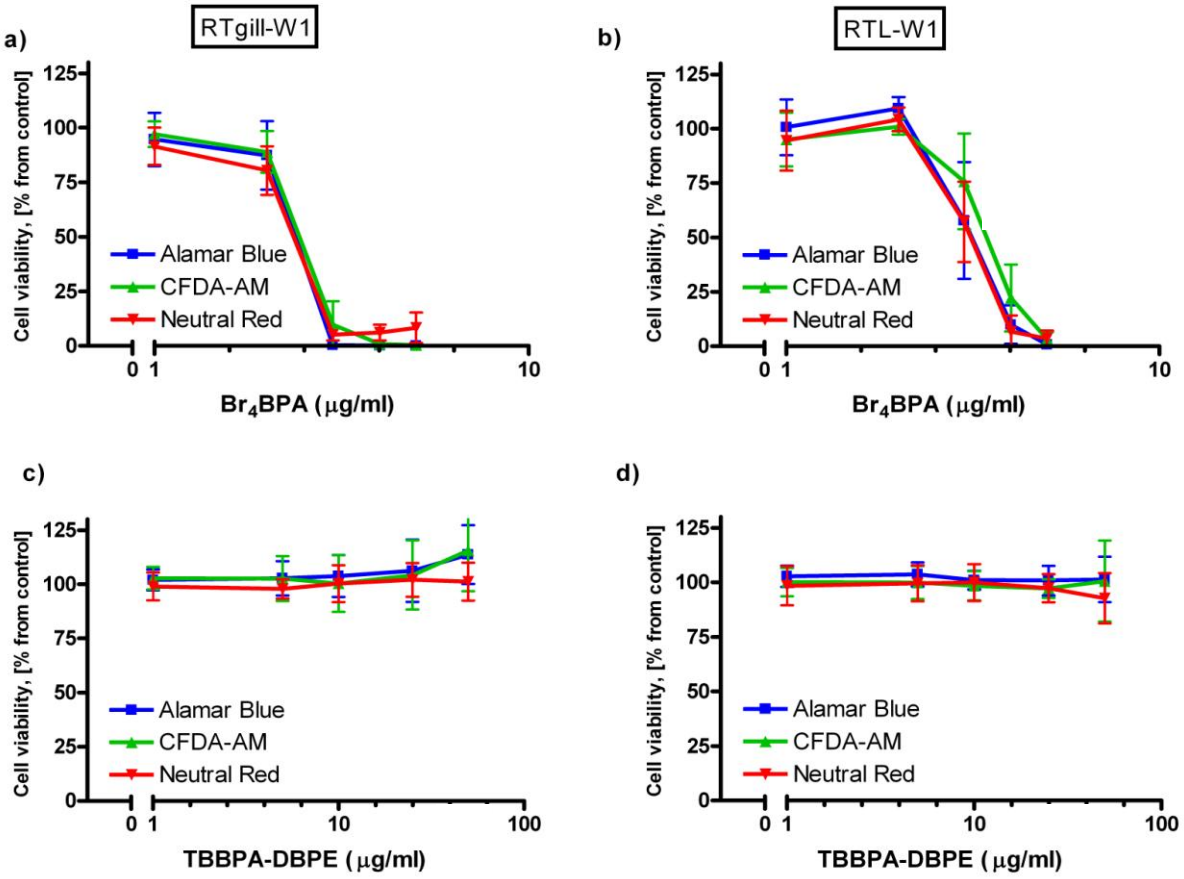


Figure 3.3: (a) RTgill-W1 exposed to Br_4BPA in L15; (b) RTL-W1 exposed to Br_4BPA in L15; (c) RTgill-W1 exposed to TBBPA-DBPE in L15; (d) RTL-W1 exposed to TBBPA-DBPE in L15 (n=4-6).

Table 3.1: Cytotoxicity of BPA and brominated BPAs to fish cell lines in L-15

	[a]EC_{50} (µg/ml) for RTgill-W1			[a]EC_{50} (µg/ml) for RTL-W1		
Chemical Name	**[1]AB**	**[2]CFDA AM**	**[3]NR**	**[4]AB**	**[5]CFDA AM**	**[6]NR**
Tetrabromobisphenol A (Br_4BPA or TBBPA)	2.33 ± 0.29 (n=4)	2.42 ± 0.28 (n=4)	2.30 ± 0.11 (n=4)	3.11 ± 0.23 (n=5)	3.46 ± 0.27 (n=5)	3.11 ± 0.18 (n=5)
Tribromobisphenol A (Br_3BPA)	6.96 ± 0.55 (n=4)	8.50 ± 1.73 (n=4)	7.04 ± 0.60 (n=4)	12.76 ± 1.55 (n=6)	12.56 ± 1.36 (n=6)	14.27± 2.32 (n=6)
Monobromobisphenol A (BrBPA)	7.78 ± 0.99 (n=6)	9.26 ± 0.79 (n=6)	8.16 ± 1.57 (n=6)	13.34 ± 2.86 (n=4)	11.52 ± 1.45 (n=2)	15.14 ± 2.98 (n=4)
Bisphenol A (BPA)	10.59 ± 0.81 (n=3)	11.32 ± 0.75 (n=3)	10.42 ± 0.84 (n=3)	8.02 ± 1.18 (n=4)	9.43 ± 0.35 (n=2)	7.49 ± 0.70 (n=4)
Dibromobisphenol A (Br_2BPA)	12.32 ± 1.81 (n=4)	12.54 ± 1.99 (n=4)	11.71 ± 1.31 (n=4)	5.14 ± 0.94 (n=4)	*7.13 ± 1.13 (n=4)	5.07 ± 0.88 (n=4)

[a] When the EC_{50}s for each compound in each viability test were compared between cell lines by the unpaired t test (p <0.05), the EC_{50}s with RTgill-W1 and RTL-W1 were statistically different in all cases except for CFDA AM in BrBPA-treated cultures.

*** ANOVAs were use to compare EC_{50}s within row for each cell line and significance (p<0.05) was found only for RTL-W1 with Br_2BPA and the mean significantly different from the others (Tukey-Kramer multiple comparison test, p < 0.05) is identified with an asterisk.**

[1,2,2,4,5,6] The means within a column were statistically different (ANOVA, p<0.05) and the statistically different pairs identified by the Tukey-Kramer multiple comparison test (p < 0.05)

[1] All means in this column were statistically different from each other except the following pairs: BPA and Br_2BPA; BrBPA and Br_3BPA.

[2] All means in this column were statistically different from each other except the following pairs: BrBPA and Br_3BPA; BPA and Br_3BPA; BPA and Br_2BPA; BPA and BrBPA.

[3] All means in this column were statistically different from each other except the following pairs: BrBPA and Br_3BPA; BPA and BrBPA; BPA and Br_2BPA.

[4] All means in this column were statistically different from each other except the following pairs: Br_4BPA and Br_2BPA; Br_3BPA and BrBPA; BPA and Br_2BPA.

[5] All means in this column were statistically different from each other except the following pairs: Br_4BPA and Br_3BPA; BPA and BrBPA; BPA and Br_2BPA.

[6] All means in this column were statistically different from each other except the following pairs: Br_2BPA and Br_4BPA; BrBPA and Br_3BPA; BPA and Br_2BPA.

Table 3.2: Significance difference among chemicals for RTL-W1

Chemical of interest (below column) is significant different with:	BPA	BrBPA	Br_2BPA	Br_3BPA	Br_4BPA
BPA		AB, NR		AB, CFDA, NR	AB, CFDA, NR
BrBPA	AB, NR		AB, NR, CFDA		AB, NR
Br_2BPA		AB, NR, CFDA		AB, CFDA, NR	CFDA
Br_3BPA	AB, CFDA, NR		AB, CFDA, NR		AB, NR
Br_4BPA	AB, CFDA, NR	AB, NR	CFDA	AB, NR	

"AB" indicates a significance difference ($p<0.05$) for EC_{50} values between the two chemicals as measured by Alamar blue.

"CFDA" indicates a significance difference ($p<0.05$) for EC_{50} values between the two chemicals as measured by CFDA AM.

"NR" indicates a significance difference ($p<0.05$) for EC_{50} values between the two chemicals as measured by Neutral Red.

Empty cells represent no significant difference ($p>0.05$) for EC_{50} values between the two chemicals as measured by all three dyes.

For example, BrBPA Vs Br_3BPA and Br_2BPA Vs BPA.

Table 3.3: Significance difference among chemicals for RTgill-W1

Chemical of interest (below column) is significantly different with:	BPA	BrBPA	Br_2BPA	Br_3BPA	Br_4BPA
BPA		AB,NR		AB, CFDA, NR	AB, CFDA, NR
BrBPA	AB, NR		AB, CFDA, NR		AB, CFDA, NR
Br_2BPA		AB, CFDA, NR		AB, CFDA, NR	AB, CFDA, NR
Br_3BPA	AB, CFDA, NR		AB, CFDA, NR		AB, CFDA, NR
Br_4BPA	AB, CFDA, NR	AB, CFDA, NR	AB, CFDA, NR	AB, CFDA, NR	

"AB" indicates a significance difference ($p<0.05$) for EC_{50} values between the two chemicals as measured by Alamar blue.

"CFDA" indicates a significance difference ($p<0.05$) for EC_{50} values between the two chemicals as measured by CFDA AM.

"NR" indicates a significance difference ($p<0.05$) for EC_{50} values between the two chemicals as measured by Neutral Red.

Empty cells represent no significant difference ($p>0.05$) for EC_{50} values between the two chemicals as measured by all three dyes.

For example, Br_2BPA Vs BPA and Br_3BPA Vs BrBPA.

3.4 Cytotoxicity as measured with AB vs CFDA AM vs NR

For most compounds, no consistent differences were found among the three measures of cell viability (Table 3.1). For example, the EC_{50}s for TBBPA among AB, CFDA AM, and NR were not significantly different (ANOVA, $p < 0.05$), regardless as to whether the testing had been done with the cell line RTgill-W1 or RTL-W1 (Table 3.1). This also was true for Br_3BPA, Br_2BPA and BrBPA. For Br_2BPA, the EC_{50}s of the three fluorescent assays did not differ in RTgill-W1, but in RTL-W1 the EC_{50} with AB and NR were significantly lower than the EC_{50} measured with CFDA AM.

3.5 Cytotoxicity as evaluated with RTgill-W1 vs RTL-W1

In both cell lines, TBBPA had the lowest EC_{50}s whereas TBBPA-DBPE was not cytotoxic. The ranking of the other compounds was slightly different between the two cell lines. Br_2BPA was the second most potent in RTL-W1 whereas it was the least toxic in RTgill-W1. In addition, Br_3BPA was more toxic than BrBPA in all cases except when tested with CFDA AM in RTL-W1. For RTL-W1, the rank order was the same with AB and NR but with CFDA AM, BPA was the least potent rather than being the 3rd most potent. For RTgill-W1, the rank order was the same with each viability assay.

When the EC_{50}s for each compound in each viability test were compared between cell lines by the unpaired t test ($p < 0.05$), the EC_{50}s with RTgill-W1 and RTL-W1 were statistically different in all cases, except for BrBPA with the CFDA AM assay. Despite EC_{50}s being statistically different between cell lines, the fold differences between the two cell lines were relatively small. This was illustrated by expressing the mean EC_{50} for each compound in each

assay with RTL-W1 as a % of the value in RTgill-W1. These values ranged from a low of 39% for Br_2BPA to a high of 192 % (2 folds) for Br_3BPA, both in the NR assay. Overall, these results suggest that for the cytotoxicity of BPA and the four brominated BPAs the differences between RTgill-W1 and RTL-W1 were slight.

3.6 Cytotoxicity as evaluated in cultures without or with fetal bovine serum

In addition to being toxic in L-15 without FBS (Figure 3.2a), BPA also caused a dose-dependent loss of viability in cultures with L-15 and 10% FBS (Figure 3.4a). In contrast, Br_4BPA, Br_3BPA, B_{r2}BPA, BrBPA and TBBPA-DBPE caused little or no loss of viability when exposures were done in L-15 with FBS (Figure 3.4b-f).

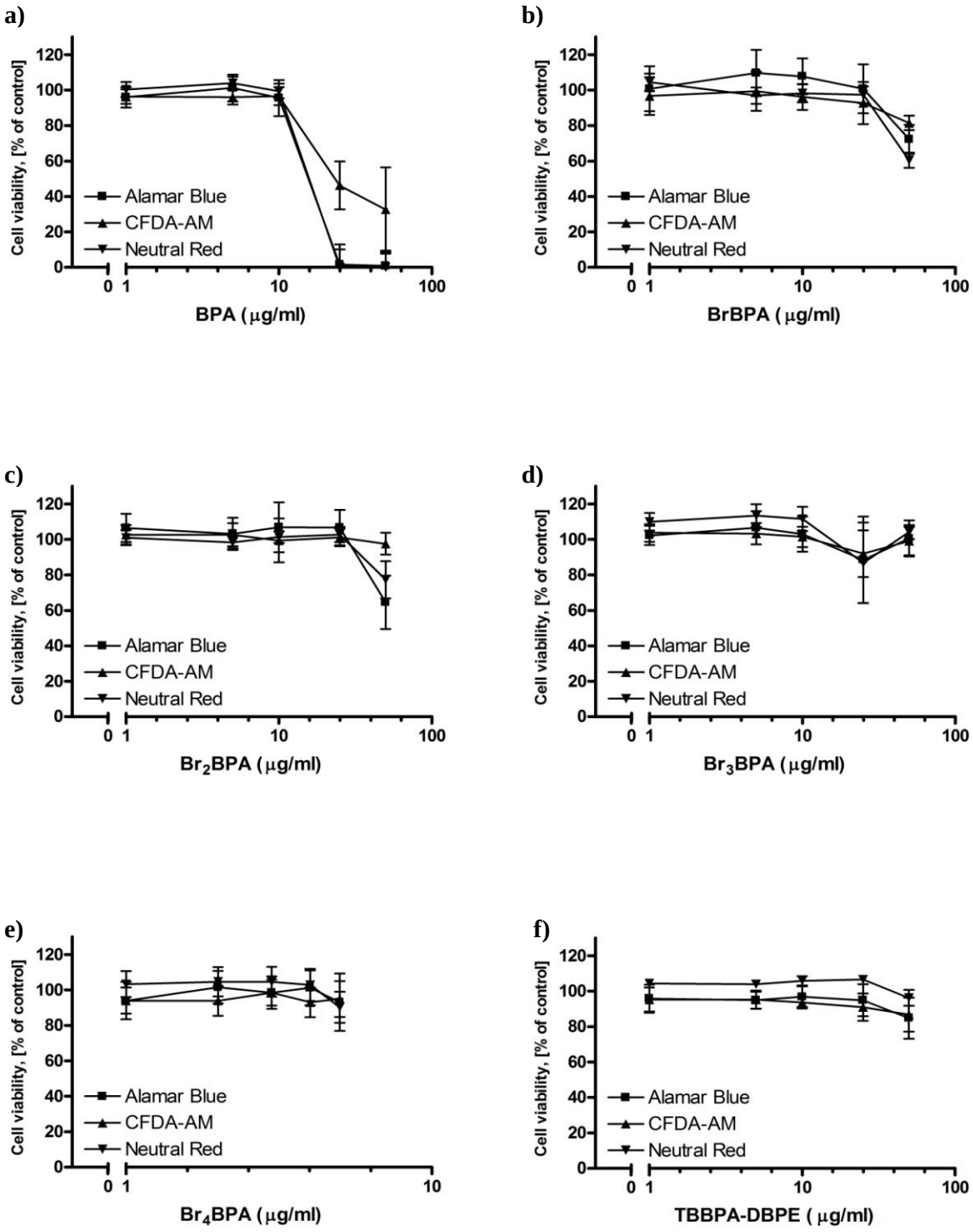


Figure 3.4: (a) RTgill-W1 exposed to BPA in L15/FBS; (b) RTgill-W1 exposed to BrBPA in L15/FBS; (c) RTgill-W1 exposed to Br_2BPA in L15/FBS; (d) RTgill-W1 exposed to Br_3BPA in L15/FBS; (e) RTgill-W1 exposed to Br_4BPA in L15/FBS; (f) RTgill-W1 exposed to TBBPA-DBPE in L15/FBS.

3.7 Cell morphology

TBBPA and other tested bisphenols such as BPA induced morphological changes as compared to the solvent control. RTgill-W1 cells were at their normal elongated, epithelial-like morphology in the solvent control [DMSO + Cells] (Figure 3.5a). When dosed with 3 µg/mL of TBBPA (Figure 3.5b), cells lifted off, leaving behind debris. This suggested there was a loss in the cells' ability to attach to each other and remain as a monolayer. In the case of BPA, cells seemed to have changed considerably in morphology when exposed to a sub-lethal concentration. There were considerable shrinkage and decrease in cell volume. The nucleus also becomes apparent (Figure 3.5c). In contrast with these two compounds, TBBPA-DBPE formed crystals. However, the cells seemed normal in areas without crystals (Figure 3.5d) and viability was not affected by the presence of crystals as proven by the three fluorescent dyes (Figure 3.3).

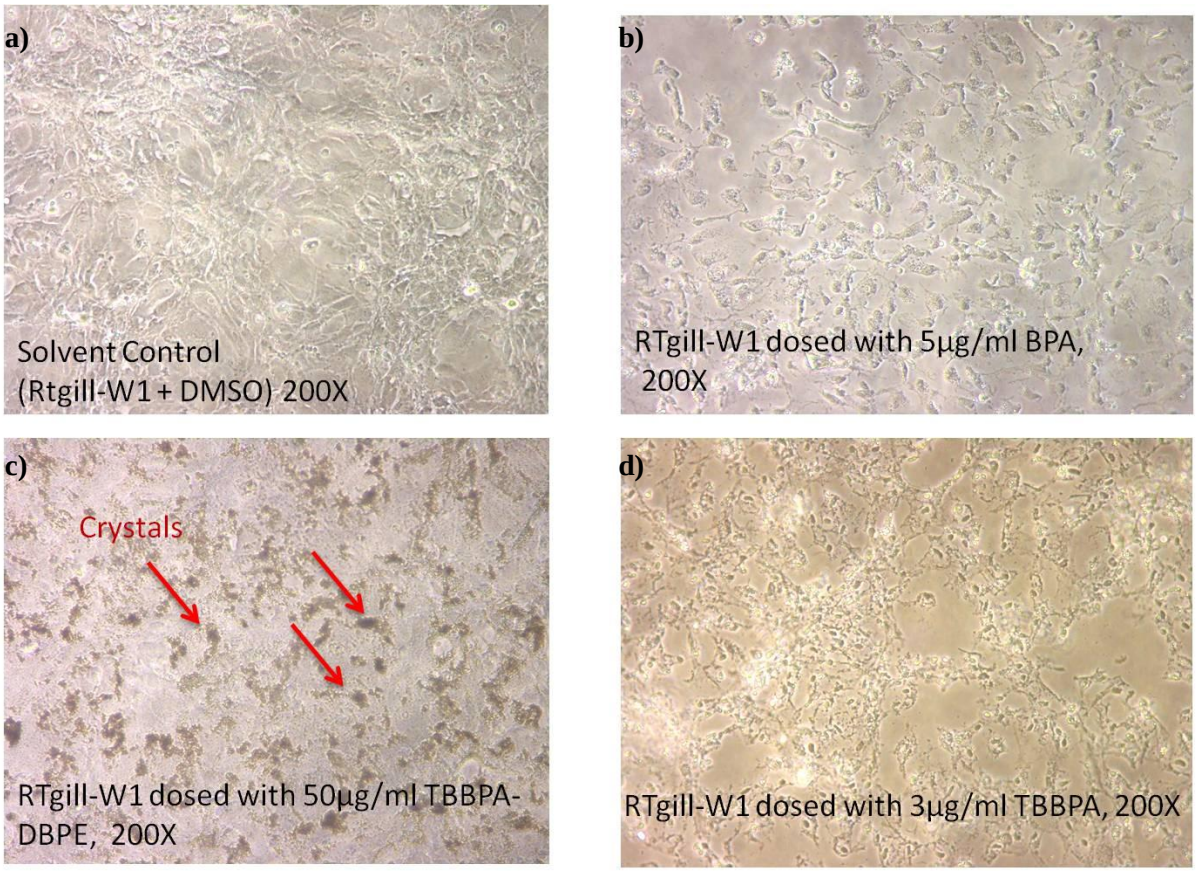


Figure 3.5: Morphology of RTgill-W1 dosed with sub-lethal concentration of (a) solvent control, (b) BPA, (c) TBBPA-DBPE and (d) TBBPA.

3.8 Evaluating cytotoxicity immediately after the dosing of cultures

The cytotoxicity of BPA and four BFRs was evaluated in a novel manner. The BFRs are added to the mixture of AB/CFDA in L15/ex. Immediately, cell viability was monitored and continued for 60 minutes. Initially BPA and TBBPA were compared. BPA caused a profound dose-dependent decline in the reduction of AB by RTL-W1 cells, whereas in TBBPA up to 50 µg/ml the cells continued to reduce AB to the same extent as the control cells (Figure 3.6 versus Figure 3.7). At 60 minutes, with 50 µg/ml of BPA, the reduction of AB was inhibited by approximately 70% (Figure 3.6c). Surprisingly, BPA also caused cell cultures to more vigorously convert CFDA AM to CF, with approximately a 3 fold rise seen after 60 minutes in cultures dosed with 50 µg/ml of BPA (Figure 3.6d). This observation was not seen with cultures dosed with TBBPA (Figure 3.7c,d). In fact with TBBPA, the CFDA AM readings were unchanged up to 25 µg/ml, but at 50 µg/ml of TBBPA, the CFDA AM readings were reduced approximately 50% (Figure 3.7d).

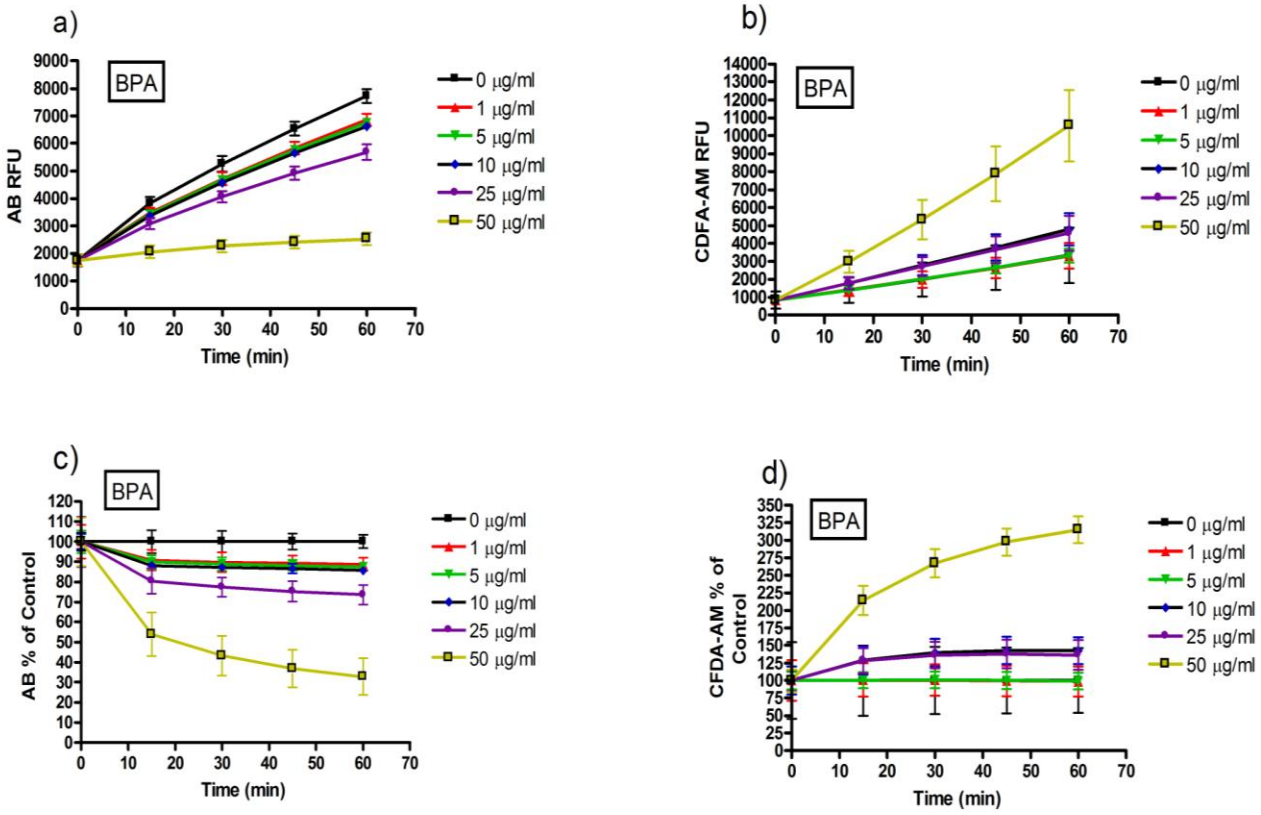


Figure 3.6: RFUs of RTL-W1 dosed with BPA as measured by (a) AB and (b) CFDA AM and the corresponding percentages of control for (c) AB and (d) CFDA AM from time = 0 to 60 min.

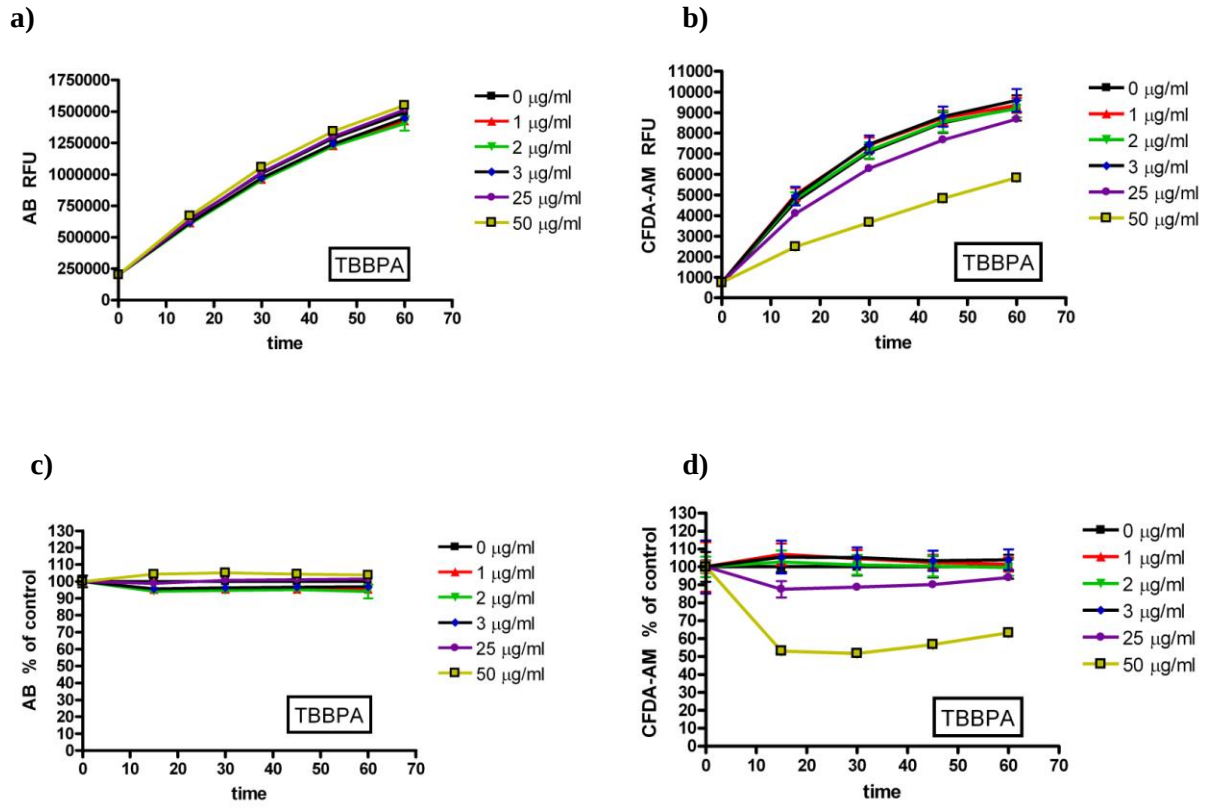


Figure 3.7: RFUs of RTL-W1 dosed with TBBPA as measured by (a) AB and (b) CFDA AM and the corresponding percentages of control for (c) AB and (d) CFDA AM from time = 0 to 60 min.

BrBPA and Br_3BPA had similar but less severe effects on AB reduction and CFDA AM conversion (Figure 3.8 & Figure 3.10) In contrast, Br_2BPA, like TBBPA had no effect on Alamar blue readings (Figure 3.9 & Figure 3.7). These results suggest that the number and positioning of the bromine groups influences the ability of these compounds to impair the ability of cells to reduce AB and to increase their capacity to convert CFDA AM to CF. As ultimately, TBBPA was the most cytotoxic compound after 24 h in L-15, these immediate changes elicited by BPA, BrBPA, and Br_3BPA might not either be expressed in the complex medium of L-15 or contributed little to the loss of cell viability seen at 24 h. However, these changes point out that these compounds can elicit different cellular responses and in an acute way.

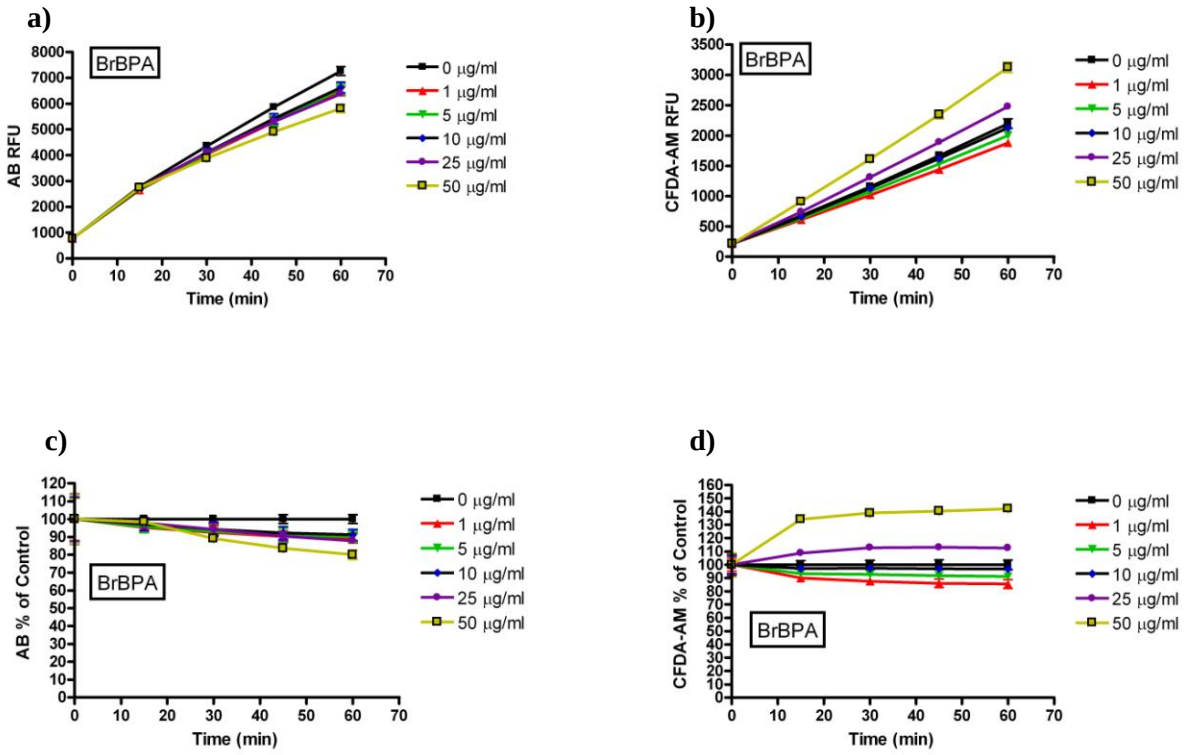


Figure 3.8: RFUs of RTL-W1 dosed with BrBPA as measured by (a) AB and (b) CFDA AM and the corresponding percentages of control for (c) AB and (d) CFDA AM from time = 0 to 60 min.

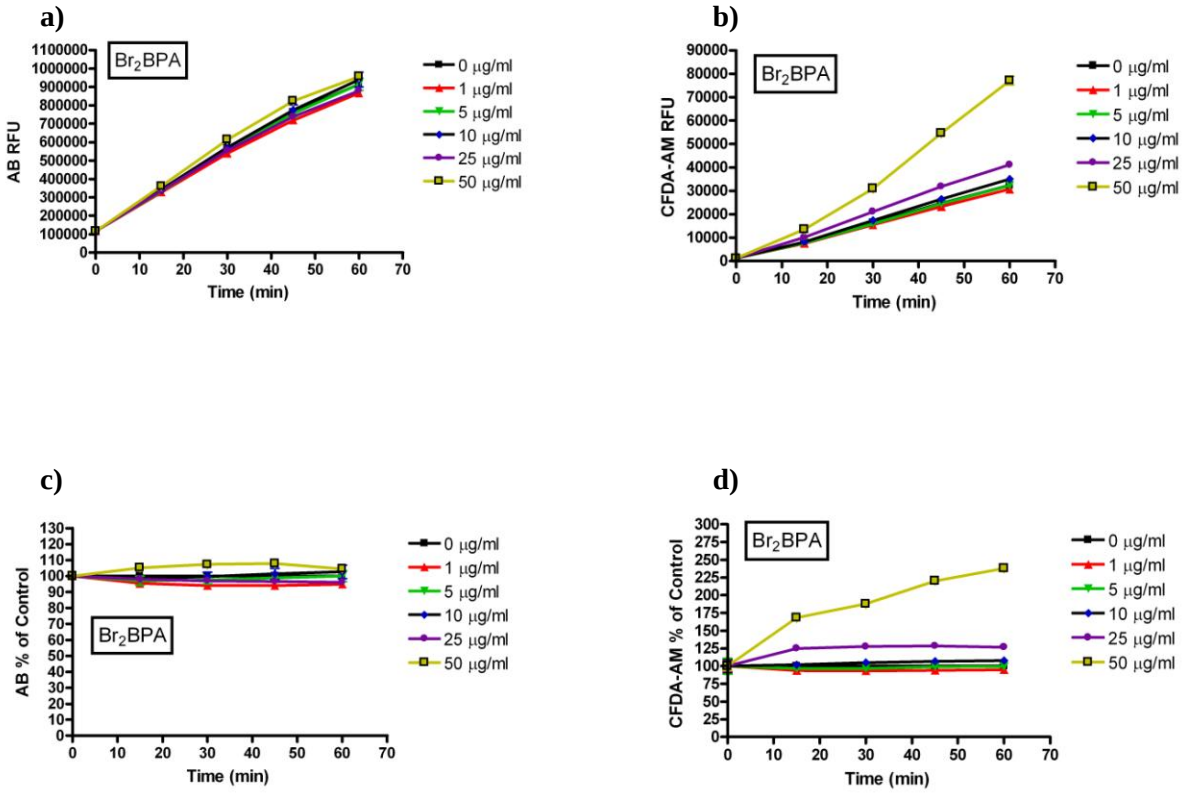


Figure 3.9: RFUs of RTL-W1 dosed with Br_2BPA as measured by (a) AB and (b) CFDA AM and the corresponding percentages of control for (c) AB and (d) CFDA AM from time = 0 to 60 min.

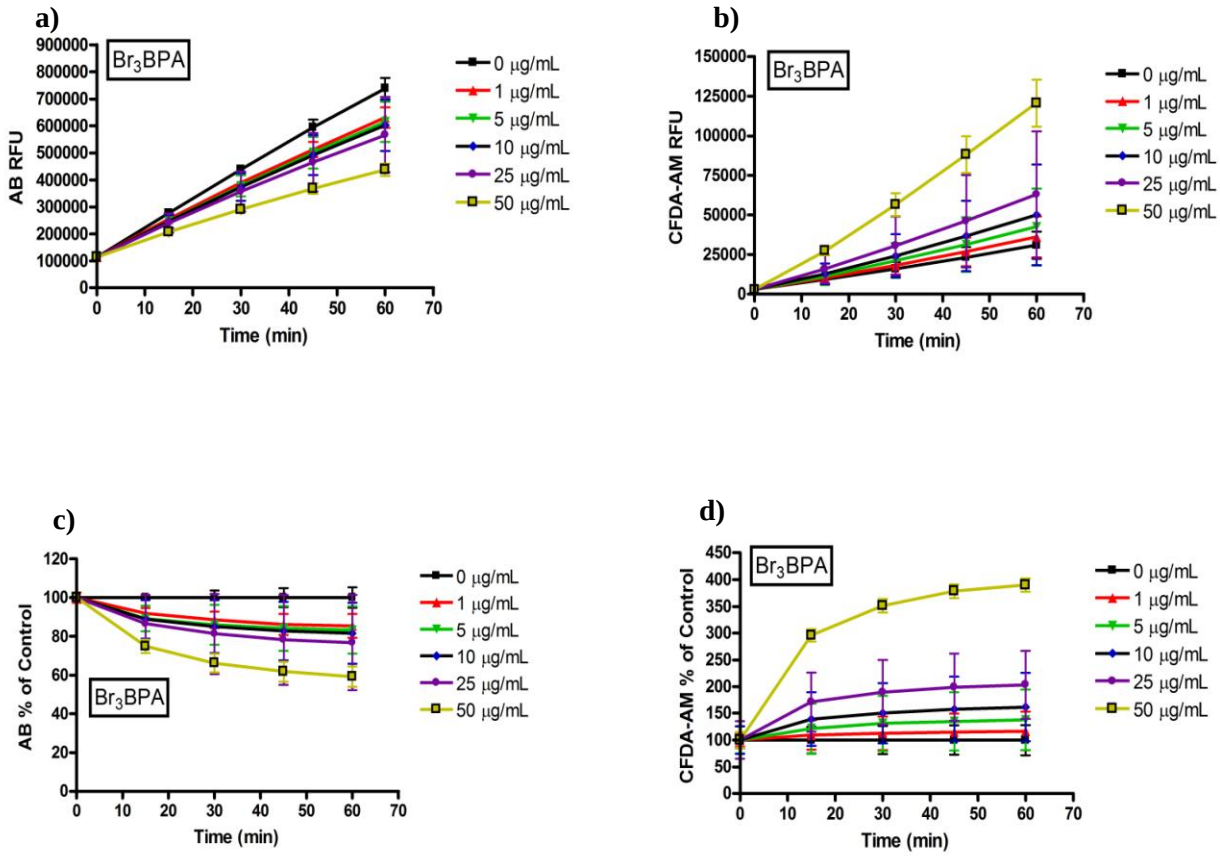


Figure 3.10: RFUs of RTL-W1 dosed with Br_3BPA as measured by (a) AB and (b) CFDA AM and the corresponding percentages of control for (c) AB and (d) CFDA AM from time = 0 to 60 min.

Possible mechanisms behind these changes were investigated briefly with menadione, which is known to cause cytotoxicity through the generation of reactive oxygen species (ROS) (Criddle *et al*, 2006); and sodium dodecyl sulfate (SDS), which kills cells by disrupting cell membranes (Woldringh & Van Iterson, 1972). Menadione brought about a rapid dose-dependent decline in AB reduction but no change in the conversion of CFDA AM to CF or in the uptake of NR (Figure 3.11, Figure 3.12 & Figure 3.13). SDS caused a dose-dependent decline in AB reduction and an increase in CFDA AM conversion (Figure 3.14, Figure 3.15 & Figure 3.16). These results suggest an increase in the production of ROS and/or a loss of plasma membrane integrity might account for the immediate changes in AB and CFDA AM readings.

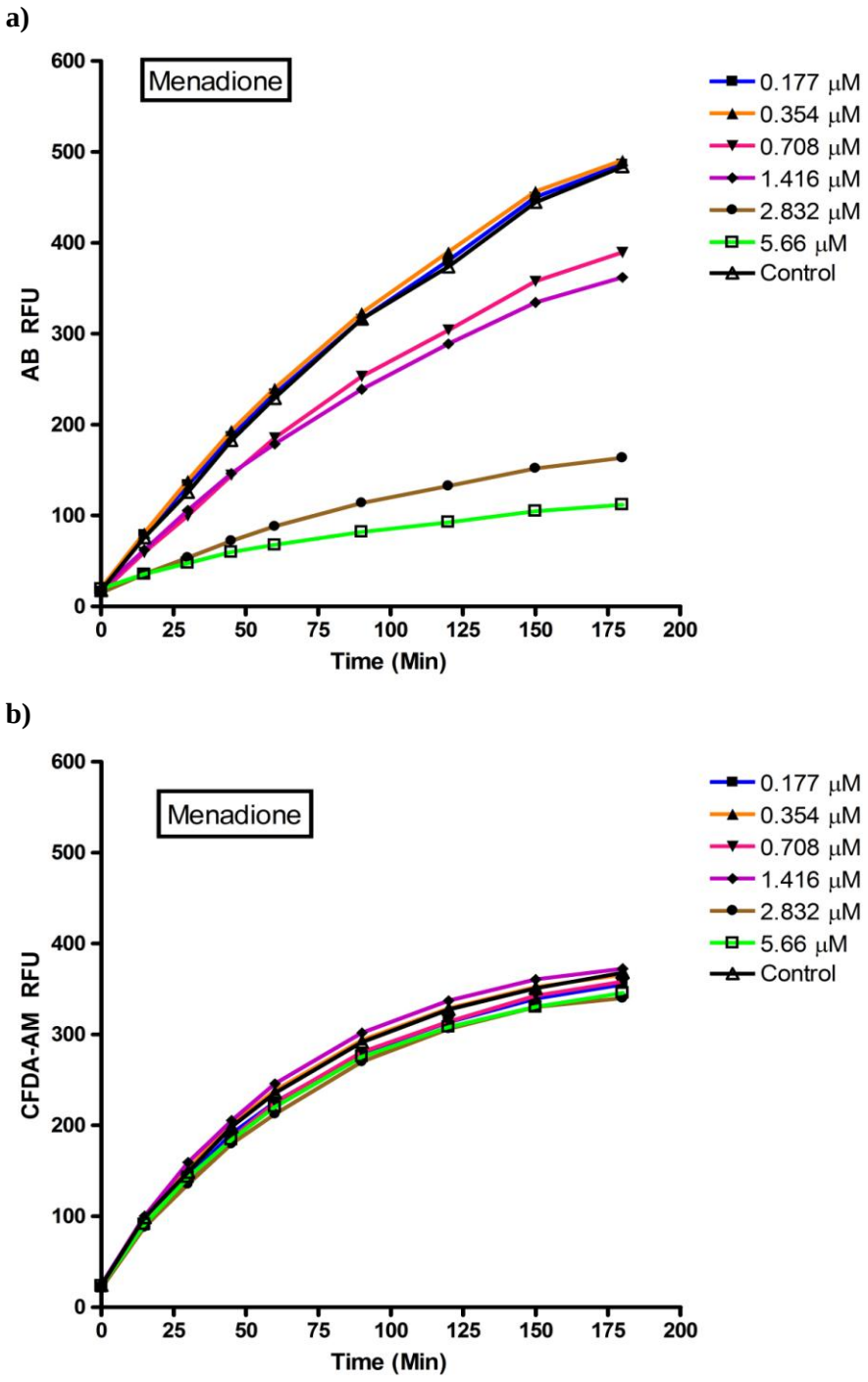


Figure 3.11: RFUs of RTL-W1 exposed to Menadione from t = 0 to 180 min as measured by (a) AB and (b) CFDA AM.

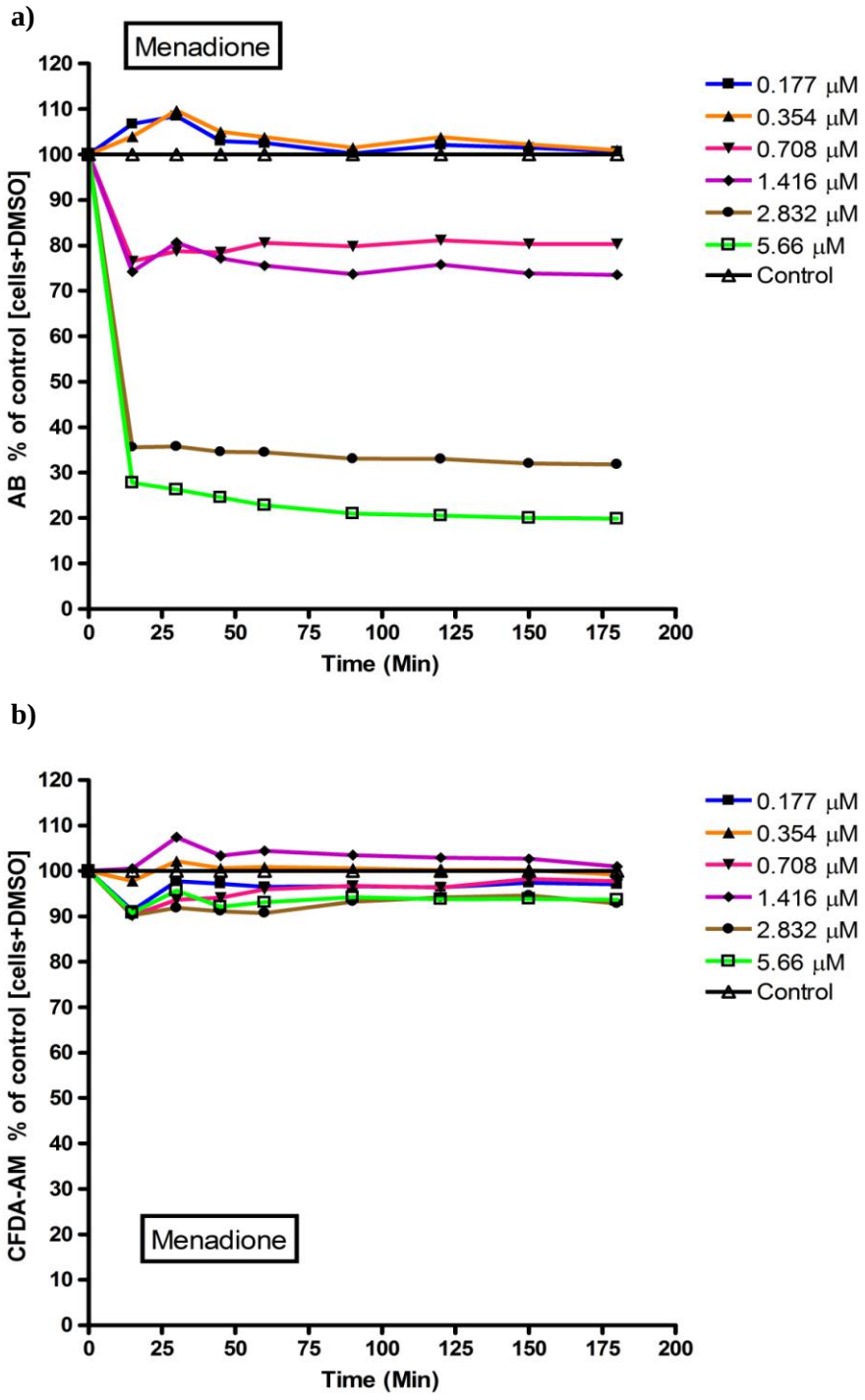
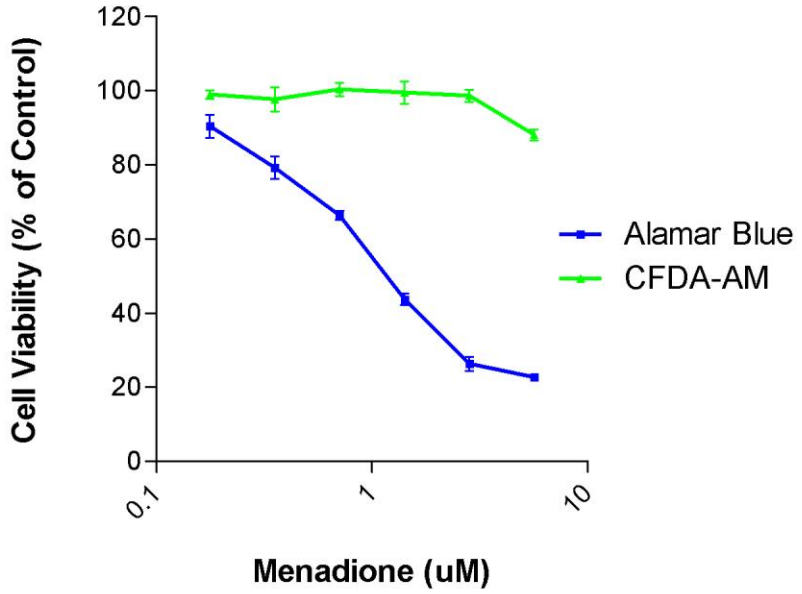


Figure 3.12: RFUs expressed as % of control of RTL-W1 exposed to Menadione from t = 0 to 180min as measured by (a) AB and (b) CFDA AM.

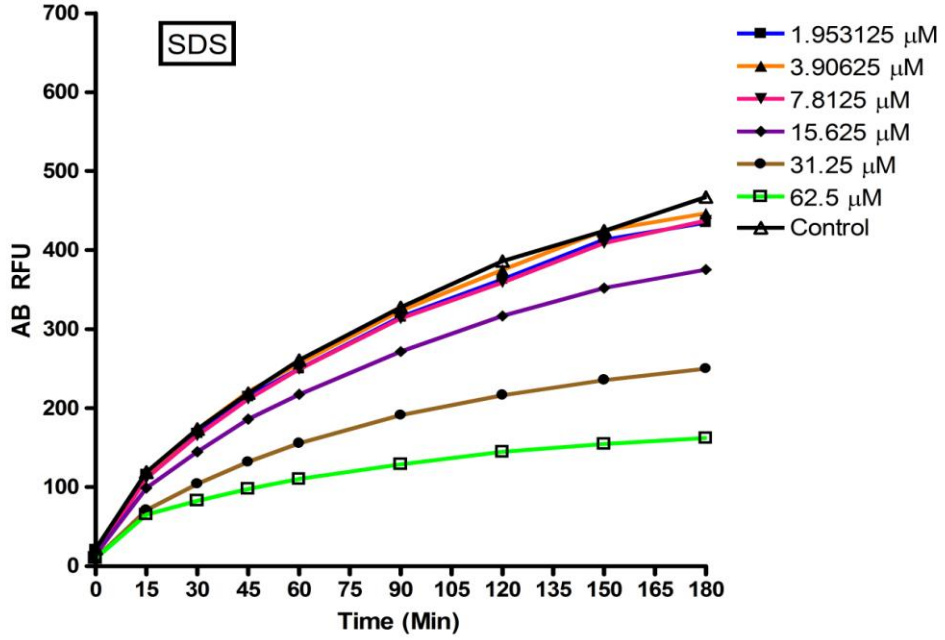


EC50AB: 0.9528 CFDA: Not Available

24Hr:
EC50 AB: 0.34733 CFDA: 0.633833

Figure 3.13: Dose response curve for Menadione in the above experiments measured at t = 180 min.

a)



b)

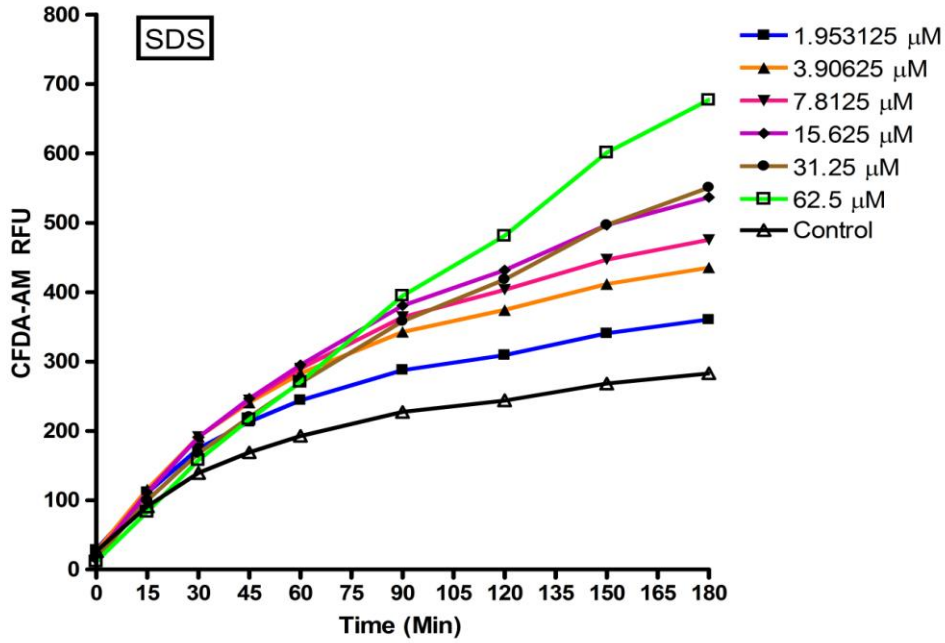


Figure 3.14: RFUs of RTL-W1 exposed to SDS from time = 0 to 180 min as measured by (a) AB and (b) CFDA AM.

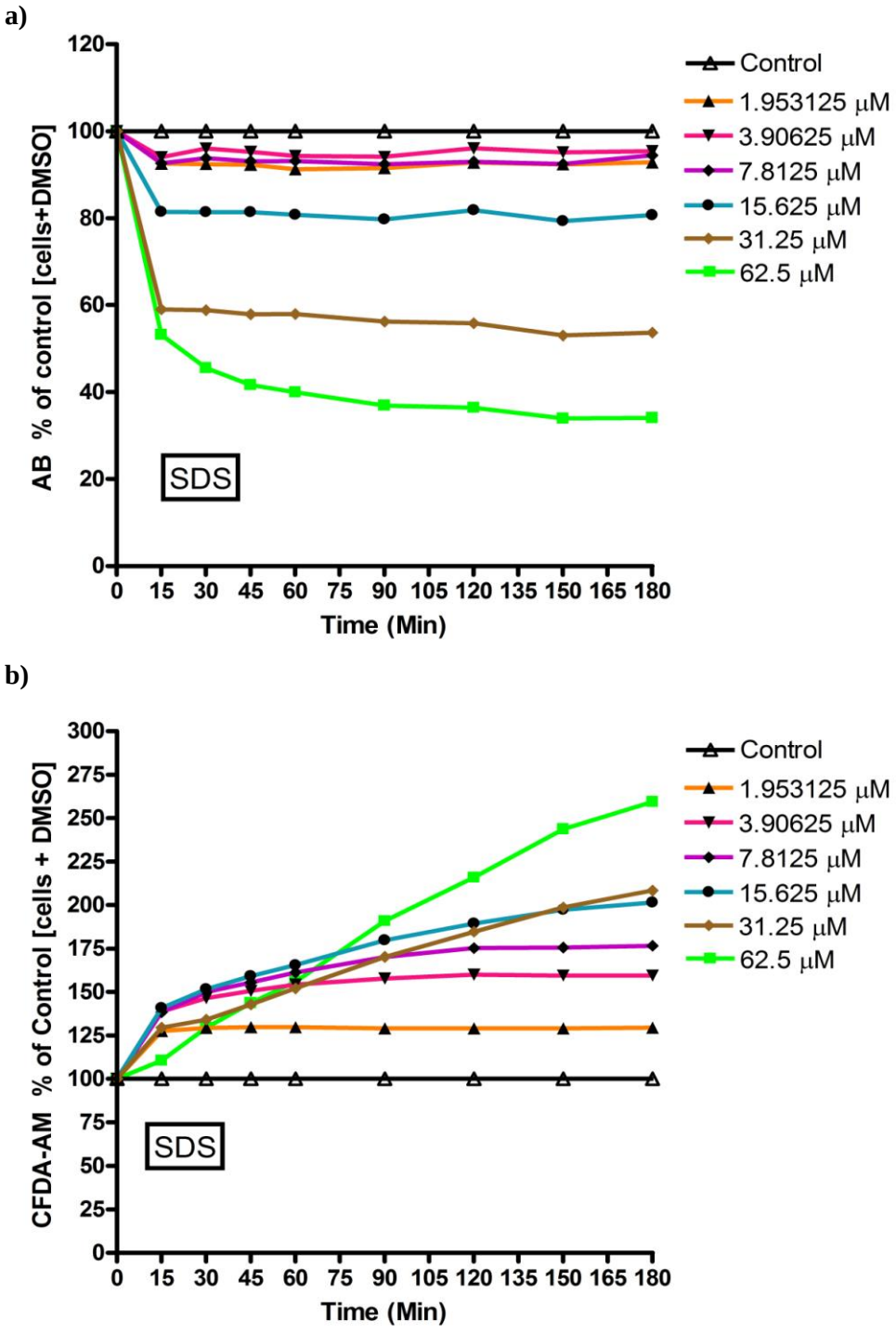
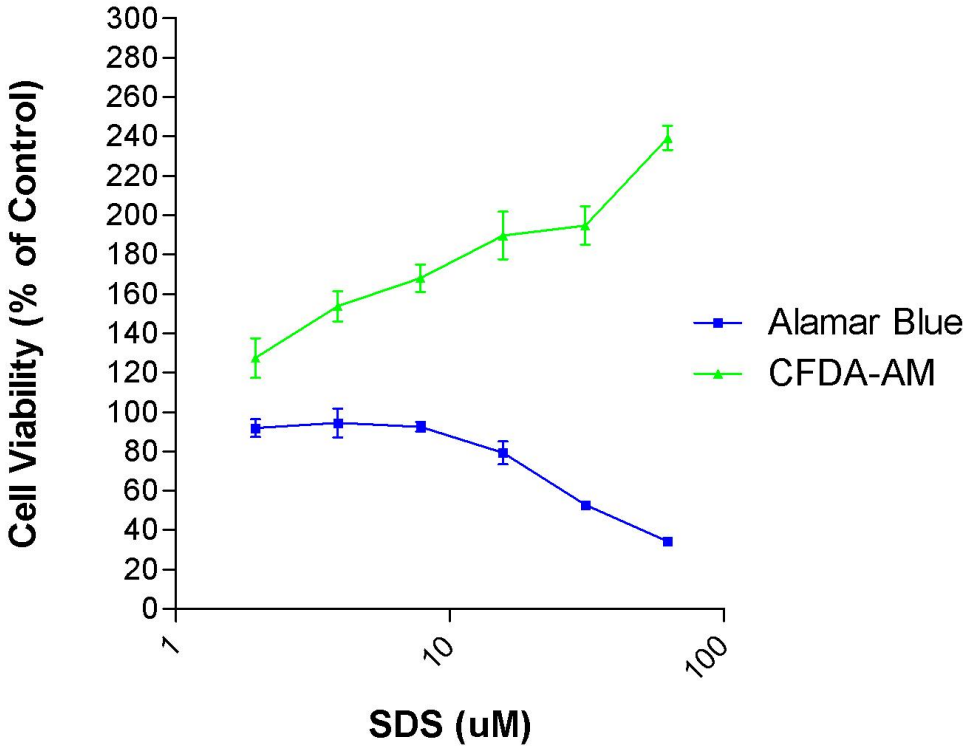


Figure 3.15: RFUs expressed as % of control for RTW-W1 exposed to SDS as measured by (a) AB and (b) CFDA AM.



EC50AB: 25.21 CFDA: Not available

24 hr:
EC50 AB: 26.09 CFDA: 44.56

Figure 3.16: Dose response curve of RTL-W1 exposed to SDS in the above experiments measured at t = 180 min.

Chapter 4 Discussion

4.1 Capacity of BPA and brominated flame retardants (BFRs) to induce EROD

BPA did not induce 7-ethoxyresorufin o-deethylase (EROD) activity in RTL-W1, which agrees by in large with other studies on the inability of BPA to induce the expression of the gene CYP1A for this enzyme in different biological systems. At the whole organism level, BPA failed to induce EROD activity in Atlantic salmon (Arukwe *et al*, 2000) and CYP1A transcripts in Atlantic cod (Olsvik *et al*, 2009). In both these cases, EROD activity and CYP1A levels actually appeared lower in fish that had received BPA. A similar story has emerged from *in vitro* studies with the mouse hepatoma cell line, Hepa-1c1c7. BPA failed to induce EROD activity and CYP1A1 transcripts in these cells (Jeong *et al*, 2000). Furthermore, BPA antagonized the action of TCDD, which is a strong inducer of EROD in many systems, including RTL-W1 (Bols *et al*, 1999). With BPA in Hepa-1c1c7 cultures, lower levels of CYP1A1 mRNA and EROD activity were induced by TCDD (Jeong *et al*, 2000). BPA also was found by others to act antagonistically to TCDD in a Hepa-1c1c7 strain that was stably transfected with an inducible luciferase express vector (Bonefeld-Jørgensen *et al*, 2007; Krüger *et al*, 2008). Although these interfering actions of BPA could have come about in several ways, all mechanisms focus on the aryl hydrocarbon receptor (AhR) because TCDD elicits responses through binding and activating this transcription factor (Furness & Whelan, 2009). BPA could inhibit TCDD-induced gene expression by interfering with TCDD binding to the AhR, movement of the AhR to the nucleus, and/or binding of the AhR to the dioxin response element. As CYP1A participates in xenobiotic metabolism, collectively these results suggest that BPA

could influence xenobiotic metabolism by interfering with the induction of key cytochrome P450 mono-oxygenase enzymes. In this way BPA might influence the toxicity of other environmental contaminants and this might be an interesting angle to explore with RTL-W1 in the future.

Br_4BPA, Br_3BPA, Br_2BPA, BrBPA and TBBPA-DBPE also did not induce EROD activity in RTL-W1 cells, but only Br_4BPA and TBBPA-DBPE have been studied for this property in other systems. Neither Br_4BPA nor TBBPA-DBPE activated the AhR in a cell bioassay, CALUX, for this receptor (Hamers *et al*, 2006). The intraperitoneal injection of Br_4BPA into juvenile rainbow trout decreased EROD activity in liver microsomes in a dose-dependent manner (Ronisz et al., 2004). This led to the suggestion that TBBPA may compete with the substrate 7-ethoxyresorufin and impede the EROD assay (Ronisz et al., 2004). Therefore, in the current study TBBPA (5 μg/ml) was added to the EROD assay for RTL-W1 that had been induce with TCDD, but EROD activity was not inhibited. Thus, the failure to see an increase in EROD activity appears to be a lack of induction, and this is supported by several other studies. EROD activity failed to be induced in livers of rats fed Br_4BPA (Germer *et al*, 2006) and of European flounders exposed to Br_4BPA for 105 days in water (Kuiper *et al*, 2007). Overall, the results suggest that Br_4BPA, Br_3BPA, Br_2BPA, BrBPA and TBBPA-DBPE have little capacity to activate the AhR.

4.2 Cytotoxicity of tetrabromobisphenol A bis(2,3-dibromopropylether) (TBBPA-DBPE)

Very little information is available on the toxicity of TBBPA-DBPE, and the results of this thesis are the first with fish material.

One *in vivo* study has been done on rodents. Acute toxicity was found to be low (LD_{50} = 20g/kg in rats and > 20g/kg in mice) (Knudsen *et al*, 2007). In a mouse cell bioassay, CALUX, for activation of the AhR, TBBPA-DBPE failed to activate the receptor (Hamers *et al*, 2006). To date, no studies have been done on whole aquatic organisms. The only cell culture study is this one with two rainbow trout cell lines, RTgill-W1 and RTL-W1. Exposure concentrations were up to concentrations where TBBPA-DBPE precipitated out of solution in the exposure medium. No cytotoxicity was observed at any concentration with any of three endpoints: Alamar Blue for energy metabolism, CFDA AM for plasma membrane integrity, and neutral red for lysosomal activity. Therefore, TBBPA-DBPE does not disrupt metabolism, cell membrane integrity or lysosomal activity in both RTL-W1 and RTgill-W1. TBBPA-DBPE failed to induce EROD activity in RTL-W1, which suggests that TBBPA-DBPE fails to bind agnostically with the AhR. Overall, the results suggest that TBBPA-DBPE has little toxicity to fish cells and could be considered as a replacement for other harmful BFRs because as to date its cytotoxicity seems minimal.

4.3 Cytotoxicity of Brominated BPAs vs BPA

The change in morphology of cells when exposed to sub-lethal concentrations of BPA and TBBPA correspond to the results of the three indicator dyes. The decrease in the ability to convert AB, CFDA and NR into their fluorescent products indicates the lost of ability to maintain normal homeostasis in metabolism and proton gradients on the membranes.

The only other *in vitro* study, besides the current one, to have compared the cytotoxicity of Br_4BPA, Br_3BPA, Br_2BPA, BrBPA, and BPA used primary rainbow trout hepatocytes

(Debenest *et al*, 2010), and the results were similar in some aspects and different in others. The hepatocytes were exposed to the compounds for 48 h and viability was assayed with 5-carboxyfluorescein diacetate (CFDA AM) (Debenest *et al*, 2010). The EC_{50}s for these compounds in the hepatocytes and in our cell lines, RTL-W1 and RTgill-W1, had roughly similar magnitudes. For hepatocytes, these ranged from 6.4 μg/ml to 15.0 μg/ml. For the rainbow trout cell lines, the values for three different endpoints ranged 2.3 μg/ml to 20.1 μg/ml. Where the studies differ is in the order in which these compounds are ranked for their cytotoxicity. From most to least cytotoxic, the order with the primary hepatocytes cultures was Br_2BPA, Br_3BPA, BrBPA, Br_4BPA, and BPA. With RTgill-W1 and RTL-W1, the broad order was Br_4BPA, Br_3BPA, BrBPA, and Br_2BPA; and Br_4BPA, Br_2BPA, BPA, Br_3BPA, and BrBPA, respectively. Thus, Br_2BPA stands out being the most cytotoxic with the primary hepatocytes while it was not with the rainbow trout cell lines. This could be due to differences between primary hepatocytes and cell lines in the capacity for xenobiotic metabolism. However, despite the different rankings by the two *in vitro* systems, the EC_{50}s are quite similar, especially when the hepatocytes are compared with the liver cell line, RTL-W1. The EC_{50} with hepatocytes was 6.4 μg/ml, and when the EC_{50}s for the three endpoints with RTL-W1 were expressed as a mean the value was 5.8 μg/ml. Therefore, the cytotoxicity tests with fish cells might not be sensitive enough to definitely rank these degradation compounds that have broadly similar cytotoxicities.

Interestingly, the hepatocyte study was done together with five other small-scale bioassays which had similar results (Debenest *et al*, 2010). These were a microtox test with *Vibrio fischeri*, an algal test with *Pseudokirchneriella subcapitata*, a LuminoTox assay with

photosynthetic enzyme complexes isolated from spinach leaves, a micro-crustacean test with *Thamnocephalus playturus,* and a cnidarian test. The exposure times and conditions were different for each test and different from those used in the fish cell studies so differences in the EC_{50}s can be expected to differ, but, the rank order of the compounds in these bioassays was similar: Br_2BPA was the most toxic; Br_4BPA and BPA were the least toxic. This contrasted with the current study where Br_4BPA always stood out as being the most cytotoxic.

However, at least one other *in vitro* study also found that Br_4BPA was more cytotoxic than BPA. When rat hepatocytes were exposed in a simple buffer to BPA or Br_4BPA for 3 h, a dose-dependent decline in cell viability as measured with Trypan blue was seen with both compounds (Nakagawa *et al*, 2007). More cells died with Br_4BPA.

4.4 Cytotoxicity as measured with AB vs CFDA AM vs NR

Several measures of cell viability were used in this study in order to reveal possible cytotoxic mechanisms but for the most part the different endpoints gave similar results.

Several *in vitro* studies with mammalian cells have sought the mechanisms behind the cytotoxicity of Br_4BPA but few have compared the actions with the other brominated BPAs and BPA. One of the few reports in which the cytotoxicity of both Br_4BPA and BPA has been studied side by side is the work of Nakagawa et al (2007). They found that Br_4BPA acts differently than BPA with respect to impacts on the mitochondria. Br_4BPA rather than BPA

disrupted oxidative phosphorylation and caused ATP depletion but others have thought BPA also could uncouple oxidative phosphorylation (Ooe *et al*, 2005).

In the current study, the reduction of AB was the measure of changes in metabolism and Br_4BPA was more effective than BPA in decreasing AB reduction (the EC_{50}s were lower). Disrupting oxidative phosphorylation will inhibit the reduction of AB (Ambrose *et al*, 2007). However, AB, CFDA AM and NR had very similar EC_{50}s, and AB was no more sensitive as a measure of cell viability. Hence, if Br_4BPA kills by first impairing mitochondrial functions, perhaps examining cultures early in the exposures might have revealed differences between AB and the other viability endpoints.

4.5 Cytotoxicity as evaluated with RTgill-W1 vs RTL-W1

Although the two cell lines generally responded very similarly to the test compounds, Br_4BPA, Br_3BPA, and BrBPA seemed slightly more cytotoxic to RTgill-W1 than to RTL-W1, while Br_2BPA was slightly more cytotoxic to RTL-W1 than to RTgill-W1. The two cell lines differ in their capacity for xenobiotic metabolism; so possibly, they metabolize brominated BPAs differently (Schirmer *et al*, 1997; Bols *et al*, 1999). In the case of rat hepatocytes, Br_4BPA was rapidly metabolized and metabolism appeared to protect against a low dose of Br_4BPA (Nakagawa *et al*, 2007). Therefore, RTL-W1 might have metabolized Br_4BPA, Br3BPA, and BrBPA slightly better than RTgill-W1, resulting in these compounds being slightly less cytotoxic to RTL-W1. On the other hand, RTgill-W1 metabolized Br_2BPA better than RTL-W1, making it less cytotoxic to RTgill-W1.

4.6 Cytotoxicity as evaluated in cultures without or with fetal bovine serum

The presence of serum (10% FBS) in the medium completely blocked the cytotoxicity of the BFRs but not the cytotoxicity of BPA, pointing to possibly distinctly different cytotoxic actions for BPA than for the brominated BPAs. Serum could protect the cells from the cytotoxicity of brominated BPAs in at least two different ways, acting singly or together. One possibility is that the brominated BPAs bind to serum proteins, making them less available to elicit cytotoxicity. BPA was cytotoxic in the presence of serum possibly because it binds less well to serum proteins and continues to be available to the cells.

Another possibility is that the serum proteins better support cellular protective mechanisms against the toxic actions of brominated BPAs more than of BPA. One protective mechanism that could be supported by the presence of serum could be more vigorous xenobiotic metabolism. However, other protective mechanisms might also be at play. In the study of rat hepatocytes, Br_4BPA caused a depletion of intracellular glutathione (GSH), an increase in oxidized glutathione (GSSG), a loss of protein thiols, and an increase in lipid peroxidation (Nakagawa *et al*, 2007). Serum, which contains GSH and protein thiols, might have prevented brominated BPAs from causing this sequence of events which seems ultimately to cause cell death. However, other cellular disturbances by Br_4BPA have been thought to contribute to cytotoxicity as well. One of these is dysregulation of calcium (Ogunbayo *et al*, 2008). Again, dysregulation of calcium might be less pronounced in the presence of serum. Therefore, in the case of BPA, other cellular disturbances which occurred in the presence or absence of serum could be the cause of cell death.

4.7 Evaluating cytotoxicity immediately after the dosing of cultures

The cellular responses elicited by BPA, BrBPA and Br_3BPA immediately upon being added to cultures brought about new observations. Some possible mechanisms can be inferred.

One possibility is that these compounds rapidly enhance the production of ROS and ROS impair the ability of the cells to reduce AB. This is supported by some observations, but contradicted by others. Menadione, which is known to generate ROS, also caused an immediate decrease in the ability of cells to reduce AB. However, BPA is known to generate ROS but *only* after metabolism (Kovacic, 2010). In addition, in cultures of mouse neural cell lines, Neuora2a and GC1, an increase in ROS was seen 12 h after the addition of BPA (Ooe *et al*, 2005). Our study suggested otherwise, the decline in Alamar Blue reduction was seen within 15 minutes. Also, BPA, but not menadione, caused an increase in the conversion of CFDA AM to CF. This represents an increase in esterase activity and suggests that other mechanisms besides just overproduction of ROS are in play.

Another possibility builds on a suggestion by Ooe et al (2005) that BPA accumulates in mitochondrial membranes and uncouples oxidative phosphorylation by inhibiting complex I in the electron transport chain. Disrupting oxidative phosphorylation inhibits energy metabolism and thus the reduction of AB (Ambrose *et al*, 2007). Also, it is possible that uncoupling oxidative phosphorylation makes plasma membranes leaky. Leaky membranes could speed up CFDA conversion to CF by allowing the substrate CFDA AM more rapid entry into the cells or, at the same time, permitting the esterases to leak out into medium where CFDA AM is most

abundant. So this way, BPA would not act directly on the esterases but the milieu in which they were operating.

In additional, according to Nakawaga et al (2007), Br_4BPA disrupts oxidative phosphorylation more profoundly than BPA and yet Br_4BPA elicited none of these early changes in AB and CFDA AM. This would tell us that BPA is acting in some other way. Although the mechanism of these early changes cannot be explained, they do point out that bromination of BPA does alter its effects on cells.

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