Evaluating the toxicity of eight reactive environmental contaminants by monitoring three measures of cell viability in two fish cell lines

by

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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.

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Abstract

As fish cell cultures continue to be explored as alternatives to whole fish for evaluating the toxicity of environment chemicals, technical issues have emerged that influence results and thus need to be understood and standardized. These include carrier solvents, dosing protocols, exposure vessel, exposure media, viability endpoints, and cell lines. Some of these factors have been explored in this thesis for eight reactive contaminants exhibiting varied physicochemical properties using the rainbow trout cell lines RTgill-W1 and RTL-W1. Sodium dodecyl sulphate (SDS) was used as a reference (control) chemical. Cell viability was evaluated with alamar Blue, carboxyfluoroscein diacetate acetoxymethyl ester and neutral red as measures respectively of metabolic activity, plasma membrane integrity, and lysosomal function. Experimental *in vitro* EC_{50} values were compared to 1) pre-existing *in vivo* LC_{50}s from the fathead minnow database and 2) pre-existing *in vitro* EC_{50}s from the Halle database. Results point to good *in vitro*/*in vivo* correlations for menadione, dichlorophene, hexachlorophene, and acrolein. Poor correlations were observed for allyl alcohol, 4-fluoroaniline, acetaldehyde, and 2,3-dimethyl-1,3-butadiene due to a combination of solubility and volatility problems. Overall, the results suggest that the impact of different technical approaches on the evaluation of acute toxicity *in vitro* depends very much on the chemical class being investigated and less on the characteristics of the cell line. The *in vitro* cytotoxicity of reactive chemicals is challenging due to the nature of the chemicals' physicochemical properties. Further improving the *in vitro* toxicity of reactive chemicals is a prerequisite for the ultimate goal of using fish cell cultures as acceptable, standard alternatives to the use of fish acute lethality assays.

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Dedication

To a cleaner, healthier, and greener environment...

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AB	alamar blue
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
BCF	bioconcentration factor
BD	1,3-butadiene
CFDA-AM	carboxyfluoroscein diacetate acetoxymethyl ester
CYP450	cytochrome P450
DCP	dichlorophene
DMBD	2,3-dimethyl-1,3-butadiene
DMSO	dimethyl sulfoxide
EC_{50}	effective concentration of test chemical that reduces cellular functions by 50%
EPA	Environmental Protection Agency
FBS	fetal bovine serum
FDA	Food and Drug Administration
FHM	fathead minnow
G6PD	glucose-6-phosphate dehydrogenase
GSH	glutathione
HCP	hexachlorophene
HLC	henry's law constant
LC_{50}	lethal concentration of test chemical that kills 50% of exposed fish/animals
LDH	lactate dehydrogenase
MD	menadione
MEIC	multicentre evaluation of *in vitro* cytotoxicity
NR	neutral red
NRU	neutral red uptake
OECD	Organization for Economic Co-operation and Development
p,p'-DDE	*p,p'*-dichlorodiphenyl dichloroethylene
ppm	parts per million

RCF	relative centrifugal force
RFU	relative fluorescent unit
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
TSCA	toxic substances control act
WHO	World Health Organization

Chapter 1
INTRODUCTION

As the world's population continues to grow in the midst of a globalization movement, our reliance on industrial and synthetic chemicals for purposes ranging from pesticides to pharmaceuticals grows as well. Surely most of these chemicals have helped mankind in many ways that eventually revolutionized the way we live. But consequently, many of these chemicals become of environmental concern as they reach levels that pose serious threats to living systems in ways that may threaten their survival, ultimately disrupting the balance of ecosystems. These chemicals may reach species from both point and nonpoint sources of discharge, but in order for these chemicals to cause toxicity, they must first come into contact with an organism.

Contaminants must first be taken up by organisms to initiate toxicity. Absorption first takes place at an organ-level: gills, lungs, gut, and/or skin, depending on the organism and the exposure route. General routes of absorption at the cellular level depend on the chemical nature of the compound(s). Lipid compounds diffuse across the plasma membrane through both passive and facilitated diffusion; those aqueous ones are taken up by carrier and channel proteins; larger molecules can enter via endocytosis (Newman & Unger, 2003). Once inside, contaminants can now be transported all over the body via the circulatory system. Interestingly, all blood leaving the digestive system heads directly to the liver, which is the major detoxifying organ in vertebrates (Boelsterli, 2003). The lungs and gut act as secondary sites of metabolism. The series of reactions, known as Phase I and Phase II, involved in the

metabolism of a compound from toxic to non-toxic is known as biotransformation, and is mainly mediated in the liver by the hepatocytes (Mothersill & Austin, 2003; Newman & Unger, 2003). Phase I reactions are concerned with the addition of various functional groups to xenobiotics to essentially increase their hydrophilicity for their eventual elimination. This functionalization is done by the large, diverse family of Cytochrome P450 (CYP450) enzymes; when needed, the induction of these enzymes can be many-fold higher than basal levels. Phase II reactions act on Phase I metabolites by conjugating endogenous compounds with them, allowing for xenobiotic elimination; glutathione-S-transferase is one of the main players involved. Interestingly, some already-hydrophilic xenobiotics bypass Phase I reactions, conserving energy and resources (Boelsterli, 2003; Mothersill & Austin, 2003; Newman & Unger, 2003). These reactions, however, can sometimes biotransform a xenobiotic into a more toxic metabolite; this is known as bioactivation. For example, parathion induces toxicity when first bioactivated to paraoxon (Pope, 1999). Ideally, once detoxified, the now-hydrophilic xenobiotic can be excreted via urine, bile, lungs, and/or gills.

1.1 The Problem: identify toxicity of environmental contaminants

The US EPA's Toxic Substances Control Act (TSCA) covers more than 83,000 chemicals, but it is estimated that 500-1000 new chemicals are submitted to TSCA each year (http://www.epa.gov/oppt/newchems/pubs/invntory.htm). The traditional way of assessing the impact of these contaminants on the aquatic environment has been through the use of single species tests involving fish (fathead minnow, zebrafish, and rainbow trout), small invertebrates (*Daphnia magna*), bacteria, and algae (Fentem & Balls, 1993). The most common of these is the 96h fish acute lethality test. It is standardized under the Organization for Economic Co-operation and Development (OECD testing protocol 203, 1992) and is also

used for the environmental assessment of industrial effluents. The test requires the exposure of 10 fish per chemical concentration, with a minimum of 5 concentrations in addition to a control. The preferred species are fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), and zebrafish (*Danio rerio*) (Weyers et al., 2000). The endpoint of this *in vivo* test is aimed at identifying the concentration that is lethal to 50% of the exposed fish, referred to as LC_{50}.

This approach, however, has numerous limitations. It is too laborious, expensive, and is also limited to a few species. Whereas it is well-documented that ecosystems encompass a great range of species that may differ in their sensitivity to environmental contaminants (Cairns, 1986; Chapman, 1981). Additional factors such as behaviour, food preference and nutritional status, and life stage may further influence the sensitivity of these species to toxic agents (Mothersill & Austin, 2003). Since the endpoint of the test represents integrative toxicity, it lacks the ability of identifying a chemical's potentially unique mode(s) of action. It also requires the use of a large number of fish, which contradicts the strong societal desire to reduce the use of fish/animals in toxicity testing (Schirmer, 2006). According to EU directive 86/609/EEC, the use of animals in toxicity testing should be avoided provided that an alternative, scientifically satisfactory, method is available. Lethality itself as an endpoint is also of concern. Many contaminants maybe present in the aquatic environment at sublethal concentrations, but they may however be present at high enough levels to cause sublethal adverse effects that may not always be associated with overt symptoms (Mothersill & Austin, 2003). It is for these political, financial, and technological reasons that the use of fish cell cultures as alternatives to the use of whole fish has been widely explored in environmental toxicology. Such *in vitro* technique was initially slow at catching scientists' attention, but

world-wide efforts using multiple cell lines and chemical classes greatly picked up in the 1980s with the advancement of culturing techniques and cell line availability.

1.2 A solution: *in vitro* toxicity testing

In 1968 Rachlin and Perlmutter first used a fish cell line in the toxicity testing of individual chemicals. Advancements in culturing techniques and availability of cell lines have took *in vitro* toxicology a long way since. In 1979, Barker and Rackham first used a fish cell line for genotoxic studies. Ahne, in 1985, was the first to propose the use of fish cell lines as alternatives to the use of whole fish in the toxicity testing of industrial effluents. Fish cell cultures from rainbow trout positively identified toxic effluent samples from a pulp and paper mill and nitrogen product producer when compared to whole fish toxicity tests (Dayeh et al., 2002, 2009). Toxicity is first manifested or initiated at the cellular level, before going on to disrupt tissues and/or organs, ultimately impacting the whole organism. The type of cell line used should not make a difference in its sensitivity to a given chemical based on Ekwall's basal cytotoxicity concept (Ekwall, 1983a). It is now clear, however, that exceptions to this concept include chemicals that are species and/or organ-specific. An example is carbon tetrachloride (CCl_4), which must first be metabolized by CYP2E1 enzymes of liver cells into the trichloromethyl radical ($CCl_3^{\cdot}$). This metabolite initiates a series of lipid peroxidation reactions ultimately causing toxicity in the organism (Boelsterli, 2003). This example of chemical-organ selectivity in animals highlights the need of grouping the various environmental contaminants out there into well-defined, organized classes. The classification system used for this thesis is described in section 1.3.

In general terms, cytotoxicity can be defined as adverse effect(s) observed in a cell involving structural and/or functional processes essential for survival, proliferation, and

function due to exposure with an exogenous chemical (Freshney, 2001; Seibert et al., 1996). One can divide cytotoxicity into three categories. Basal cytotoxicity refers to chemicals that attack cellular structures and/or functions that are essentially common to all cell types, regardless of their origin. Examples of basal cytotoxicity include effects on energy metabolism, plasma membrane integrity, and ion regulation. Thus, all cell types would be expected to react with equal sensitivity to such basally-acting toxic agents (Ekwall, 1983a; Ekwall & Ekwall, 1988). Selective cytotoxicity refers to those chemicals that are preferentially more toxic to certain cell types than others due to their unique, differentiated properties. The biotransformation capabilities of specific cell types and the specificity of membrane receptors to their ligands is an example (Mothersill & Austin, 2003; Seibert, 1996). Functional cytotoxicity refers to chemicals that interfere with cellular processes in ways that threaten the survival of a particular organ or even the organism as a whole. The disruption in cell-to-cell communication and/or widespread hormonal imbalance is an example (Mothersill & Austin, 2003).

When confronted with xenobiotics, cells employ four main strategies in dealing with the chemical (Sheehan et al., 1995). 1) Sequestration takes place when a cell actively concentrates a chemical in a particular subcellular compartment. Hydrophobic chemicals are typically sequestered in biological membranes, which allows for their bioaccumulation over time. 2) Scavenging takes place when a xenobiotic is detoxified or neutralized by non-enzymatic reactions with endogenous chemicals (Mothersill & Austin, 2003). Examples include the conjugation of Reactive Oxygen Species (ROS) with various antioxidants (e.g. glutathione, GSH) that render them harmless. 3) Proteins such as the metallothioneins allow for the binding of many metallic xenobiotics, preventing them from binding at other places

that may otherwise be toxic. 4) The most versatile way is enzymatic detoxification of a xenobiotic by specialized systems such as the CYP450 systems employed by hepatocytes. One should keep in mind, however, that a particular xenobiotic may actually be metabolized (bioactivated) into a more toxic form when going through one or more of the above mentioned strategies (Mothersill & Austin, 2003).

Cytotoxicity assays using fish cell cultures can be performed using both primary cultures and cell lines. Primary cultures are directly initiated from a tissue or organ. Their main advantage is that they retain many of their differentiated *in vivo* properties (albeit for short amount of time, ~ 48h), and as such can be used to screen chemicals that are known to be tissue or organ-selective (Castaño et al., 2003; Mothersill & Austin, 2003). However, there are disadvantages to primary cultures, including fluctuating sensitivity to chemicals, which depends on the status of the source fish and the nature of culture initiation, and difficulty in initiation. Provided optimal culture conditions are followed, it is thought that primary cultures can be initiated from a wide range of tissues and organs. These have included hepatocytes, respiratory cells, and neuronal cells (Castaño et al., 2003; Mothersill & Austin, 2003).

Cell lines, in contrast, are made up of primary cells that are further subcultivated (or passaged) to give rise to new cells. Many species give rise to finite cell lines that, by definition, undergo a limited number of subcultivations before their growth is arrested (Mothersill & Austin, 2003). Some mammalian and fish species, however, give rise to continuous cell lines that appear to immortalize spontaneously, and as such can be subcultivated indefinitely (Castaño et al., 2003; Mothersill & Austin, 2003). To date, more than 150 continuous cell lines have been established from 74 fish species, representing 34

families, most of which have fibroblastic or epithelial-like morphologies (Fryer & Lannan, 1994). Also, most of them are anchorage dependent, requiring a substrate for attachment, and grow in media initially intended for mammalian cell lines (Bols & Lee, 1994; Segner, 1998). Some of these cell lines can be purchased from the American Type Culture Collection (ATCC) and/or the European Collection of Cell Cultures (ECACC). In addition to greatly reducing our dependence on whole fish, the use of fish cell lines offers rapid, reproducible, and much more economic means of toxicological assessment than traditional *in vivo* systems. And by utilizing various endpoints, one can possibly elucidate cellular modes of action; this is especially important for emerging contaminants. Mechanistic studies would be better to interpret because of the lack of *in vivo* complexities such as bioaccumulation and depuration. Further, one can also manipulate experimental conditions by modifying exposure conditions and the external environment in a way that is more representative of the natural environment (Baksi & Frazier, 1990). Cell lines also offer us with a stable source of cells since they can be cryopreserved for long periods of time, which allows for the continued availability of a homogeneous supply of cells. Endpoints of *in vitro* toxicology are aimed at identifying the effective concentration of a chemical that impacts cellular functions by 50%, referred to as EC_{50}. It is important to note however, that the use of cell lines also comes with disadvantages. Over time, the many cell divisions may cause the cells to lose their differentiation in a way that may make them unrepresentative of their parent tissue (Castaño et al., 2003; Mothersill & Austin, 2003). Another problem is that the genomes of cell lines may deviate from the normal diploid karyotype. Finally, a cell line may overestimate the toxicity of a compound (false positives) due to the absence of compensatory, repair, and/or feedback mechanisms that would otherwise offer protection *in vivo*. But likewise, the use of

cells may underestimate (false negatives) the toxicity of a given chemical due to its rapid volatility and/or poor solubility in the test system (Castaño et al., 2003; Mothersill & Austin, 2003).

1.2.1 Cell line selection for *in vitro* toxicity testing

Selected cell lines should be widely available, characterized, and relevant (Riddell et al., 1986a; Schirmer, 2006; Stark et al., 1986). To assess the cytotoxicity of reactive chemicals, a gill (RTgill-W1) and a liver (RTL-W1) cell line from rainbow trout (*Oncorhynchus mykiss*) were chosen because their respective organs execute paramount roles *in vivo* due to the first line of exposure and metabolizing capabilities, respectively.

Gill damage is often the primary cause of death in many acute lethal fish toxicity tests, perhaps a reflection of gills' functional complexity. The epithelia are involved in transport of respiratory gases, electrolytes and essential ions, acid-base equivalents, and nitrogenous waste (Mothersill and Austin, 2003). There are two major types of cells in gill epithelia. The respiratory (pavement) cells make up the most at 90-95% of cell population and are involved mostly for gas exchange. The remaining are mostly chloride cells that are involved in iono-regulation and are characteristic with a multitude of mitochondria, endoplasmic reticulum (ER), and Na^+/K^+ ATPase. Due to complexity of gill organ, gill cell cultures have been used for many physiological and toxicological studies, including regulation of cell volume and intracellular pH, electrochemical coupling via cell connections, detoxification via Phase I and Phase II metabolism of steroids and lipophilic xenobiotics (Mothersill and Austin, 2003).

RTgill-W1, initiated from the gills of rainbow trout, was chosen because of the role the gill has as the first line of exposure and a major route of uptake for many chemicals in the

aquatic environment. The cells in the RTgill-W1 culture exhibit an epithelial-like morphology, predominantly taking on irregular polygonal shapes.

The RTL-W1 cell line, initiated from rainbow trout liver, was chosen because unlike most liver cell lines it is able to induce cytochrome P450 enzymatic systems, which are a body's main detoxifying agents (Lee et al., 1993). The cells in the RTL-W1 culture exhibit an epithelial-like morphology and are polygonal in shape.

1.2.2 Selection of exposure medium for *in vitro* toxicity testing

The nature of the exposure medium in a cytotoxicity assay is of great significance since it can greatly influence the bioavailability of a given chemical to cells, potentially increasing or decreasing the cellular response (Schirmer et al., 2006; Seibert et al., 2002; Stark et al., 1986). For example, the addition of serum to exposure media was found to reduce fluoranthene uptake by cells because most of the fluoranthene was being bound to serum proteins and as a result the chemical's toxicity to cells may have been underestimated (Schirmer et al., 1997). On the other hand, cytotoxicity assays aimed at identifying CYP1A-inducing chemicals were found to be more effective when serum is added to exposure media (Schirmer et al., 1997). Thus, a simplified exposure medium containing essential salts, pyruvate, and galactose at concentrations matching those found in L-15, known as L-15/exposure or L-15/ex, has been selected for investigating the cytotoxicity of reactive chemicals (Schirmer et al., 2004). This simplicity in incubation medium is meant to maximize the bioavailable fraction of the toxicant in question to the cells, so that any protection observed would be intrinsic to the cells. Thus, protective molecules such as toxicant-binding serum proteins and antioxidants would be absent. L-15/ex is also easy to prepare and is significantly less expensive than complete culture media.

1.2.3 Method of dosing for *in vitro* toxicity testing

There are two main ways of exposing cells in culture to a given chemical: 1) direct dosing and 2) indirect dosing.

Direct dosing refers to the direct addition of a small volume of the chemical that is 200 times concentrated to microplate wells that would already contain confluent cells along with their exposure medium (Figure 1-1 – Panel A). This is done by letting the desired chemical volume to mix with the exposure medium right at the surface to prevent the disruption of the underlying cells. Indirect dosing refers to the addition of 200 times concentrated chemical to a volume of exposure medium to make up the final concentration of the chemical in the well. The diluted chemical in exposure medium is then added directly to the wells of a microplate containing a confluent layer of cells (Figure 1-1 – Panel B).

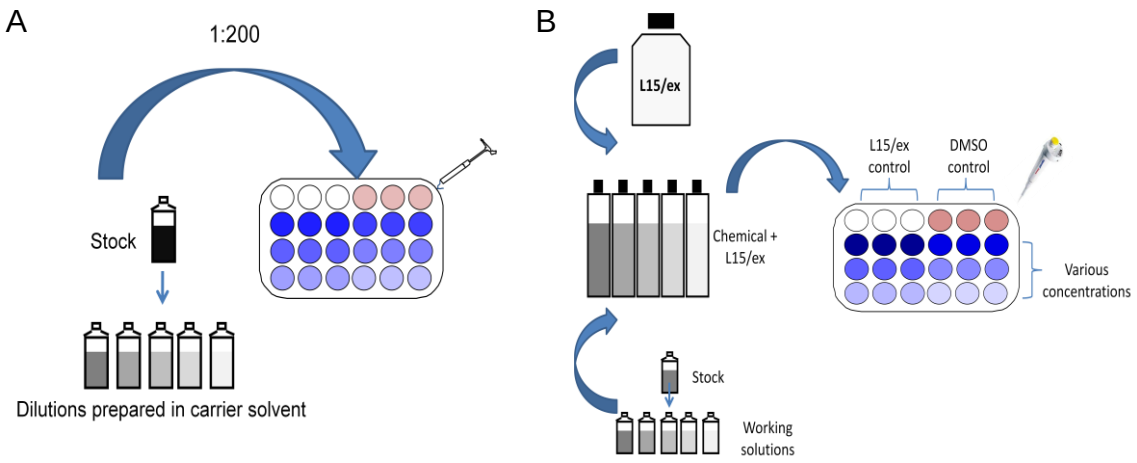

Figure 1-1: Schematic representation of cell culture dosage.

Cells were initially plated in 24-well microplates and then incubated for 24h to allow for cell attachment. After which, cells were either directly (A) or indirectly (B) dosed with a selected chemical. In direct dosing, the chemical dilutions were separately prepared in their carrier solvent at a concentration that is 200 times more concentrated than the desired final concentration. After vigorous vortexing, a small volume of each dilution was then directly added to its respective microplate well(s) that already contained a confluent layer of cells along with their exposure medium. In indirect dosing, the chemical dilutions were also prepared separately, but the desired final concentration was then achieved by further diluting the chemicals in the exposure medium L-15/ex. After vigorous vortexing, the chemical-L-15/ex mixture was then added to its respective microplate well(s).

1.2.4 *In vitro* toxicity testing cell viability endpoints

The nature of the endpoint used to measure cell viability after toxicant exposure is also of significance because it can have a profound impact on the derivation of effective concentrations (Fotakis & Timbrell, 2006; Gulden et al., 2005; Schirmer, 2006). To date, most of the endpoints of the *in vitro* assays deal with cellular changes at the molecular, biochemical, histological, or physiological level (Mothersill & Austin, 2003). These cellular changes are numerous and cover a wide range of responses, ranging from subtle molecular alterations, for example CYP induction and GSH depletion to cell death. Additional criteria should be taken into account before a particular endpoint is chosen to evaluate a specific cellular response. The ideal endpoint should not only be relevant, reliable, and sensitive, but also simple, rapid, and cost-effective (O'Brien et al., 2000).

The most common endpoint of *in vitro* assays is cell death as measured by a single indicator dye 24h after exposure of cells to a chemical, usually targeting a single cellular response (Schirmer, 2006). Clearly, this would be inappropriate for the cytotoxicity testing of reactive chemicals since, as previously mentioned, these chemicals act through various modes of action. Thus, in this thesis, a battery of three fluorescent indicator dyes has been employed: alamar Blue (AB) tests for metabolic activity (O'Brien et al., 2000), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) tests for cell membrane integrity (Schirmer et al., 1997), and neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride; NR) tests for lysosomal function (Borenfreund & Puerner, 1985). An economic advantage to using these three dyes is that they can all be used on the same set of cells, but their use has also been shown to help deduce possible cellular modes of action (Boaru et al., 2006; Dayeh et al., 2009).

Alamar Blue, a commercial preparation of the resazurin dye, was initially believed to be reduced by mitochondrial enzymes of viable cells, but it is now known to be reduced more generally by diaphorases, enzymes that can found in both mitochondria and cytoplasm (O'Brien et al., 2000). Thus, the dye is a measure of cellular metabolism as opposed to mitochondrial function. Alamar Blue added to cells in the oxidized form, resazurin, becomes reduced by cellular diaphorases of viable cells into resorufin, which is fluorescent and pink in colour. The resulting relative fluorescence can be quantified using a variety of fluorescence microwell plate readers.

CFDA-AM dye is an esterase substrate used to measure cell membrane integrity. The initial non-polar, non-fluorescent 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is added to cells which diffuses into cells rapidly and is converted by non-specific cellular esterases of viable cells into the polar, fluorescent 5-carboxyfluorescein (CF), which then diffuses out of cells slowly (Schirmer et al., 1997). The resulting relative fluorescence can be quantified using a fluorescence microwell plate reader.

The Neutral Red dye accumulates in the lysosomes of viable cells, which upon extraction can be quantified using a fluorescence microwell plate reader. The accumulation of NR in lysosomes, however, requires an intact plasma membrane, sufficient energy (ATP) levels, and a functioning lysosome. Accordingly, the assay can also be thought of as a general endpoint against all three cellular parameters (Borenfreund & Puerner, 1985). Nonetheless, it can still be used to detect damage specific to lysosomes. For example, Schirmer et al. (1998) found that acenaphthylene, acenaphthene, and phenanthrene were photocytotoxic to RTgill-W1 cells immediately after their UV irradiation as measured by the NR assay, whereas no such toxicity was detected by other indicator dyes.

1.3 Classifying environmental contaminants

In order to be able to predict the nature of the impact of different types of chemical contaminants on the environment, they have been classified according to structure and/or function in various ways. One such classification system, used in this thesis, was detailed by Verhaar et al. (1992) where environmental contaminants have been divided into four distinct classes that are dependent on the presence or absence of certain functional groups.

A) Inert chemicals. These chemicals bring about toxicity through narcosis, defined as a non-specific interaction between a chemical and the cell plasma membrane. As such, the potency of an inert chemical is directly related to its hydrophobicity, which is a function of log*P*. Interestingly, because these chemicals do not have specific modes of action, they act as models of baseline (or minimum) toxicity. This means that in the absence of specific modes of action all chemicals are as toxic as their hydrophobicity suggests. It is estimated that 80% of all chemicals exhibit baseline or basal toxicity (Ekwall, 1983a,b) and the results from the international Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) study support this estimation (Clemedson et al., 1996). Common examples of inert chemicals include ethanol, acetone, benzene, and toluene (Verhaar et al.,1992).

B) Less inert chemicals. These chemicals bring about toxicity at slightly lower levels than predicted by baseline toxicity, making them slightly more toxic. They typically posses hydrogen bond donor acidity and thus tend to have polar functional groups. Hence, their mechanism of action is polar narcosis. Common examples of less inert chemicals include phenols (e.g. phenol and its derivatives) and aromatic amines (e.g. aniline and many of its derivatives) (Verhaar et al., 1992).

C) **Specifically acting chemicals**. Such chemicals belong to a varied set of chemicals, but they all essentially interact with specific cellular receptors to induce toxicity. Examples include the inhibition of acetylcholinesterase activity by the organophosphate pesticides (e.g. parathion) and CYP450 induction by polycyclic aromatic hydrocarbons and other dioxin-like compounds that act as ligands for the aryl hydrocarbon receptor (e.g. rifampin) (Verhaar et al., 1992).

D) **Reactive chemicals**. Such chemicals attack biomolecules in various targets by employing various modes of action. Also in this class are chemicals that require conversion to more toxic products in order to exhibit toxicity; this phenomenon is known as bioactivation. One of the main differences in the toxicity of narcotic chemicals and those reactive is the fact that the interaction of reactive (alkylating) chemicals with the target is typically irreversible, while narcosis is due to a reversible interaction. As opposed to reversible interactions, in an irreversible interaction it is not just the concentration at the target site that is of relevance, but also the amount of target that is occupied or depleted (Freidig et al., 1999). Examples of reactive chemicals include many epoxides (e.g. propylene oxide and 1,2-epoxybutane) and aldehydes (e.g. propanal or acrolein and benzaldehyde) (Verhaar et al., 1992).

1.3.1 Selected reactive chemicals

The reactive class of chemicals is of special interest since it has been greatly underrepresented in *in vitro* toxicology. For this thesis, eight reactive chemicals that exhibit a wide range of physicochemical properties are all of environmental concern and have been chosen for analysis (Schirmer et al., 2008). In the following section a brief overview is given, where possible, for each of the reactive chemicals with respect to their *in vitro* toxicity, their *in vivo* toxicity, and why they are of environmental concern.

Menadione (2-methyl-1,4-naphthoquinone; MD) is a vitamin K3 analog that belongs to the quinone class of chemicals (Figure 1-2). As a bifunctional quinone, it causes cytotoxicity via two distinct pathways (Scott et al., 2005). The first is through oxidative stress that follows the cyclic pattern known as redox cycling, generating ROS such as superoxide ($O_2^{\cdot}$-), hydrogen peroxide (H_2O_2), and the highly damaging hydroxyl radical ($OH^{\cdot}$**)**. In fact, it is used as a model chemical for studies focusing on ROS-related toxicity *in vitro* (Scott et al., 2005). The second pathway is by covalently modifying cellular nucleophiles, such as the case with sulphur-containing cysteine residues that go on to form dangerous protein arylation adducts. Many of these proteins are enzymes involved in glutathione (GSH) and ATP metabolism, and thus menadione toxicity is typically preceded by GSH depletion and great ATP loss; eventual cell death is mainly mediated by apoptosis (McAmis et al., 2003; Shi et

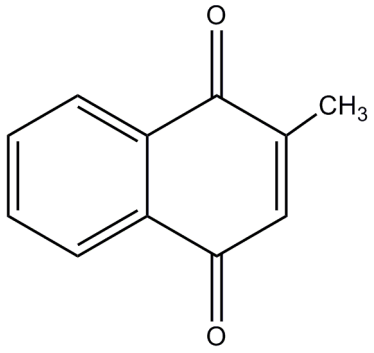

Figure 1-2: Molecular structure of menadione.

al., 1994). Menadione can also modify plasma membrane proteins, thereby altering a membrane's permeability to ions and other unwanted molecules.

Menadione can be highly toxic to mammals *in vivo*. The problem is that a body's endothelial cells are especially sensitive to ROS; hence, enhanced ROS production due to menadione toxicity alters vascular endothelial barriers, which can lead to inflammation, oedema, and overall organ dysfunction (Stevens et al., 2000). In fact, the US Food and Drug Administration banned the use of menadione from over-the-counter supplements and from most food intended for human consumption. Menadione toxicity causes haemorrhages in kidneys, lungs, livers, and can lead to severe haemolytic anaemia. Moreover, menadione's pathways of toxicity had been explored for their chemotherapeutic potential to treat various cancers, but its use has now been largely replaced with safer agents (McAmis et al., 2003).

Menadione is of environmental concern because of its widespread use in several human activities. As vitamin K itself is essential for proper blood coagulation and bone strength, synthetic forms of the vitamin, namely menadione and its derivatives, are being used as nutritional supplements in several industries. These include the pet food industry and aquaculture feed industry. In aquaculture, the additive is used in fish feed (in the form of menadione sodium bisulfite) but mainly in feed for shrimp in Asian countries (Shiau & Liu, 1994; Udagwa, 2001).

Dichlorophene [2,2'-methylenebis(4-chlorophenol); DCP] is a halogenated phenolic compound (Figure 1-3). DCP causes toxicity by inhibiting the activity of a key enzyme involved in the metabolic pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD). Dichlorophene caused 50% inhibition of G6PD under *in vitro* conditions at a concentration of 34μM forty to fifty seconds after reaction initiation. G6PD deficiency can lead to haemolytic anaemia (Yamarik & Andersen, 2004). The hydrophobic nature of dichlorophene allows it to freely diffuse through cell membranes, attracting and transporting protons on its way in by its hydroxyl groups, which leads to disruptions in proton gradients. Dichlorophene alters the permeability of erythrocytes to K^+ ions ultimately causing anaemia (Yamarik & Andersen, 2004). Previous *in vitro* studies have demonstrated a 50% inhibition of phosphorus uptake and a 50% increase in ATPase activity at concentrations of 7.9 and 2.7μM dichlorophene, respectively (Nakaue et al., 1972). When orally administered, most of this compound is metabolized by the gut wall and liver cells into more soluble forms for excretion *via* the kidneys (Dixon, 1982; Yamarik & Anderson, 2004). Nonetheless, with a log*P* of 4.34, dichlorophene would be expected to bioconcentrate in adipose tissue.

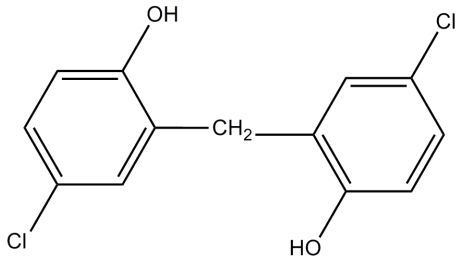

Figure 1-3: Molecular structure of dichlorophene.

Dichlorophene poses serious threats to humans *in vivo*. Symptoms of dichlorophene poisoning include nausea, diarrhoea, vomiting, and liver, renal, and myocardial failures. Irritation of the respiratory tract is one of the first overt symptoms. Dichlorophene has also been reported as a developmental toxicant, causing low birth rates, birth defects, and

psychological and behavioural deficits. It has even been recognized as an immunotoxicant, hindering the ability of the body to response to infectious substances (Yamarik & Anderson, 2004).

Dichlorophene is of environmental concern because of its widespread use by many industries. It is mainly used as a bacteriocide and fungicide in cosmetics, but it can also be found in deodorants, hair sprays, hair growth formulations, toothpaste, dermatologic preparations, construction material, and paper mill products (Schorr, 1971). Because of its biocidal properties, it is also the main ingredient in such formulations as that against Athlete's foot and is commonly used against infestations of intestinal worms in veterinary medicine, namely those infecting cats and dogs (Kintz et al., 1997). It is also considered an indirect food additive because it can be found in some adhesives (Rothschild, 1990). Overtime, most of this utilized dichlorophene eventually makes its way into aquatic environments (Toxnet Hazardous Substance Data Bank).

Dichlorophene has been recognized as very toxic to aquatic organisms and may cause long-term effects in the aquatic environment (EC Directive 2001/58/EC). This property stems from the inherent hydrophobicity of dichlorophene (log*P* of 4.34). Once released to the environment, it adsorbs strongly to sediments. An important degradation pathway in natural water is that of phototransformation because of its absorption of sunlight (Mansfield & Richard, 1996). DCP's hydrophobicity allows it to bioconcentrate in marine organisms, bioaccumulate, and eventually biomagnify in potentially ecosystem-disruptive ways, although none of these properties have been studied in the field.

Hexachlorophene [2,2'-methylenebis(3,4,6-trichlorophenol); HCP] is a halogenated neurotoxic phenol (Figure 1-4). HCP poisoning inhibits central and peripheral nervous systems metabolism by uncoupling the oxidative phosphorylation responsible for the many glycolytic and oxidative pathways taking place in various tissues (Rajendra et al., 1992). But the chemical can also attack extraneuronal targets, specifically the erythrocytes. It has been shown to inhibit both the Na^+/K^+ pump and acetylcholinesterase activity located within erythrocyte membranes. In fact, an HCP concentration of 50 µM can cause 50% haemolyses within a matter of a couple of hours (Matsumura, 1997; Miller & Buhler, 1974). Moreover, several studies have reported on the inhibitory action of hexachlorophene on various dehydrogenases. One study focusing on a key player in the citric acid cycle reported a hexachlorophene-induced IC_{50} on brain succinate dehydrogenase activity of 0.65mM (Lokanatha, 1999).

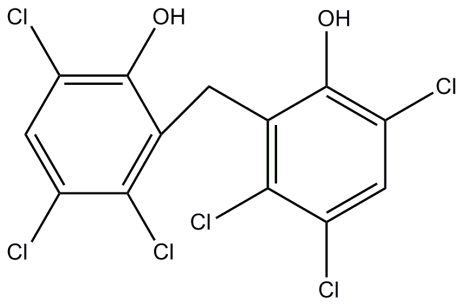

Figure 1-4: Molecular structure of hexachlorophene.

Hexachlorophene can be highly toxic to mammals and other vertebrates *in vivo*. HCP toxicity causes brain oedema, characterized by intracellular fluid influx, increase in intracranial pressure, and vacuolation of myelin that results in a spongy degeneration of white matter (Andreas, 1993). Symptoms of HCP poisoning are in accordance with other uncoupling phenols such as 2,4-dinitrophenol (Kaiser, 1964) and pentachlorophenol (Kehoe et al., 1939), namely depression, diarrhoea, increased respiratory rate, and fever, but unlike them, HCP can also cause convulsions and paralysis (Nakaue et al., 1973).

Hexachlorophene is of great concern because of its widespread use by many industries. As is typical of hydroxychloroaromatic chemicals, HCP is known for its biocidal properties. As such, it had been widely used as a disinfectant in various gels, soaps, deodorants, shampoos, mouthwashes, toothpaste products, hospital equipment, cosmetics, and many other personal care products (Lockhart, 1972). It was even widely applied by the pesticide industry for many years (Lockhart, 1972). In the early 1970s, however, many regulatory agencies, including the US FDA, limited the use of hexachlorophene in consumer, medical, and agricultural products as the toxicological implications of its use over three decades were widely being reported. Today, HCP is used as a model to study demyelinating diseases, by hospitals to control for *Staphylococcus aureus* infections, and by some countries as a biocide in cosmetics (Allen et al., 1994; Nicolas & Taylor, 1994).

Hexachlorophene is of significant environmental concern. In fact, it has been characterized as a very toxic substance to aquatic organisms (EU directive R24/25-50/53). Much of HCP from the various human activities eventually makes its way to the aquatic environment (Toxnet Hazardous Substance Data Bank). The environmental concern stems from hexachlorophene's inherent hydrophobicity (log*P* of 6.92). Once released into soil, it adsorbs very strongly to soil particles. It does not leach to groundwater, hydrolyze, or evaporate to any appreciable extent. Similarly, HCP released to water adsorbs very strongly to sediments, and does not hydrolyze or evaporate to any appreciable extent (Toxnet Hazardous Substance Data Bank). It may, however, undergo slow photodegradation due to its absorption of light above 290 nm. Crucially, its hydrophobic nature allows it to bioconcentrate in many aquatic organisms, bioaccumulate, and eventually biomagnify in potentially ecosystem-disruptive ways. The half-life for the biodegradation of

hexachlorophene in river sediments has been estimated to be ~290 days (Toxnet Hazardous Substance Data Bank).

4-fluoroaniline (1-amino-4-fluorobenzene) is a halogenated aromatic amine (Figure 1-5). 4-fluoroaniline is metabolized by CYP450 systems to various hydroxylated products, some of which are destined for excretion *via* the kidneys (Rietjens & Vervoort, 1991); flavin-containing monooxygenases may also help catalyze such compounds (Boersma et al., 1993). These hydroxylated metabolites, however, can be toxic to spleens, livers, and kidneys (Hong et al., 2000; Okazaki et al., 2003; Rankin et al., 1995). 4-fluoroaniline toxicity is similar to that of aniline and its derivatives. It can be highly destructive to tissues of the mucous membranes and upper respiratory tract, eyes, and skin. Inhalation may result in spasm, inflammation, and oedema of the larynx and bronchi, chemical pneumonitis, and pulmonary oedema. Absorption into a body may lead to the formation of methaemoglobinaemia and eventually cyanosis. Symptoms include headache, nausea, confusion, vertigo, ataxia, weakness, drowsiness and coma (Gosselin, 1976; US EPA, 1994).

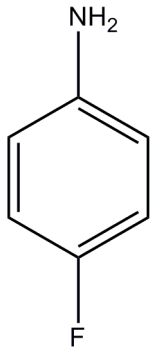

Figure 1-5: Molecular structure of 4-fluoroaniline.

4-fluoroaniline is a widely used intermediate in the synthesis of dyes, perfumes, plant growth factors, drugs, and preservatives (Okazaki et al., 2003). It is also used in the synthesis of some pesticides, but it is also a common metabolic product of pesticide breakdown (Lyons

et al., 1995). It is also a metabolic product of aromatic amine breakdown by many species (Ehlhardt, 1991). Along with other haloanilines, it gets discharged as industrial waste to the environment (Games & Hites, 1977).

Haloanilines in general, including 4-fluoroaniline, are of great concern because not much is known about the ecotoxicological impact of these haloanilines on both organisms and the environment. The problem is not so much because of their toxic potency, but rather because of their widespread abundance in the environment. In other words, 4-fluoroaniline and other aniline derivatives pose threats to both terrestrial and aquatic organisms because of their increased potential of coming into contact with these organisms. 4-Fluoroaniline may be released to the environment in waste effluents generated from its commercial production or use as chemical intermediate. When released to the atmosphere**,** 4-fluoroaniline degrades rapidly by reacting with photochemically-produced hydroxyl radicals with a half-life of ~6.5h. When released to soil or water, adsorption to humic materials and sediments may occur, but leaching is also possible (Toxnet Hazardous Substance Data Bank).

Allyl alcohol (2-propen-1-ol) is the smallest representative of the allylic alcohols (Figure 1-6). Alcohol dehydrogenases (ADHs) are metabolic enzymes that specialize in the oxidation of an alcohol's hydroxyl group into an aldehyde. The problem is that the resulting aldehydes are much more reactive than their parent compounds. For example, methanol gets bioactivated to formaldehyde, ethanol to acetaldehyde, and in the case of allyl alcohol to acrolein (Koerker et al., 1976; Reid, 1972). The metabolism to acrolein is mediated by the hepatic class I isoform of alcohol dehydrogenase, which is found to localize in post-mitochondrial fractions of the periportal regions of liver lobules (Serafini-Cessi, 1972). The nature of the subsequent events leading to cell death is poorly understood. What is known is that the ADH involved strictly uses NAD^+ as a cofactor (Arslanian et al., 1971) and that the activation of protein kinase C seems to be critical for the cytotoxicity of hepatocytes (Jaeschke et al., 1987; Maddox et al., 2003; Maddox et al., 2004; Rikans & Moore, 1987; Serafini-Cessi, 1972). Acrolein is a potent electrophile that depletes GSH levels, forms many covalent adducts, and causes oxidative stress (Belinsky et al., 1986; Miccadei et al., 1988).

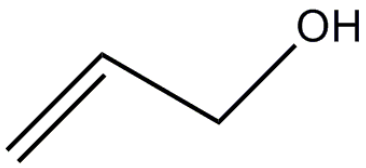

Figure 1-6: Molecular structure of allyl alcohol.

Allyl alcohol is widely used in the manufacture of food flavourings, perfumes, pharmaceuticals, pesticides, organic chemicals, plastics, various polymers, and other allylic compounds. Exposure to allyl alcohol is toxic to mammals *in vivo*. It is primarily a potent hepatotoxin due to its conversion to the highly reactive, unstable acrolein, and thus can lead to liver failure. But the parent compound itself is also toxic. It can readily be absorbed through skin, causing severe irritation of the eyes (at concentrations as low as 25ppm) and

nose (10-15ppm). If absorbed deep enough, can cause muscle spasms and aching. Other symptoms include abdominal pain, nausea, vomiting, and diarrhea (Jaeschke et al., 1987; Maddox et al., 2003).

Not much is known about the ecotoxicological impact of allyl alcohol, but it has been recognized as dangerous for the environment. Once released into the aquatic environment, it does not volatilize quickly due to its high solubility in water (but it tends to concentrate in surface waters since it is lighter than water, d=0.845), nor does it photooxidize. Biodegradation is expected to be the major route of allyl alcohol elimination. Release of allyl alcohol into soil is expected to leach and possibly enter groundwater (Toxnet Hazardous Substance Data Bank).

Acrolein (2-propenal) is a potent α, β-unsaturated aldehyde (Figure 1-7). Acrolein's electrophilic, reactive nature is attributed to the conjugation of a vinyl group with a carbonyl group within its structure. In fact, compared to other α, β-unsaturated aldehydes, acrolein is by far the strongest electrophile. It rapidly attacks nucleophilic sites such as the sulfhydryl group of cysteine, imidazole group of histidine, and the amino group of lysine (Esterbauer et al., 1991; Marnett et al., 2003). Accordingly, enzymes with such amino acids in their active site are particularly sensitive to acrolein toxicity. One such enzyme is O6-MeG-DNA methyltransferase, which is crucial for DNA repair (Krokan et al., 1985). In fact, acrolein is known for its genotoxicity and mutagenecity. It causes DNA single strand breaks (Graftstrom, 1988), chromosome aberrations, and sister

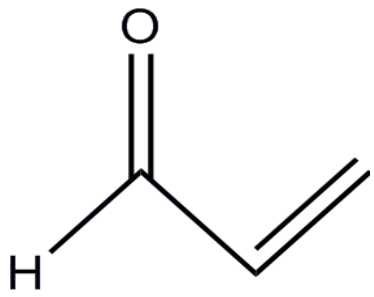

Figure 1-7: Molecular structure of acrolein.

chromatic exchanges (Au, 1980). Acrolein forms covalent adducts with proteins. It causes a rapid depletion of cellular GSH levels by forming glutathionylpropionaldehyde adducts. In fact, *in vitro* studies have shown that an increase in GSH levels could attenuate acrolein toxicity (Tanel & Averill-Bates, 2007). Both acrolein and its GSH adduct have been associated with further production ROS, which go on to attack lipid membranes and thus produce more lipid peroxidation products; thus, acrolein also acts as a redox cycler (Krokan et al, 1985). Metabolic products of acrolein metabolism include mercapturic acids, acrylic acid, and glyceraldehydes.

Acrolein is a ubiquitous pollutant in the environment. It is commonly used as a herbicide against aquatic weeds, as a rodenticide fumigant, and as a microbiocide by oil and paper mill industries. It is also used in large amounts in the synthesis of polymers and industrial chemicals (acrylate polymers and acrylic acid) (Eisler, 1994; Ghilarducci & Tjeerdema, 1995). Acrolein is also a major by-product of forest fires, fossil fuels, car exhaust, overcooked fats and oil, cigarette smoke, and, biologically, of lipid peroxidation and the breakdown of allyl compounds. It is also a product of cyclophosphamide metabolism, and is thought to be responsible for the toxic side effects from this anti-tumour drug (US EPA, 1993).

Acrolein is very toxic to humans *in vivo*. It has been associated with the development of Alzheimer's disease (Calingasan et al., 1999), atherosclerosis, diabetic nephropathy (Suzuki & Miyata, 1999), and many respiratory diseases; its genotoxic properties have been linked with many respiratory tract cancers. It is also teratogenic and embryolethal (Slott & Hales, 1986).

Acrolein is of environmental concern because of its highly toxic nature to fish and aquatic invertebrates. A 96h LC_{50} of 22 µg/L has been determined for bluegill sunfish, <31 for rainbow trout, whereas an LC_{50} of only 14 µg/L has been determined for white sucker and fathead minnow (US EPA, 2003). The EC_{50} for many aquatic plants and algae, however, are typically higher (ranging from ~30 µg/L to 26 mg/L), and thus the use of acrolein as a herbicide is of concern for the many sensitive fish species (US EPA, 2003). The environmental fate of acrolein follows two major routes: volatilization and hydration. The major hydration product of acrolein is 3-hydroxypropanal, with other aldehydes also being formed (WHO, 1991). Eisler (1994) summarizes the half-time persistence of acrolein in freshwater as usually less than 50h; in seawater it is less than 20h and in the atmosphere less than 3h. Accordingly, acrolein poses the greatest risk to aquatic organisms at or near sites of industrial discharges, spills, and biocidal use within the first few days of its release (Toxnet Hazardous Substance Data Bank).

Acetaldehyde (ethanal) is a toxic short-chain aldehyde (Figure 1-8). Acetaldehyde is the principal metabolic product of ethanol, catalyzed primarily by alcohol dehydrogenase and to a less extent by *CYP2E1* in the liver, but also in extrahepatic tissues such as in the oesophagus (Quertemont, 2004; Yokoyama & Omori, 2003). The resulting acetaldehyde is much more reactive than its parent compound, and thus this enzymatic catalysis is more of a bioactivation step. But in attempt to keep acetaldehyde levels in check, the chemical itself is further oxidized to acetate by aldehyde dehydrogenase (ALDH) (Quertemont, 2004; Yokoyama & Omori, 2003).

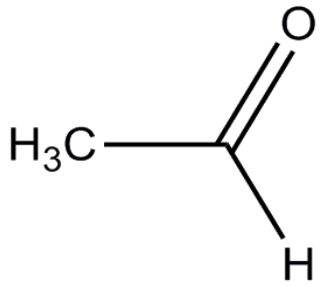

Figure 1-8: Molecular structure of acetaldehyde.

Reports on acetaldehyde's genotoxic effects are well-documented (Singh & Khan, 1995). Acting as an electrophile, its attacks on DNA causes both single and double strand breaks, gene mutations, and increases in chromosomal aberrations and sister-chromatid exchanges. The xenobiotic also forms covalent adducts and crosslinks with DNA, proteins, and lipids, all of which alter cellular structure and function (Jennett et al., 1990; Ristow & Obe, 1978). Its reactions also generate free radicals that go on to attack lipid membranes and release lipid peroxidation products. In fact, acetaldehyde has been recognized as a carcinogen (Woutersen et al., 1984) and as a teratogen (Oshea & Kaufman, 1979).

Clearly, exposure to acetaldehyde poses serious threats to mammals *in vivo*. Nonetheless, it is of widespread use by many industries. It is used in the synthesis of food flavourings, dyes, perfumes, resins, plastics, rubbers, and as an intermediate in the synthesis

of many organic chemicals. Additionally, acetaldehyde itself is a natural product of combustion, photooxidation of commonly found hydrocarbons in the atmosphere and surface water, and as natural ingredient in many fruits. But the most common route of exposure to acetaldehyde by humans is through their consumption of alcoholic beverages. It is worth noting, however, that, with the exception of livers, most tissues of the body do not have the ADH necessary to convert ethanol into the real hangover chemical, acetaldehyde (Olivares et al., 1997). This would explain the hepatotoxicity associated with the overuse of alcoholic beverages.

Not much is known about the ecotoxicological effects of acetaldehyde as most of its studies have been conducted on mammals *in vivo* or mammalian cell lines *in vitro*. Nonetheless, acetaldehyde is of environmental concern to both terrestrial and aquatic life due to its adverse interactions with common cellular structures and functions. Once released into water, it rapidly biodegrades and volatilizes with a half-life ranging from 3-9h for a typical river (Toxnet Hazardous Substance Data Bank). Once released into soil or ground, it may leach into groundwater, but would also be expected to rapidly biodegrade and evaporate. As for its release in the atmosphere, it degrades within hours by reacting with hydroxyl radicals and photolysis (Toxnet Hazardous Substance Data Bank). After all, acetaldehyde has a boiling point of 21°C, or room temperature.

2,3-dimethyl-1,3-butadiene (diisopropenyl; DMBD) is used as an intermediate in the synthesis of many organic and industrial chemicals (Chiang et al., 1998; Mikhael et al., 1996) (Figure 1-9). Not much is known about its toxicity to biological systems or its ecotoxicological impact. A close relative of 2,3-dimethyl-1,3-butadiene is the shorter 1,3-butadiene (BD). It is a well-known hazardous air pollutant and carcinogen (Doyle et al., 2004). Of the top 50 most produced chemicals within the United States, BD ranks 36th with levels reaching 3000 tonnes per year; the chemical is mainly used for the synthesis of polymers (International Agency for Research on Cancer, 1992; Occupational Safety and Health Administration, 2002). It also ranks within the top 33 in the US Toxic Release Inventory (Doyle et al., 2004; US EPA, 2001). Although forest fires are the only natural source of BD production, many anthropogenic sources include exhaust emissions, cooking oils, and cigarette smoke (Doyle et al., 2004; Hughes et al., 2001; International Agency for Research on Cancer, 1992). BD is a photochemically-active chemical that reacts in the atmosphere with hydroxyl and other radicals to produce a multitude of known respiratory irritants. These photochemical degradation products include acrolein, formaldehyde, acetaldehyde, butadiene monoxide, CO, CO_2, ozone, glycolaldehyde, and malonaldehyde (Doyle et al., 2004). Another close relative of 2,3-dimethyl-1,3-butadiene is isoprene. It is thought that the three mentioned dienes (1,3-butadiene, 2,3-dimethyl-1,3-butadiene, and isoprene) all follow similar reaction pathways (Skov et al., 1992).

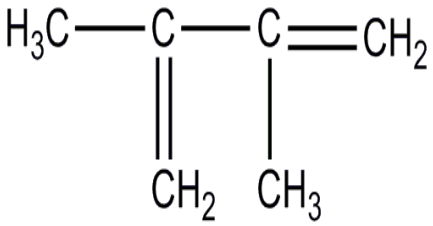

Figure 1-9: Molecular structure of 2,3-dimethyl-1,3-butadiene.

Accordingly, one can conclude that 2,3-dimethyl-1,3-butadiene's environmental concern stems from its ability to generate hazardous photochemical degradation products in the atmosphere.

1.4 Selection of reference chemical and established databases

The toxicity of the chosen reactive chemicals has to be compared with a control chemical. For this, sodium dodecyl sulphate (SDS) has been selected as a positive control for several reasons. Its high solubility (log*P* of 1.60) eliminates the need of using a special solvent; serial dilutions can thus be prepared using the exposure medium itself. It is also of low volatility (logHLC of -6.74) and so loss of the compound to evaporation is of no concern. The mode of action of SDS, cell membrane damage, allows for easy observation using simple phase-contrast microscopy. Accordingly, SDS toxicity can be easily measured using any nonspecific bioassay (Schirmer et al., 2008). An additional advantage is that SDS itself is also of environmental concern as it is heavily used in many industrial processes as an anionic surfactant (Cserhati et al., 2002).

In order to evaluate the effectiveness of the modified *in vitro* approach, experimental EC_{50}s for each chemical will be compared with 1) previously recorded *in vitro* EC_{50}s and 2) previously recorded *in vivo* LC_{50}s. For acute fish lethality data, the US EPA fathead minnow database (http://www.epa.gov/med/Prods Pubs/fathead minnow.htm) was chosen as it was found to be the most comprehensive and appropriate. Listed LC_{50} values are all based on flow-through exposures of fathead minnow to analytically-determined chemical concentrations for 96h. As for cell line data, the Halle Registry of Cytotoxicity is the chosen database (Halle, 2003). A limitation of this database, however, is that its listed chemical

EC_{50}s are all based on mammalian (mouse, rat, human) cell lines that had been assayed by various endpoints (Schirmer et al., 2008).

1.5 Physicochemical properties and cytotoxicity data of selected chemicals

As a general rule, the uptake of xenobiotics from the aquatic environment into biological membranes requires these chemicals to be available in a dissolved form in the surrounding water. But many environmental factors influence the availability, transport, and eventual uptake of these chemicals by aquatic organisms (Rand, 1995). Table 1-1 summarizes key parameters that are of ecotoxicological relevance along with Halle *in vitro* and fathead minnow *in vivo* cytotoxicity data and is followed by a detailed discussion.

Table 1-1: Physicochemical properties and cytotoxicity data of selected chemicals

Chemical	Log*P**	LogHLC* (atm-m^3/mol)	LogKoc*	LogBCF* (L/kg wet-wt)	Halle EC_{50}[1] (µM)	FHM LC_{50}[2] (µM)
Sodium dodecyl sulphate	1.60	-6.74**	1.97	1.85	230.9	23.4
Menadione	2.21	-8.51	3.04	0.53	7.94	0.65
Dichlorophene	4.34	-11.54	3.62	2.48	8.32	1.15
Hexachlorophene	6.92	-12.07	5.57	3.86	7.94	0.051
4-fluoroaniline	1.28	-5.65	1.54	0.43	unknown	151.36
Allyl alcohol	0.21	-5.25	0.61	0.50	8511.38	5.50
Acrolein	0.19	-4.45	0.69	0.50	46.77	0.36
Acetaldehyde	-0.17	-4.17	0.51	0.50	2454.7	691.8
2,3-dimethyl-1,3-butadiene	3.13	-0.72	2.72	1.73	unknown	83.2

* Calculated with EPI Suite http://www.epa.gov/oppt/exposure/pubs/episuite.htm.

** Taken from PhysProp database http://www.syrres.com/esc/physdemo.htm

[1] Halle *in vitro* database summarizes toxicity tests with mammalian cell lines using various endpoints and exposure conditions. EC_{50} = effective concentration of test chemical that reduces cellular functions by 50%.

[2] Fathead minnow (FHM) *in vivo* database summarizes toxicity tests using fathead minnow. LC_{50} = concentration causing death in 50% of fish after 96h.

Log*P* (logKow): Hydrophobic chemicals can easily pass through cellular lipid membranes whereas those hydrophilic require active uptake processes. The log*P* parameter is a function of hydrophobicity: it measures the extent of a chemical's ability to partition between water and octanol at equilibrium. Octanol is used as a surrogate for lipids (fats). The greater the log*P* of a chemical, the more likely it is to partition to octanol and thus it is more hydrophobic. Log*P* is also used to predict a chemical's ability to bioaccumulate in aquatic organisms. The US EPA uses the following log*P* ranges in its prediction of a chemical's tendency to bioaccumulate:

< 2.7 = Low
2.7 – 3 = Moderate
> 3 = High

LogKoc: Although hydrophobic chemicals can diffuse easily through lipid membranes, their actual availability in water is usually limited due to their tendency to adsorb to suspended solids and sediments. The parameter logKoc measures the extent of a chemical's ability to partition between organic carbon and water at equilibrium. The higher the logKoc of a chemical, the more likely it is to adsorb to solids and sediments than remain in water. According to the National Technical Information Service (Review of Exposure Assessment Guidelines, September 1996), a chemical's potential to adsorb to soil particles can be predicted using the following logKoc ranges:

0-2 = low
> 2-4 = moderate
> 4-7 = high

LogHLC: The availability of chemicals in aqueous media is also influenced by their ability to volatilize out of solution. The parameter logHLC (Henry's law Constant) measures the extent of a chemical's ability to partition between air and water at equilibrium. The higher the logHLC of a chemical, the more likely it is to volatilize than remain in water. According to the National Technical Information Service (Review of Exposure Assessment Guidelines, September 1996), logHLCs in atm-m^3/mol can be interpreted as follows:

< -6.5	= non-volatile
-6.5 – -5	= slow volatilization
>-5 – -3	= significant volatilization
> -3	= rapid volatilization

LogBCF: Each of the above three parameters essentially influences a chemical's availability for uptake by aquatic organisms. Specifically, logBCF (bioconcentration factor) measures a chemical's ability to partition between an organism (organic phase) and water (aqueous phase). The higher the logBCF of a chemical, the more likely it is to bioconcentrate in organisms' tissues and thus bioaccumulate and eventually biomagnify up the food chains. The following logBCF threshold ranges are used by the US EPA to predict a chemical's potential to bioconcentrate in organisms:

< 2	= Low
2 – 3.7	= Threshold for concern
> 3.7	= High

1.6 Objectives

The objectives of my thesis are the following:

1) Do reactive chemicals posses any unique problems for *in vitro* toxicology? In other words, do reactive chemicals have their own special concerns, or do they share the same problems posed by other classes of environmental chemicals?

2) How repeatable is our proposed *in vitro* approach, and how well can it predict *in vivo* toxicity? Can we reliably use it to investigate the cytotoxicity of reactive chemicals?

These objectives will be explored by manipulating some of the basic, yet essential techniques in *in vitro* toxicology, with special emphasis on the following:

a) Cell line selection

b) Selection of exposure medium

c) Method of dosing

d) Selection of cell viability endpoints

Chapter 2
MATERIALS AND METHODS

2.1 Material for cell culture

The following were purchased from BD Falcon, NJ, USA: 75cm^2 tissue culture flasks (catalogue no. 353110), and both 15mL (352096) and 50mL (352070) centrifuge tubes.

Purchased from Sigma-Aldrich in Oakville, ON: Leibovitz's L-15 media (L-15), Fetal Bovine Serum (FBS), penicillin-streptomycin solution, tissue-culture grade water, Dulbecco's Phosphate Buffered Saline (DPBS), trypsin from bovine pancreas, all L-15/ex salts, pyruvate, galactose, dimethyl sulfoxide (purity of ≥99.9%; catalogue no. D8418), and neutral red solution (3.3g/L in DPBS; N2889). Alamar blue (DAL1100) and CFDA-AM (C1354) were purchased from Molecular Probes, Eugene, OR, USA. TrypLE (12604021) was purchased from Invitrogen Corporation, Carlsbad, California, USA. The 24-well microplates (3527), CellBIND plates, and plate sealants (6575) were purchased from Corning Life Sciences (Costar®), NY, USA.

2.2 Reactive chemicals and sodium dodecyl sulphate

All of the following chemicals were purchased from Sigma-Aldrich in Oakville, ON: sodium dodecyl sulphate (purity of ≥99.0%; catalogue #71725), menadione (98%; M57405), dichlorophene (95%; 133221), hexachlorophene (Pestanal; 45526), 4-fluoroaniline (≥99%; F3800), allyl alcohol (≥99%; 240532), acrolein (Supleco; 5S06230), acetaldehyde (99.5%; ≥402788), and 2,3-dimethyl-1,3-butadiene (≥98%, containing 100ppm butylated hydroxytoluene as inhibitor of polymerization; 145491).

2.3 Cell lines and maintenance of cells in culture

RTgill-W1 was initiated from the gill epithelium of rainbow trout (*Oncorhynchus mykiss*) (Bols et al., 1994). It is available from the American Type Culture Collection (ATCC #CRL2523). RTL-W1 was initiated from the livers of rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 1993).

Cultures of both RTgill-W1 and RTL-W1 were routinely maintained in 75cm^2 tissue-culture treated flasks. The cultures were left to grow for 7-10 days at room temperature in L-15 medium supplemented with 10% FBS (v/v) and 1% penicillin-streptomycin solution (v/v). Once confluent, defined by a cell monolayer covering 80-90% of the flask, the cultures were passaged (1:2) into new flasks as described in Dayeh et al., 2003. Flasks were routinely checked for contamination using an inverted phase contrast microscope (Nikon Eclipse TS100, Nikon Instruments Inc., Melville, NY). Briefly, under sterile conditions, the old medium was aspirated out and replaced by 2mL of trypLE and placed on an orbital shaker for 5-7 minutes to allow for cell dissociation. The addition of 6mL of complete L-15 medium arrested the enzymatic action of TrypLE. Cell clumps were loosened by gently pipetting the solution in and out several times. The cell suspension was then transferred to a 15mL eppendorf tube and centrifuged at 440 relative centrifugal force (RCF) units for five minutes. Fresh complete medium (6 mL) was added to each of the old flask and a new flask. After centrifugation, the supernatant was replaced with 8mL of complete medium and gently pipetted to resuspend the cells. The cell suspension was equally divided into the two flasks that were labelled and stored at room temperature for later use.

2.4 Methodology of the *in vitro* assay

The endpoint of cell viability as measured with three cytotoxicity assays were conducted on RTgill-W1 and RTL-W1. The following is a general outline of the common procedures involved in performing the assay (Figure 2-1). Cells were plated in 24-well microplates (Corning Life Sciences, NY) at a density of 1.5 x 10^5 cells/well in 1mL L-15 medium supplemented with 10% FBS and 1% Pen/Strep solutions. The cells were allowed to attach and form a monolayer for 24h at room temperature. Afterwards, the media was removed by inverting the plate over a catch basin and subsequently blotted on paper towel. The plates were then either directly or indirectly dosed with various concentrations of the reactive or reference chemicals.

For direct dosing, the plates were washed with 1mL of L-15/ex per well, which was then removed by inversion. After which each well received 2mL of fresh L-15/ex to which 10 µL of a 200x concentrated stock solutions of the reactive toxicants (or control chemical) were added to achieve the final desired concentration. For indirect dosing, the plates were also washed with 1mL of L-15/ex per well, which was then removed by inversion. But each well then received 2mL of L-15ex that already contained the chemical at its desired final concentration.

With the exception of the control chemical, DMSO was used as the solvent always at a final concentration in the exposure medium of 0.05% (v/v). After 24h of exposure at room temperature and in the dark, cells were assayed using a battery of three fluorescent indicator dyes. Alamar blue and CFDA-AM were used concurrently and prepared in PBS (total volume of 400 µL/well) to give final concentrations of 5% (v/v) and 4 µM, respectively. After the AB/CFDA-AM exposure the cells were then treated with NR also prepared in PBS

(total volume of 400 µL/well) at a final concentration of 1.5% (v/v). Cells were incubated in the AB/CFDA-AM or the NR solution for 1h in the dark at room temperature after which fluorescence was quantified using a SPECTRAmax® GEMINI XS microplate reader (Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths for AB were 530nm and 595nm, 485nm and 530nm for CFDA-AM, and 530nm and 645nm for NR, respectively. Fluorescence was also quantified using the CytoFluor Series 4000 microplate reader (PerSeptive Biosystems, Burlington, ON, Canada).

Variations of the above general outline include: the use of L-15/ex as the solvent, extending the duration of exposure of cells to toxicants as needed, the sealing of plates with specialized sealants after the addition of test chemicals, and/or the use of microplates coated with alternative surfaces.

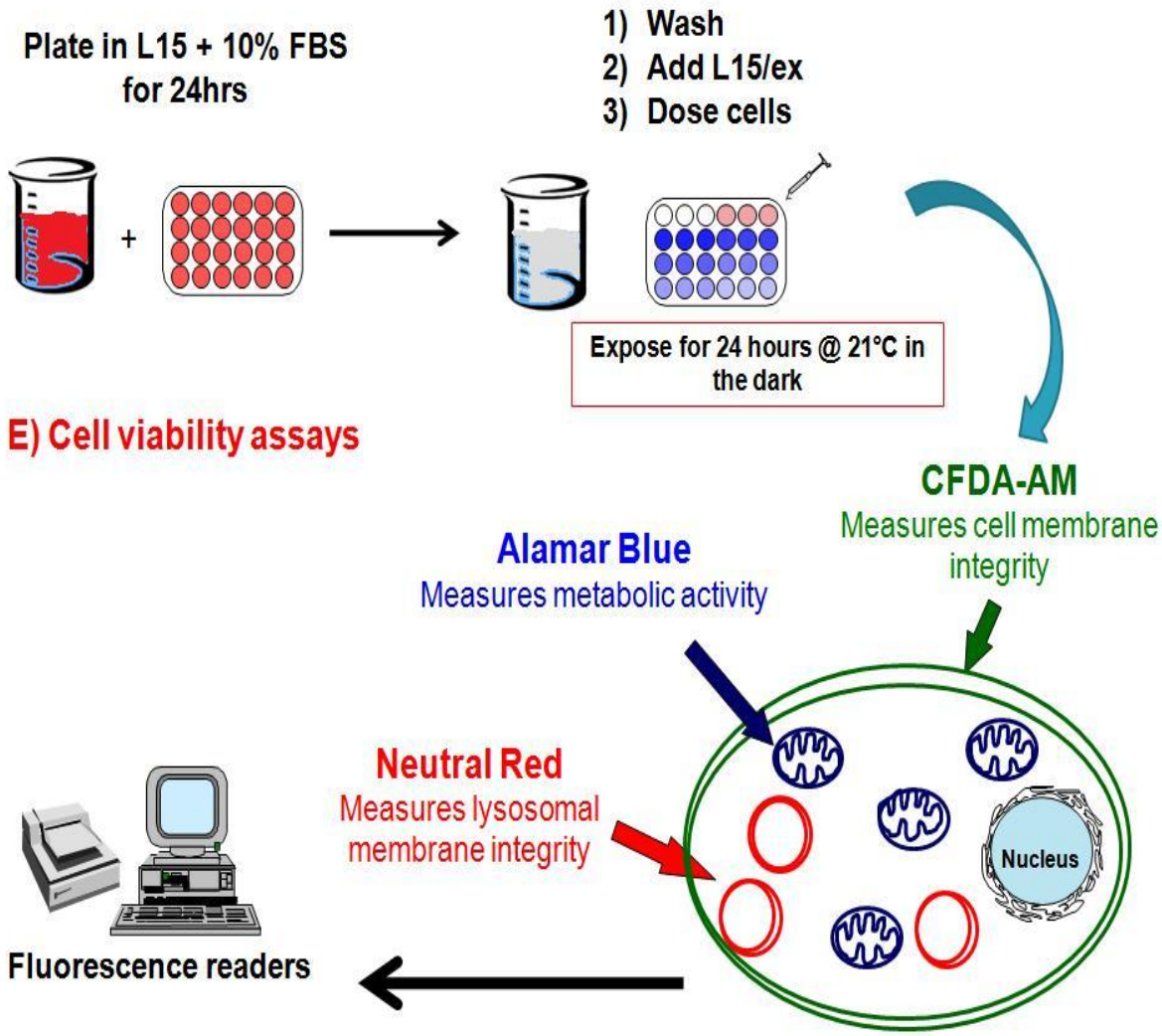

Figure 2-1: Schematic representation of the cytotoxicity assay.

RTgill-W1 and RTL-W1-W1 cells at a density of $1.5x10^5$ were plated in 24-well microplates in L-15 complete medium (10% FBS and 1% P/S) for 24h. After which, plates were washed with L-15/ex, which was then replaced with fresh L-15/ex for chemical exposure. Cells were either directly or indirectly dosed with various concentrations of each chemical before their incubation in the dark and at room temperature for 24h (or as desired). After which, cell viability was assayed by each of the three indicator endpoints: alamar Blue, CFDA-AM, and neutral red.

2.5 Analysis of data

The cytotoxicity of each chemical was expressed in terms of its EC_{50}, which is the effective concentration of test agent that reduced the viability of treated cells to 50% of the untreated controls. Raw data was first compiled and analyzed using Microsoft Excel. To account for the background fluorescence of the indicator dyes, raw florescent units (RFUs) from no-cell control plates were subtracted from the treated plates. The corrected RFUs from the treated plates were averaged (three wells per concentration) and then expressed as a percentage of the average fluorescent readings of the DMSO (or solvent) controls. Data are presented as mean EC_{50} ± standard deviation. Unless otherwise noted, each experiment was repeated at least three independent times, where wells were exposed to each chemical concentration (or reference chemical) in triplicates. To test for significance in EC_{50} values between the two cell lines, an unpaired *t*-test was used ($p \leq 0.05$). An Analysis of Variance (ANOVA) was used to compare the three EC_{50} values for each cell line (one for each cell viability endpoint). Significance was set at $p \leq 0.05$. If significance was found, the data were further examined by the Tukey-Kramer multiple comparisons test. All graphs, statistical analyses and EC_{50} values were calculated using GraphPad Prism and GraphPad InStat version 4.00 (GraphPad Software, San Diego, California, USA).

Chapter 3
RESULTS

3.1 Cytotoxicity of reference chemical: sodium dodecyl sulphate (SDS)

SDS was used as a positive control and therefore it was important to first establish its EC_{50} values using both RTgill-W1 and RTL-W1. SDS was cytotoxic to both cell lines in a dose-dependent fashion; an increase in SDS concentration led to a decrease in cell viability as measured by all three endpoints (Figure 3-1). For both cell lines the cell membrane integrity seemed to be the least affected by SDS toxicity as evident by the relatively higher CFDA-AM EC_{50} values (Table 3-1). A one-way ANOVA was performed to test for significance among the three endpoints for each cell line. For RTgill-W1, the CFDA-AM EC_{50} was significantly higher than that of AB and NR ($p<0.05$). The same was true for RTL-W1 where the CFDA-AM EC_{50} was significantly higher than that of AB and NR ($p<0.05$). Overall, RTL-W1 was significantly more sensitive to SDS toxicity than RTgill-W1 as measured by AB ($p<0.05$), CFDA-AM ($p<0.05$), and NR ($p<0.05$). Morphologically, the liver cells appeared to be more sensitive to SDS than the gill cells when exposed to concentrations higher than ~108 µM. The difference in sensitivity was most evident at the highest concentration tested of 216 µM, where the liver cells were completely lysed and disintegrated whereas the gill cells seem to have left some remnants behind. When compared to Halle's EC_{50} database, EC_{50}s for both RTgill-W1 and RTL-W1 cell lines were much closer to the LC_{50} recorded in the FHM database.

The cytotoxicity of SDS using RTL-W1 cells seeded in CellBIND plates was also investigated (Table 3-1). When compared to the EC_{50} values of RTL-W1 seeded in conventional plates, the resulting CellBIND EC_{50} values were significantly higher for AB ($p<0.05$) and NR ($p<0.05$), but not for CFDA-AM ($p>0.05$). The EC_{50}s for the three endpoints were significantly different from each other with the order of sensitivity being NR, AB, and CFDA-AM. All values, however, were still comparable to FHM's LC_{50}.

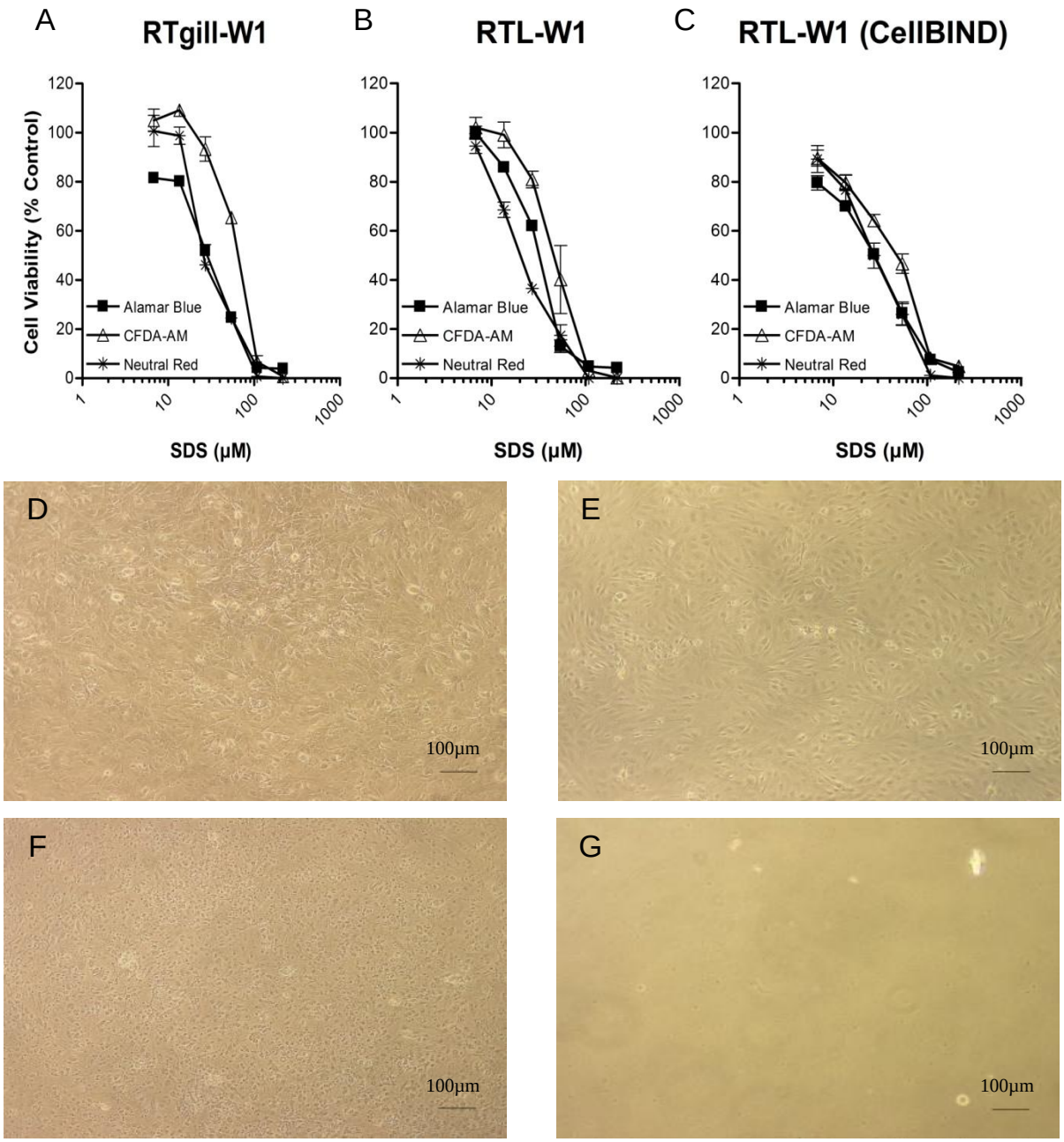

Figure 3-1: Viability of RTgill-W1 and RTL-W1 exposed to sodium dodecyl sulphate (SDS) for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to SDS in L-15/ex for 24h in conventional plates for RTgill-W1 (Panel A), RTL-W1 (B), and RTL-W1 in CellBIND plates (C). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in L-15/ex control wells. Results shown are representative graphs of 3-5 independent experiments. The data points represent the mean of three culture wells with standard deviation. Appearance of control RTgill-W1 (D) and RTL-W1 (E) cells and those exposed to the highest concentration of 216 µM SDS (F and G, respectively) are shown. Cells were photographed with an inverted phase contrast microscope at 100x magnification.

Table 3-1: Comparing the cytotoxicity of sodium dodecyl sulphate as evaluated with RTgill-W1 and RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Cell line	Culture surface	Mean EC_{50}[1] (µM) with standard deviation			Halle EC_{50}[2] (µM)	FHM LC_{50}[3] (µM)
		AB	CFDA-AM	NR		
RTgill-W1 (n=5)	normal	$36.55^{*,a} \pm 4.95$	$64.53^{*,b} \pm 5.37$	$29.67^{*,a} \pm 6.92$		
RTL-W1 (n=5)	normal	$23.82^{**,a} \pm 5.60$	$45.33^{**,b} \pm 12.65$	$18.80^{**,a} \pm 3.52$	230.9	23.4
RTL-W1 (n=3)	CellBIND	$35.06^{*,a} \pm 2.51$	$52.72^{**,b} \pm 3.14$	$26.86^{*,c} \pm 3.74$		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), neutral red (NR).
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
*EC_{50} means within a column with a different number of superscript * are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05). No * indicates no difference.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05).

3.2 Cytotoxicity of menadione

RTgill-W1 and RTL-W1 were exposed to menadione for 24 hours in microwell culture plates after which cell viability was measured with three fluorescent indicator dyes: AB, CFDA-AM and NR (Figure 3-2 – Panels A & B). Results indicated that menadione was cytotoxic to both cell lines in a dose-dependent fashion; as the concentration of the toxicant increased, cell viability decreased as measured by all three endpoints. No significant difference between the two cell lines was observed ($p>0.05$). There was also no significant difference in EC_{50} values between the three viability endpoints for RTgill-W1 ($p>0.05$). As for RTL-W1, the only difference was for AB being significantly lower than that of CFDA-AM and NR ($p<0.05$).

When compared to LC_{50} *in vivo* FHM database, the experimental EC_{50} values were much closer to the reported *in vivo* value than that *in vitro* value reported in the Halle database (Table 3-2). Morphologically, menadione toxicity was manifested in cultures by causing breaks in cellular monolayers and subsequent detachment of cells into the medium (Figure 3-2 – Panels C & D).

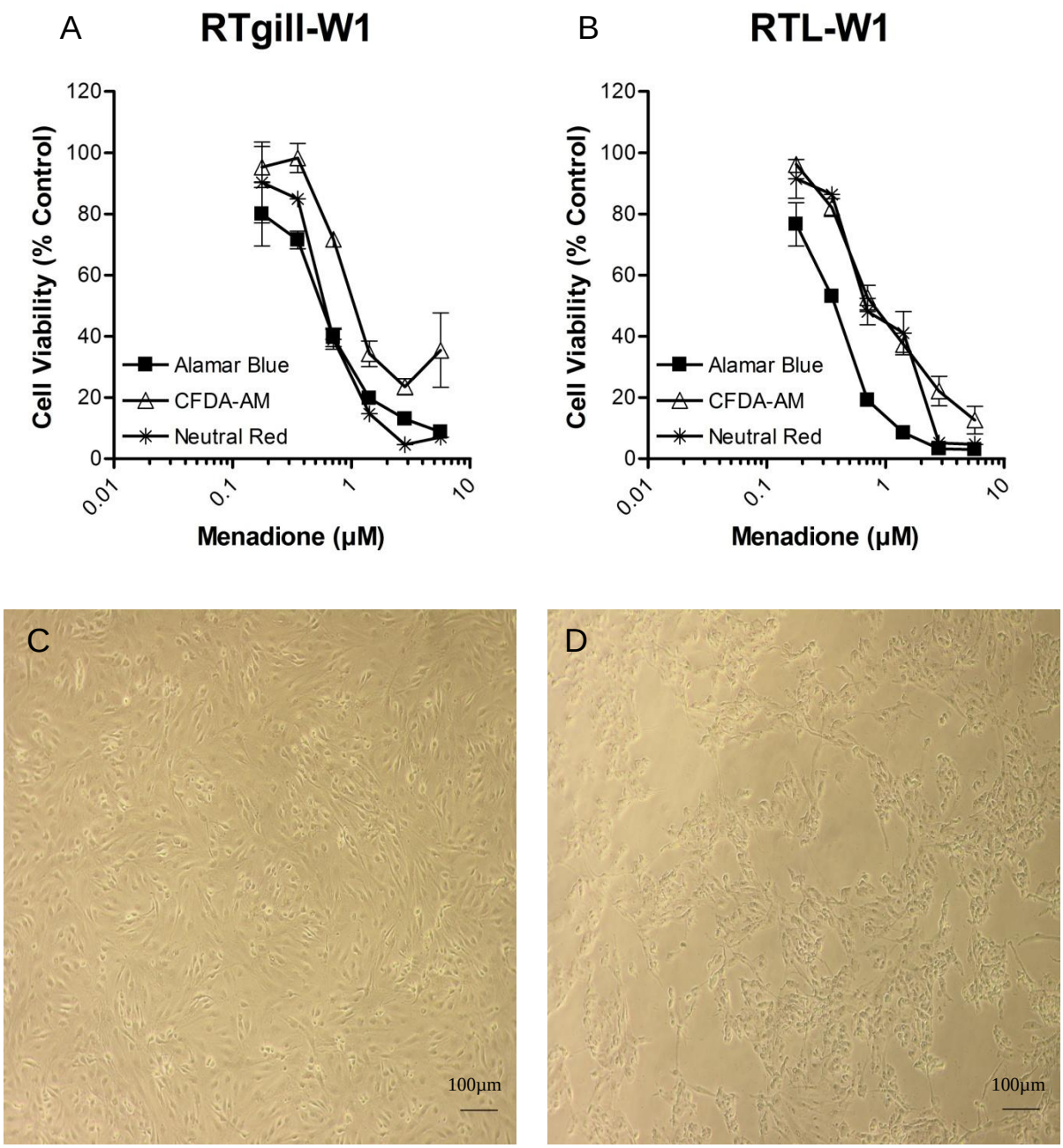

Figure 3-2: Viability of RTgill-W1 and RTL-W1 exposed to menadione for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to menadione in L-15/ex for 24h for both RTgill-W1 (A) and RTL-W1 (B). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of three independent experiments. The data points represent the mean of three culture wells with standard deviation. Appearance of control RTL-W1 cells (C) and those exposed to the highest concentration of 5.5 µM menadione (D) are shown. Cells were photographed with an inverted phase contrast microscope at 100x magnification.

Table 3-2: Comparing the cytotoxicity of menadione as evaluated with RTgill-W1 and RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Cell line	Mean EC_{50}[1] (µM) with standard deviation			Halle EC_{50}[2]	FHM
	AB	CFDA-AM	NR	(µM)	LC_{50}[3] (µM)
RTgill-W1 (n=3)	$0.58^{a} \pm 0.12$	$0.71^{a} \pm 0.17$	$0.59^{a} \pm 0.13$		
				7.94	0.65
RTL-W1 (n=3)	$0.35^{a} \pm 0.10$	$0.63^{b} \pm 0.07$	$0.81^{b} \pm 0.06$		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), neutral red (NR).
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints.
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test ($p < 0.05$).
No EC_{50} means within a column were significantly different from one another.

3.3 Cytotoxicity of dichlorophene

The cytotoxicity of dichlorophene was examined using both direct and indirect dosing. For both dosing methods results indicated that dichlorophene was cytotoxic to both RTgill-W1 and RTL-W1 cell lines in a dose-dependent fashion; as the concentration of the toxicant increased, cell viability decreased as measured by all three endpoints (Figure 3-3). For direct dosing, there was no significant difference ($p>0.05$) in toxicity between the two cell lines as measured by all three endpoints (Welch correction applied for CFDA-AM); the same is true for the indirect dosing of dichlorophene (Table 3-3). One-way ANOVA tests were conducted to test for significance between the three endpoints for each cell line and dosing condition. The only difference was for the indirect dosing of dichlorophene, where the resulting CFDA-AM EC_{50} values were significantly higher than that of AB and NR ($p<0.05$). Experimental EC_{50}s were much closer to the LC_{50} value for FHM than the EC_{50} value in the Halle database. This was true for both direct and indirect dosing methods (Table 3-3).

One-way ANOVA tests were also conducted to analyze the effect of direct versus indirect dosing on the cytotoxicity of dichlorophene. For RTgill-W1, there was no significant difference between the two dosing conditions ($p>0.05$). As for RTL-W1, the direct dosing of dichlorophene led to significantly lower EC_{50} values as measured by all three endpoints ($p<0.05$). Note that Welch correction was applied where appropriate.

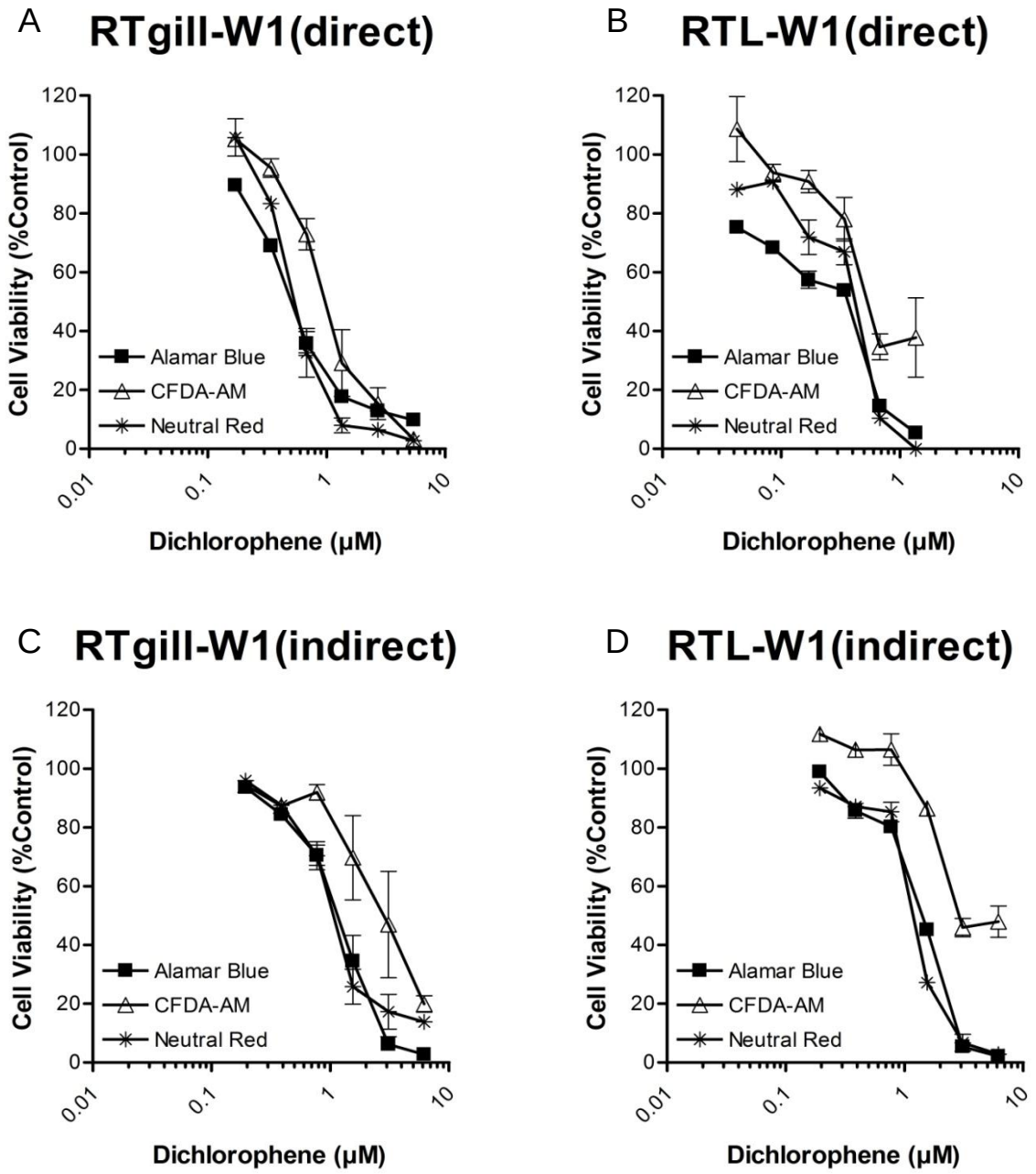

Figure 3-3: Viability of RTgill-W1 and RTL-W1 exposed to dichlorophene, either directly or indirectly, for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to dichlorophene in L-15/ex for 24h either directly for both RTgill-W1 (A) and RTL-W1 (B), or indirectly (C & D, respectively). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of three independent experiments. The data points represent the mean of three culture wells with standard deviation

Table 3-3: Comparing the cytotoxicity of dichlorophene as evaluated with RTgill-W1 and RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Cell line	Dosing method	Mean EC_{50}[1] (µM) with standard deviation			Halle EC_{50}[2] (µM)	FHM LC_{50}[3] (µM)
		AB	CFDA-AM	NR		
RTgill-W1 (n=3)	Direct	$0.52^{*,a} \pm 0.11$	$0.86^{*,a} \pm 0.39$	$0.52^{*,a} \pm 0.11$		
RTL-W1 (n=3)	Direct	$0.47^{*,a} \pm 0.06$	$0.28^{*,a} \pm 0.08$	$0.59^{*,a} \pm 0.26$		
					8.32	1.15
RTgill-W1 (n=3)	Indirect	$1.17^{a} \pm 0.47$	$5.22^{b} \pm 2.38$	$1.30^{a} \pm 0.44$		
RTL-W1 (n=3)	Indirect	$1.53^{**,a} \pm 0.23$	$7.85^{**,b} \pm 3.81$	$1.53^{**,a} \pm 0.47$		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), neutral red (NR).
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints.
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
*EC_{50} means within a column with a different number of superscript * are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05). No * indicates no difference.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05).

3.4 Cytotoxicity of hexachlorophene

Results indicated that hexachlorophene decreased the viability of RTgill-W1 and RTL-W1 in a dose-dependent fashion as measured by both AB and NR (Figure 3-4). There was no significant difference between the two cell lines in their response to hexachlorophene toxicity as measured by both AB and NR (p>0.05). There was also no significant difference between the AB and NR EC_{50} values for each of the two cell lines (p>0.05; Table 3-4). The drop in cell viability as measured by CFDA-AM was much less pronounced in both cell lines to the extent that associated EC_{50}s could not be reliably calculated (Figure 3-4). Note that due to the non-sigmoidal shape of the viability curves, constraints were used in the EC_{50} calculations so that the maximum (top) response is defined as 100% and the minimum (bottom) response is defined as 0%. Both cell lines however led to EC_{50}s that are much closer to the LC_{50} for FHM than those in the Halle database (Table 3-4).

For both cell lines, the morphology of cells exposed to the highest concentration of hexachlorophene (0.19 µM) for 24h was comparable to the morphology of control cells. The cells in both conditions appeared healthy and each monolayer in the well was confluent and intact without signs of detachment or structural alterations (Figure 3-4, Panels C & D).

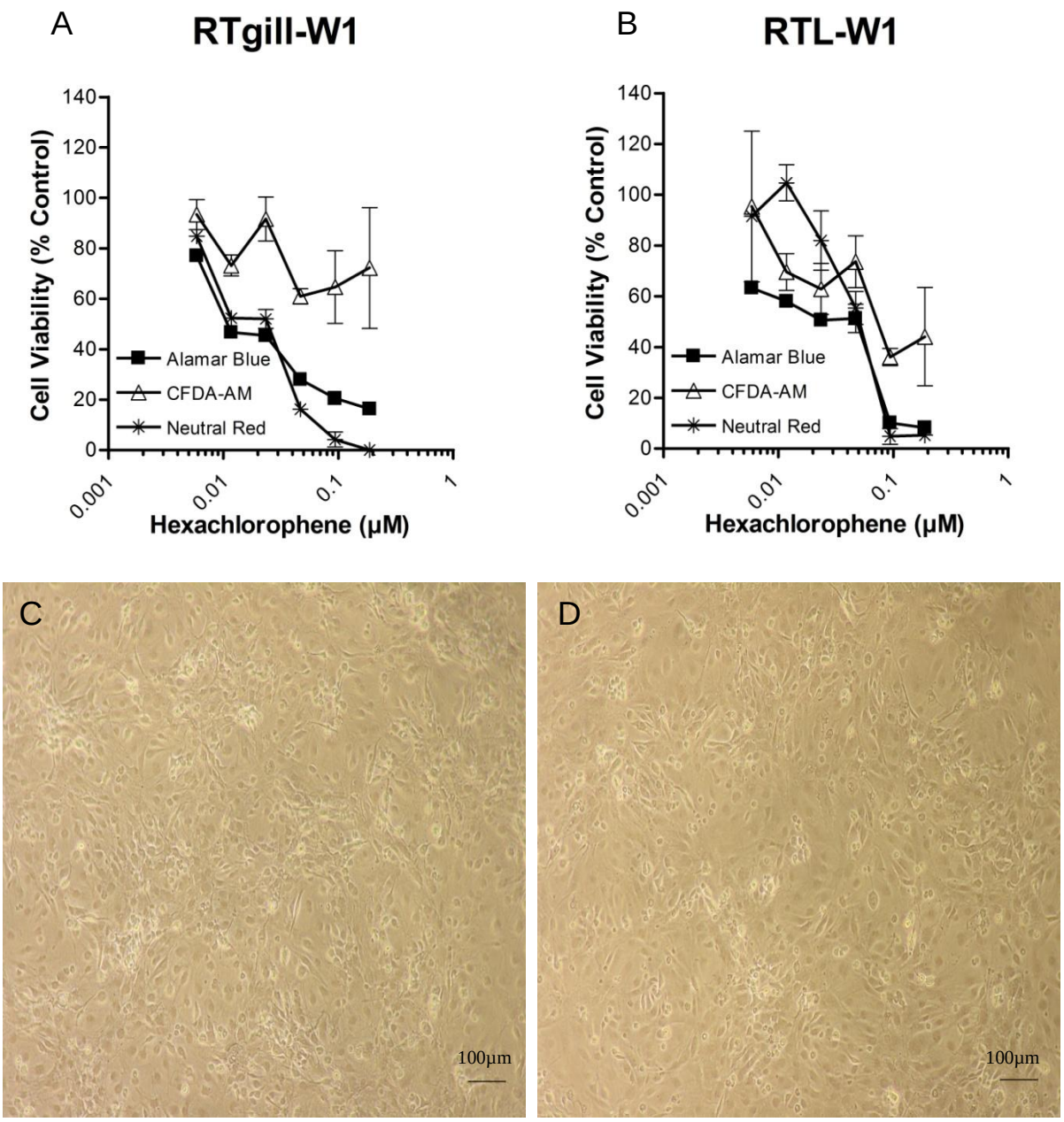

Figure 3-4: Viability of RTgill-W1 and RTL-W1 exposed to hexachlorophene for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to hexachlorophene in L-15/ex for 24h for both RTgill-W1 (A) and RTL-W1 (B). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of two or three independent experiments. The data points represent the mean of three culture wells with standard deviation. Appearance of control RTL-W1 cells (C) and those exposed to the highest concentration of 0.19 µM hexachlorophene (D) are shown. Cells were photographed with an inverted phase contrast microscope at 100x magnification.

Table 3-4:Comparing the cytotoxicity of hexachlorophene as evaluated with RTgill-W1 and RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Cell line	Mean EC_{50}[1] (µM) with standard deviation			Halle EC_{50}[2]	FHM LC_{50}[3]
	AB	CFDA-AM	NR	(µM)	(µM)
RTgill-W1 (n=2)	$0.033^{a} \pm 0.024$	not calculable	$0.026^{a} \pm 0.013$	7.94	0.051
RTL-W1 (n=3)	$0.093^{a} \pm 0.082$	not calculable	$0.069^{a} \pm 0.027$		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), neutral red (NR).
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints.
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test ($p < 0.05$).
No EC_{50} means within a column were significantly different from one another.

3.5 Cytotoxicity of 4-fluoroaniline

Results indicated that 4-fluoroaniline was cytotoxic to both cell lines in a dose-dependent fashion; an increase in concentration of 4-fluoroaniline led to a decrease in cell viability (Figure 3-5). Initial cytotoxicity assays of this chemical at a concentration range that encompassed the expected LC_{50} value of 151.36 µM (3.125-200 µM) did not lead to any appreciable decrease in cell viability (Figure 3-5, Panel A). In fact, a dose-response curve was not obtained with any of the three dyes and the values for each endpoint hovered around the 100% viability mark (Figure 3-5, Panel A). As the range of concentrations was shifted to higher values in relatively small increments, cell viability also started to decrease in small increments. This was done until cells exposed to the highest concentration of 4-fluoroaniline approached 0% viability. The chemical, however, became insoluble at the higher concentrations so that crystals formed in the medium as illustrated by the arrows in Figure 3-5, Panels B, C, & E. The EC_{50} values were 16-40 times higher the LC_{50} for FHM (Table 3-5).

There was no significant difference between the two cell lines in their response to 4-fluoroaniline toxicity ($p>0.05$). Also, for each of the cell lines, there was no significant difference in viability between the three indicator endpoints ($p>0.05$; Table 3-5).

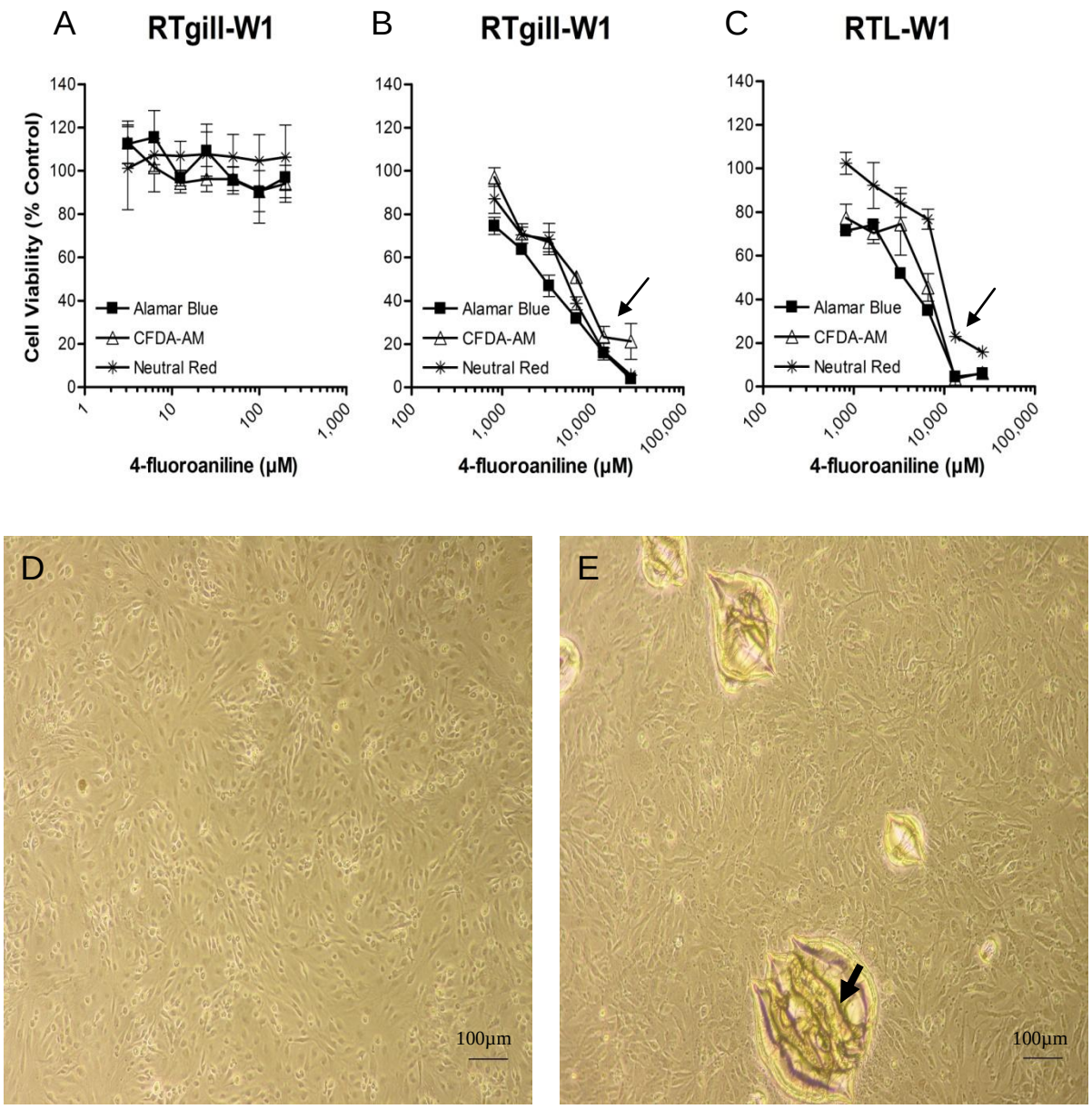

Figure 3-5: Viability of RTgill-W1 and RTL-W1 exposed to 4-fluoroaniline for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to 4-fluoroaniline in L-15/ex for 24h for RTgill-W1 at a lower range of concentrations (A), RTgill-W1 at a higher range of concentrations (B), and RTL-W1 at that same high range of concentrations (C). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of two independent experiments. The data points represent the mean of three culture wells with standard deviation. Appearance of control RTL-W1 cells (D) and those exposed to the second highest concentration of 13,195 µM (E) are shown. Cells were photographed with an inverted phase contrast microscope at 100x magnification. Arrows indicate concentration at which significant chemical crystallization is first observed.

Table 3-5: Comparing the cytotoxicity of 4-fluoroaniline as evaluated with RTgill-W1 and RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Cell line	Mean EC_{50}[1] (µM) with standard deviation			Halle EC_{50}[2]	FHM LC_{50}[3]
	AB	CFDA-AM	NR	(µM)	(µM)
RTgill-W1 (n=2)	3522[a] ± 1744	2763[a] ± 1388	4031[a] ± 3125		
				unknown	151.36
RTL-W1 (n=2)	4712[a] ± 1100	6122[a] ± 861	5937[a] ± 3715		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), and neutral red (NR) after 24h.
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints.
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05).
No EC_{50} means within a column were significantly different from one another.

3.6 Cytotoxicity of allyl alcohol

3.6.1 Exposure to allyl alcohol for 24h

Initial range-finding tests that were based on the expected LC_{50} value for allyl alcohol of 5.495 µM (0.3-9.0 µM) had no effect on RTL-W1 cell viability as measured by all three endpoints (Figure 3-6, Panel A). As the range of tested concentrations was shifted to higher values in relatively small increments, cell viability started to decrease also in small increments. This was done until cells exposed to the highest concentration of allyl alcohol approached 0% viability or until pure allyl alcohol was used as the highest concentration (73,345 µM). Even at the highest concentration tested, all three viability curves for RTgill-W1 hovered around the 100% viability mark at the end of the 24h incubation period; thus, EC_{50}s could not be calculated for RTgill-W1 (Figure 3-6, Panel B). In contrast, RTL-W1 was responsive to allyl alcohol (Figure 3-6, Panel C), but the resulting EC_{50}s were many orders of magnitude (365-1500 times) higher than the LC_{50} for FHM. There was also no significant difference between the three viability endpoints ($p>0.05$) in their response to the toxicity of allyl alcohol (Table 3-6). Nonetheless, they were still lower than the EC_{50} reported in the Halle database (Table 3-6).

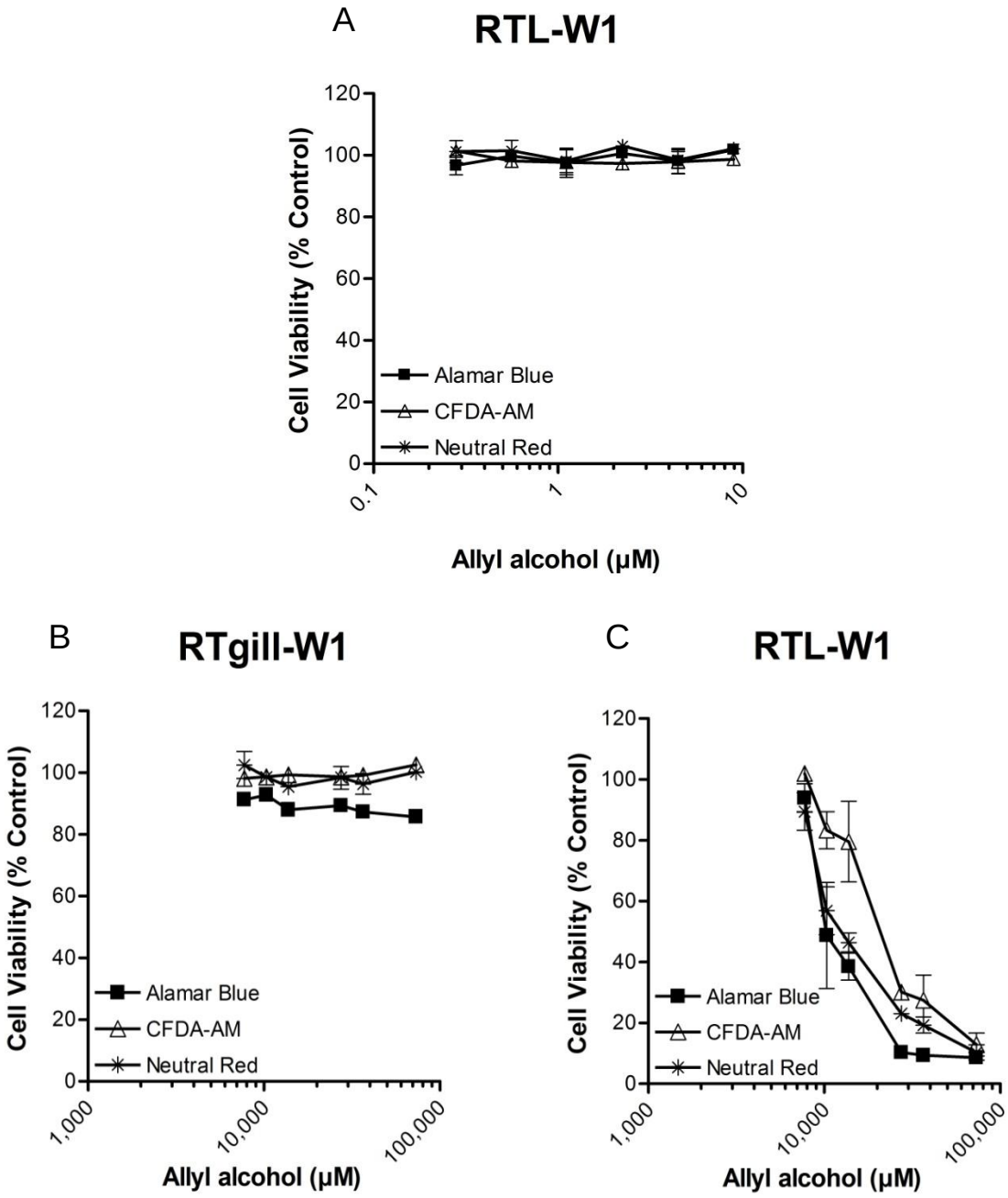

Figure 3-6: Viability of RTgill-W1 and RTL-W1 exposed to allyl alcohol for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to allyl alcohol in L-15/ex at lower concentrations for RTL-W1 (A), or at higher concentrations (7,735-73,345 µM) for both RTgill-W1 (B) and RTL-W1 (C). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of two independent experiments. The data points represent the mean of three culture wells with standard deviation.

Table 3-6: Comparing the cytotoxicity of allyl alcohol as evaluated with RTgill-W1 and RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Cell line	Mean EC_{50}[1] (µM) with standard deviation			Halle	FHM LC_{50}[3]
	AB	CFDA-AM	NR	EC_{50}[2] (µM)	(µM)
RTgill-W1 (n=2)	Not calculable	Not calculable	Not calculable		
				8511.38	5.495
RTL-W1 (n=2)	$1968^{a} \pm 1135$	$8538^{a} \pm 12018$	$2976^{a} \pm 3766$		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), neutral red (NR).
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints.
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test ($p < 0.05$).

3.6.2 Further exploring the cytotoxicity of allyl alcohol on RTgill-W1

In an attempt to further investigate the cytotoxicity of allyl alcohol on the gill cells, further trials were conducted so that the exposure period was lengthened to both 48 and 120h. The 48h exposure to RTgill-W1 caused a sharp drop in the viability of cells exposed mainly to the highest concentration of allyl alcohol (73,345 µM) as measured by all three endpoints (Figure 3-7, Panel A). As seen in Figure 3-7, Panels B & C, the decrease in viability was due to the detachment of cells from their underlying substratum. For 120h of exposure, the drop in viability as measured by all three endpoints was relatively more pronounced (Figure 3-7, Panel D). A significant number of cells were detached and floating in the medium at the higher concentrations, including, albeit to a less extent, some in the control wells (Figure 3-7, Panels E & F).

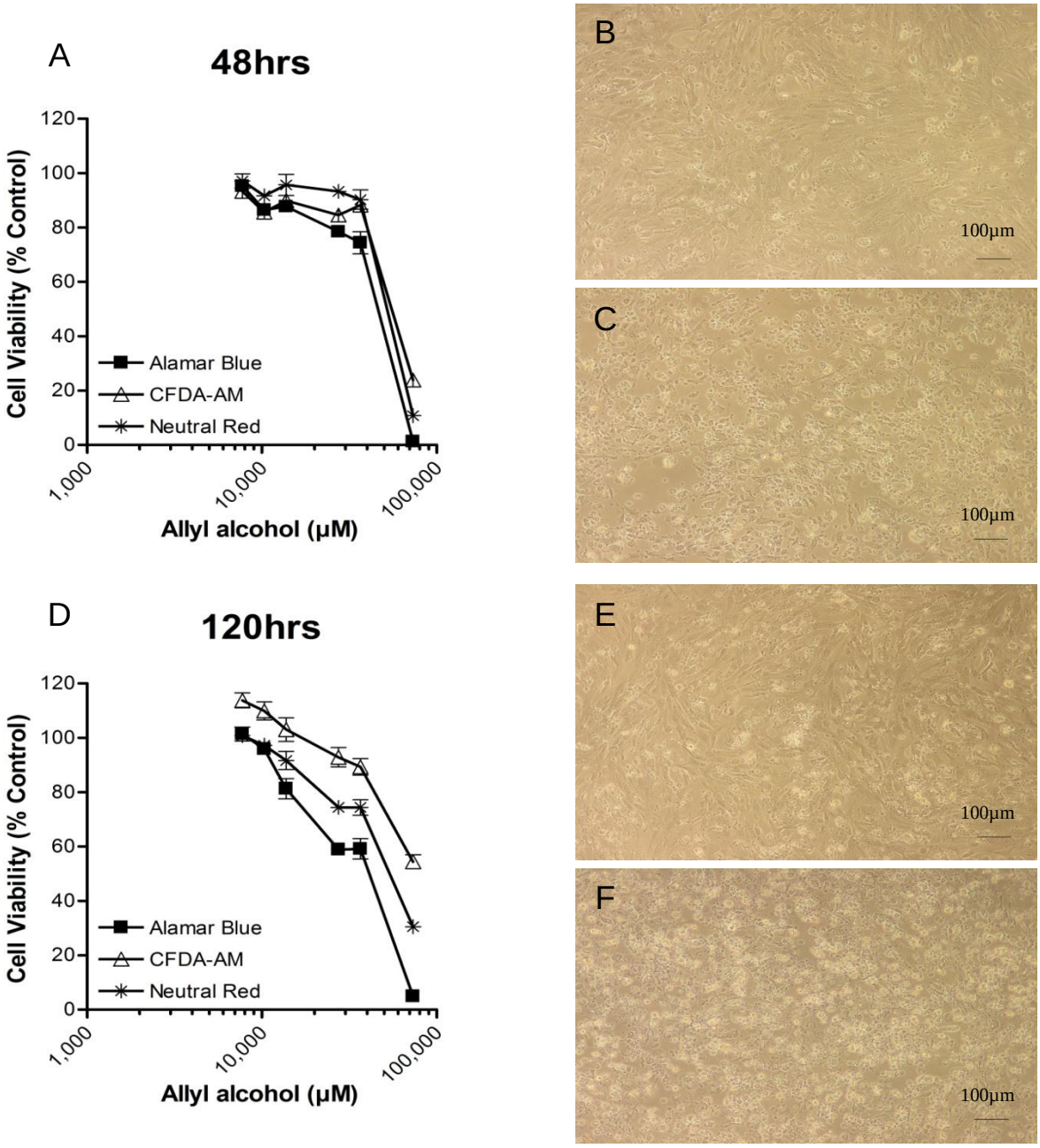

Figure 3-7: Viability of RTgill-W1 exposed to allyl alcohol for both 48 and 120h in L-15/ex.

RTgill-W1 cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to allyl alcohol in L-15/ex for 48h (A), or 120h (D). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of one independent experiment. The data points represent the mean of three culture wells with standard deviation. The diagrams at the top are for the appearance of control RTgill-W1 cells (B) and those exposed to the highest concentration of 73,345 µM allyl alcohol for 48h (C). The diagrams at the bottom are for the appearance of control RTgill-W1 cells (E) and those exposed to the highest concentration of 73,345 µM allyl alcohol for 120h (F). Cells were photographed with an inverted phase contrast microscope at 100x magnification.

3.7 Cytotoxicity of acrolein

Initial range-finding tests using acrolein at concentrations between 50-100 µM decreased RTL-W1 cell viability, but not in a defined pattern as cells exposed to all chemical concentrations resulted in low RFUs that were comparable to those of the controls (Figure 3-8, Panel A). Morphological assessment of cells using a phase-contrast microscope showed that cells from all chemical concentrations, including those of solvent controls, were either severely impaired or dead to a comparable extent (Figure 3-8, Panels D & E).

Due to the extent of the impact of acrolein on neighbouring culture wells due its volatility alternative exposure strategies were tested. Separate microwell culture plates, where each concentration was tested in an individual plate, and plate sealers, where a sealing film covered each well, were examined. Exposure of RTL-W1 to acrolein in both sealed and separated culture plates resulted in a decrease of cell viability in a dose-dependent fashion as measured by all three endpoints (Figure 3-8, Panels B & C). The only significant difference between the two experimental conditions was for CFDA-AM being significantly higher in the separate plates condition ($p<0.05$). The sealing of plates did not lead to significant differences between the three viability endpoints ($p>0.05$). The use of separate plates, however, led to a significantly lower NR value than that of AB and CFDA-AM ($p<0.05$; Table 3-7).

Note that due to the non-sigmoidal shape of the viability curves, constraints were used in the EC_{50} calculations so that the maximum (top) response is defined as 100% and the minimum (bottom) response is defined as 0%. Both experimental conditions, however, led to EC_{50}s that are much closer to FHM's LC_{50} than that obtained from the Halle database (Table 3-7).

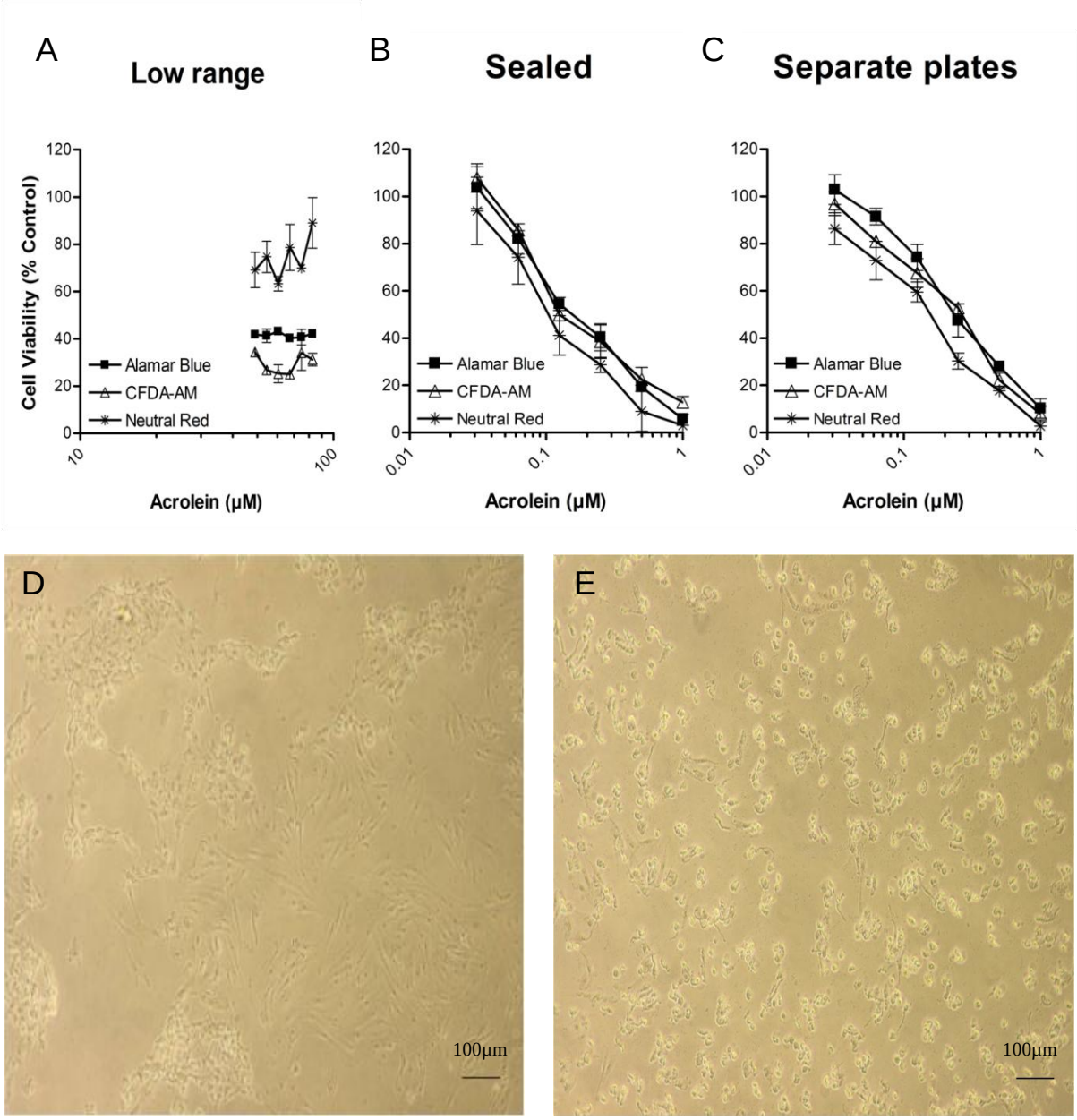

Figure 3-8: Viability of RTL-W1 exposed to acrolein for 24h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to acrolein in L-15/ex at concentrations higher than 50 µM in unsealed plates (A), at lower concentrations in sealed plates (B), and at lower concentrations where a separate plate was used for each concentration (C). The appearance of control RTL-W1 cells (D) and those exposed to the highest concentration of acrolein at 82.69 µM (E) from the initial unsealed-plate trials are shown. Cells were photographed at 100x magnification. Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in DMSO control wells. Results shown are representative graphs of two independent experiments. The data points represent the mean of three culture wells with standard deviation.

Table 3-7: Comparing the cytotoxicity of acrolein as evaluated with RTL-W1 with the toxicity as summarized in Halle and Fathead Minnow databases.

Condition	Mean EC_{50}[1] (µM) with standard deviation			Halle EC_{50}[2] (µM)	FHM LC_{50}[3] (µM)
	AB	CFDA-AM	NR		
Sealed (n=2)	$0.144^{a} \pm 0.042$	$0.165^{*,a} \pm 0.015$	$0.109^{a} \pm 0.013$	46.77	0.36
Separate plates (n=2)	$0.247^{a} \pm 0.007$	$0.229^{**,a} \pm 0.005$	$0.141^{b} \pm 0.005$		

[1]EC_{50} = concentration causing a 50% reduction in cell viability as measured with alamar Blue (AB), carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), and neutral red (NR) after 24h.
[2]Halle database summarizes toxicity tests with mammalian cell lines using various endpoints.
[3]FHM (fathead minnow) database; LC_{50} = concentration causing death in 50% of fish after 96h.
*EC_{50} means within a column with a different number of superscript * are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05). No * indicates no difference.
[a]EC_{50} means within a row with a different superscript letter are statistically different from one another by Tukey-Kramer multiple comparison test (p <0.05).

3.8 Cytotoxicity of acetaldehyde

RTgill-W1 and RTL-W1 cells were exposed to acetaldehyde for 48h in sealed microwell culture plates. Acetaldehyde did not reduce cell viability in either RTgill-W1 or RTL-W1 (Figure 3-9, Panels A & B). Cell viability as measured by all three endpoints remained at DMSO control levels. At the highest concentration of acetaldehyde (2270.3 µM), RTgill-W1 cells were comparable in morphology to those of the control (Figure 3-9, Panels C & D). Both conditions had cell monolayers that were intact without any overt signs of cell shape changes or death.

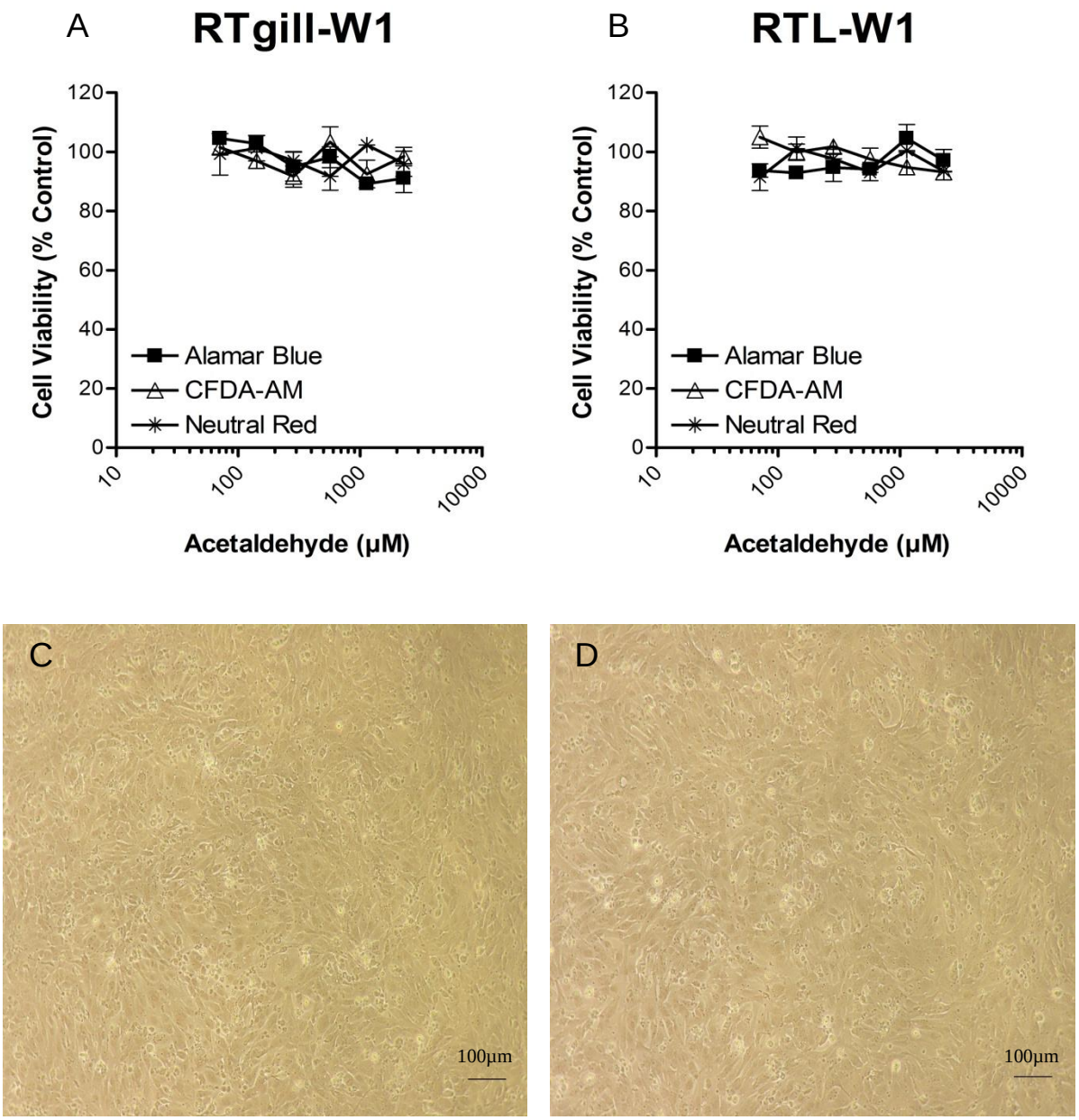

Figure 3-9: Viability of RTgill-W1 and RTL-W1 exposed to acetaldehyde in sealed plates for 48h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to acetaldehyde in L-15/ex in sealed plates for 48h for both RTgill-W1 (A) and RTL-W1 (B). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in L-15/ex control wells. Results shown are representative graphs of one independent experiment. The data points represent the mean of three culture wells with standard deviation. Appearance of control RTgill-W1 cells (C) and those exposed to the highest concentration of 2,270.3 µM acetaldehyde (D) are shown. Cells were photographed with an inverted phase contrast microscope at 100x magnification.

3.9 Cytotoxicity of 2,3-dimethyl-1,3-butadiene

RTgill-W1 and RTL-W1 were exposed to 2,3-dimethyl-1,3-butadiene for 72 hours in sealed microwell culture plates. A 72h exposure to 2,3-dimethyl-1,3-butadiene did not reduce the viability in cultures of either RTgill-W1 or RTL-W1 (Figure 3-10, Panels A & B). Cell viability as measured by all three endpoints was similar to the DMSO control levels. Cells in the highest concentration (109.5 µM) were comparable in morphology to those of the control (Figure 3-10, Panels C & D). Both conditions had cell monolayers that were intact without any overt signs of cell shape changes or death.

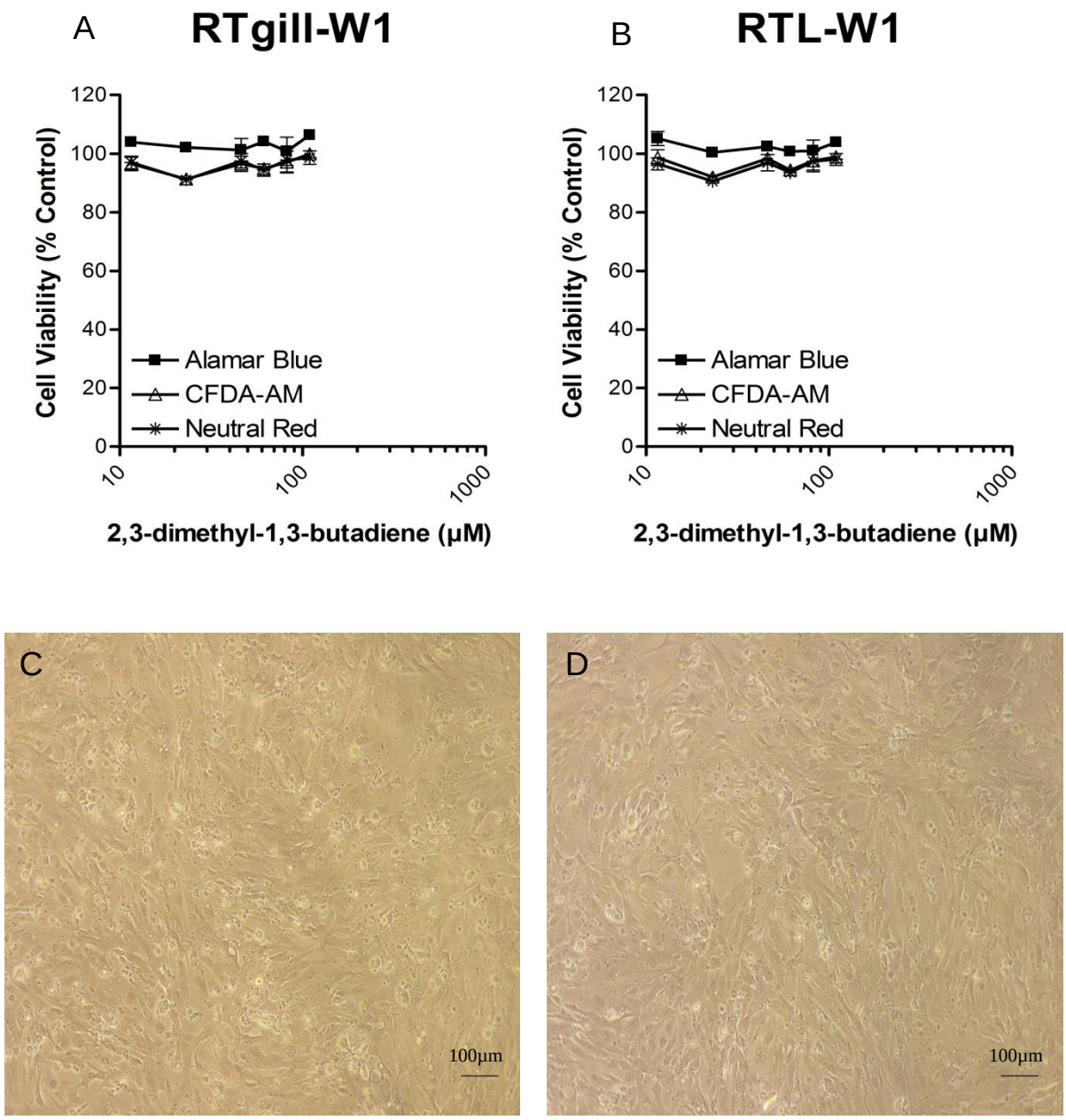

Figure 3-10: Viability of RTgill-W1 and RTL-W1 exposed to 2,3-dimethyl-1,3-butadiene in sealed plates for 72h in L-15/ex.

Cells at a density of $1.5x10^5$ cells per well were seeded into 24-well microplates and incubated for 24h in complete L-15 medium and then exposed to 2,3-dimethyl-1,3-butadiene in L-15/ex in sealed plates for 72h for both RTgill-W1 (A) and RTL-W1 (B). Cell viability was measured with fluorescent indicator dyes: alamar Blue, CFDA-AM, and neutral red. Results were expressed as a percentage of the readings in L-15/ex control wells. Results shown are representative graphs of one independent experiment. The data points represent the mean of three culture wells with standard deviation. Appearance of control RTgill-W1 cells (C) and those exposed to the highest concentration of 109.5 µM 2,3-dimethyl-1,3-butadiene (D). Cells were photographed with an inverted phase contrast microscope at 100x magnification.

Chapter 4
DISCUSSION

Screening chemicals for their toxicity *in vitro* using cells in culture offers many advantages over traditional *in vivo* methods that use whole fish. In this thesis, a total of eight reactive chemicals have been investigated for their cytotoxicity using modified dosing and exposure conditions on the rainbow trout RTgill-W1 and RTL-W1 cell lines. Some of the chemicals were straight forward to analyze, with EC_{50} values being well comparable to previously established *in vivo* LC_{50} values using fathead minnow fish. Challenges, however, were faced when investigating some of the other chemicals. Results were also compared with previously conducted *in vitro* assays by others in the field. It is worth noting at this stage that caution is warranted in our attempt to compare our experimental *in vitro* EC_{50} values with those *in vivo* LC_{50} values from the fathead minnow database. This is because 1) the selected contaminants were evaluated for their cytotoxicity (EC_{50} values) using two rainbow trout cell lines, whereas the LC_{50}s from the FHM database were based on, as the name suggests, whole fathead minnow fish, 2) the default exposure time of each contaminant in our *in vitro* approach was 24h, whereas the FHM database values were based on exposure times of 96h, 3) experimental EC_{50} values were based on nominal concentrations, whereas the FHM database values were based on analytically-determined concentrations, and 4) the cell viability endpoints employed in this thesis were not necessarily reflective of lethality on part of the cells, whereas the FHM database values were based on lethality.

4.1 Reference chemical: sodium dodecyl sulphate

Sodium dodecyl sulphate was cytotoxic to both RTgill-W1 and RTL-W1 in a dose-dependent fashion; an increase in SDS concentration led to a decrease in cell viability as measured by all three endpoints (Figure 3-1). The observation that the liver cells were significantly more sensitive to SDS toxicity than those of the gill can be explained by the suggestion that these liver cells do not secrete as much basal lamina (extracellular matrix proteins) as the gill cells; accordingly, the liver cells would be more likely to let go (detach) of their underlying plastic surfaces during the experimental manipulations and subsequent chemical insult as they do not adhere to them as strongly as the gill cells. After all, the toxicity of SDS is attributed to its disruption of cell membranes and eventual cell lysis. Thus, detachment of RTL-W1 cells from their substratum was likely accelerated due to their weaker ability to attach to it, and that observation can partly explain RTL-W1's higher sensitivity. Another contributing factor to RTL-W1's lower EC_{50}s lies within cell morphology. As seen in figure 3-1 (Panels D-G), liver cells exposed to the highest SDS concentration of 216 µM were completely disintegrated after the 24h exposure period, whereas those of the gill cells died, but left their remnants behind; in other words, the gill cells died on their feet.

To help explain the role of cell detachment (or quantify), cytotoxicity assays of RTL-W1 cells exposed to SDS were conducted using microplates optimized for cell attachment (CellBIND plates). The plates were specially treated by the manufacturers in such a way that increases the oxygen content of a well's polymer surface, which was previously demonstrated to improve hydrophilicity and wettability, both of which are thought to improve cell attachment and spreading (Dupont-Gillain et al., 2000; Van Kooten et al.,

2004). By using these alternative plates, RTL-W1 EC_{50} values were significantly increased for AB and NR so that they approached those of the gill cells; in fact, there was no significant difference between them (Table 3-1). Accordingly, RTL-W1 cells' apparent increase in sensitivity can be primarily attributed to their weaker ability to bind to their substratum.

Nonetheless, calculated EC_{50} values for both cell lines (including those done using CellBIND plates) were much closer to FHM's *in vivo* LC_{50} value than that obtained from the Halle *in vitro* database, suggesting our experimental approach is more reliable and suitable for the cytotoxicity testing of SDS *in vitro* (Table 3-1). Although Halle's averaged EC_{50} is ~10 times higher than FHM's LC_{50}, there are other *in vitro* experiments within the database that achieved comparable *in vitro*/*in vivo* results. For instance, Gueniche & Ponec (1993) exposed human SVK-14 skin cells to SDS. After 24h, cell detachment as measured by the neutral red uptake (NRU) assay led to an EC_{50} of 37.2 µM. Contrastingly, another study by Riddell et al. (1986a) exposed mouse 3T3-L1 cells also to SDS for 24h and used NRU as their endpoint. Their resulting EC_{50}, however, was much higher at 346.7 µM. Therefore, SDS may exhibit differential toxicity to certain cell types, an observation that should be kept in mind.

4.2 Menadione

The mechanism of menadione cytotoxicity to human and rodent cell lines has been intensively studied because of its potential as a chemotherapeutic agent (Calderon et al., 2002). At the most general level, menadione acts in two ways separately or together to cause cell death. The most studied way, and perhaps the most important one, is the disturbance of redox homeostatsis, resulting in oxidative stress. Metabolism of menadione involves redox cycling of the parent quinone molecule and liberates reactive oxygen species (ROS), including hydrogen peroxide (Sata et al., 1997). Secondly, menadione can form adducts with sulfhydryls and primary amines (Grant et al., 1988). The cytotoxicity of menadione depends on the exposure medium. Vitamin C (ascorbate) is an example of a medium component that potentiates redox cycling and cell killing. In this case the redox imbalance and killing is greater for cancer cells (Calderone et al., 2002). Other medium components can actually protect the cells. N-acetylcysteine (NAC) is a precursor of glutathione and blocks killing by menadione (Calderone et al., 2002). The glutathione/glutathione disulfide (GSH/GSSG) system protects cellular thiol groups and redox balance. For example, GSH reduces hydrogen peroxide to water. Menadione normally lowers GSH but NAC prevents this, aiding cell survival.

As for *in vitro* approaches using mammalian cell lines, a couple of studies investigating the effects of oxidative stress vascular injury have exposed human endothelial cells (from HUVEC and IVEC cell lines) to menadione for 24h (Schleger et al., 2004). Viability as assayed by both alamar Blue and ATP content was found to be ~10 µM, which is in agreement with Halle's overall reported value of 7.94 µM. What is common to all of these

studies is the use of exposure media that suppress, to some extent, menadione's ability to redox cycle.

One study investigating the effect of dietary vitamin E on the modulation of menadione-induced oxidative stress using neutral red as an endpoint and DMEM as exposure medium for 48h has reported an EC_{50} of 10 µg/mL (~59.1 µM) using the fathead minnow (previously carp) EPC cell line (George et al., 2000). Interestingly, the addition of 25 µM vitamin E shifted the EC_{50} to >1,000 µg/mL (>5,900 µM), which greatly highlights the role of oxidative stress in menadione's mode of toxic action. Accordingly, much of the improvement achieved in our cytotoxicity testing of menadione can be attributed to the elimination of antioxidants and vitamins from the exposure medium, L-15/ex. No other relevant studies using piscine cell lines have been found in the literature.

The sensitivity and cell death mode of mammalian cells to menadione appears to depend on the dose, exposure time, and cell type. Menadione has been reported to cause either apoptosis, necrosis, or autoschizis, which is a mode of death showing both apoptotic and necrotic characteristics (Verrax et al., 2004). In some cases the menadione concentration clearly dictates the mode of death. With the rat pancreatic acinar cell line, AR4-21, 10-20µM menadione caused apoptosis, but at 100µM cell death was by necrosis (Sata et al., 1997). A similar pattern has been seen with hepatocytes, but the concentrations were different. For hepatocytes, menadione at 50-100µM caused apoptosis, and at 200µM necrosis took place (McConkey et al., 1988).

The rainbow trout cell lines of this study appeared to be more sensitive to menadione than mammalian cell lines. Regardless of the endpoints, the EC_{50} values for RTgill-W1 and RTL-W1 were less than 1µM. For mammalian cells the EC_{50} values are approximately 10µM

(Schleger et al., 2004). In the Halle database, the value is listed at 7.94μM. The most likely explanation for the greater sensitivity of RTgill-W1 and RTL-W1 is that exposure was done in a simple buffered solution. Whereas in most mammalian studies exposure has been done in basal or even complete medium. Another possible explanation is that the antioxidant defence systems are different and/or stronger in mammalian cells. Others have found that the rat hepatoma cell line (H4IIE) was more protected from oxidative stress than the topminnow hepatoma cell line (PLHC-1) (Rau et al., 2004). Relative to mammals, fish have slightly lower GSH levels and subtle differences in GSH regulation (Filho, 1996; Filho et al., 2000; Leggatt & Iwama, 2009; Leggatt et al., 2007). Whether the rainbow trout cell lines are dying of apoptosis, necrosis or some other mechanism will be an interesting study for the future.

Regardless of the reasons for the greater sensitivity of the rainbow trout cell lines to menadione or the mechanism of cell death, the sensitivity of the cell lines does match well with the results with whole fish (Table 3-2). In the FHM database, the LC_{50} for menadione is 0.65μM. This correlation suggests that the protocols used here will be valuable for predicting the toxicity of compounds like menadione to fish.

4.3 Dichlorophene

Experimental EC_{50} values for DCP were also much closer to FHM's LC_{50} than that obtained from the Halle database (Table 3-3). Much of this improvement can be attributed to the elimination of serum from the chosen exposure medium L-15/ex. Serum albumins are known to bind to hydrophobic chemicals such as dichlorophene, thereby effectively reducing their bioavailability to cells (Yamarik & Anderson, 2004).

The liver cell line was more sensitive to dichlorophene toxicity than that of the gill as the range of concentrations tested for RTL-W1 is lower than that for RTgill-W1 (Figure 3-3).

This was necessary because detachment of the liver cells from their underlying substratum became problematic when tested at the same concentrations used for the gill cells in a way that prevented the outcome of reliable EC_{50} values. This increase in sensitivity can be explained by the earlier suggestion that RTL-W1 cells secrete lower amounts of basal lamina (extracellular matrix) proteins, which would weaken their attachment to their substratum, thereby making them more vulnerable to detachment by chemical insults. And since dichlorophene's main mode of action is by disrupting cellular membranes (Yamarik & Anderson, 2004), it would make sense for the liver cells to be more sensitive to dichlorophene exposure. To further support this conclusion, studies using plates with alternative cell binding surfaces (e.g. CellBIND) should be explored and compared with current results.

The effect of indirect dosing on dichlorophene cytotoxicity has also been explored. Indirect dosing has the advantage of evenly distributing a chemical in its exposure medium before its addition to cells. Interestingly, doing so led to AB and NR EC_{50} values that were even closer to that of FHM's LC_{50} value. What's puzzling, however, is the apparent greater insensitivity of the cell membrane integrity of both cell lines, especially RTL-W1, to dichlorophene's toxic action as measured by CFDA-AM (Figure 3-3 & Table 3-3). A plausible explanation is that at the concentrations tested dichlorophene requires more time to fully exert its effects on lipid bilayers. The lack of *in vitro* cytotoxicity studies on dichlorophene by others makes further conclusions more difficult.

One comprehensive study by Freese et al. (1979) looking into the effect on growth inhibition by lipophilic acids after a 72h exposure reported a dichlorophene-induced IC_{50} of 3.9 µM for human HeLa cells and 5.5 µM for the L132 human lung cells. These outcomes

are a little higher, but still comparable enough to our experimental EC_{50}s using the rainbow trout cell lines of ~0.5-1.5 µM. The slight difference from these values, along with Halle's averaged value of 8.32 µM, can be attributed to the fact that the authors exposed their cells in "growth medium", even though there is enough evidence that serum proteins are able to bind to dichlorophene in a way that would effectively reduce its bioavailability to the cells (Yamarik & Anderson, 2004).

With a log*P* value of 4.34 and a logKoc of 3.62, dichlorophene *in vitro* toxicity runs into problems of solubility and adsorption to plastic surfaces that can vary between trials. Additionally, much of this contaminant was likely partitioning into lipid phases as in cells' lipid bilayers. The rather low lethal and effective concentrations for dichlorophene on aquatic organisms, coupled with its tendency to strongly adsorb to sediments and biomagnify makes this contaminant a cause of concern. More attention by regulatory agencies should be paid to the chemical's actual availability in ecosystems, and efforts should be taken to keep its levels in check.

4.4 Hexachlorophene

Hexachlorophene's possession of six chlorine atoms per molecule makes it an excellent disinfectant, but its bisphenol group makes it more than $2x10^5$ times more hydrophobic than the reference chemical SDS. With a log*P* of 6.92 and a logKoc of 5.57, much of this contaminant was likely partitioning into cellular lipid membranes or adsorbing to plates' well surfaces, a combination that would be expected to greatly reduce the chemical's bioavailable fraction to the cells. Cell binding of chemicals due to their partitioning into cellular membranes increases in importance as the chemicals' hydrophobicity increases (Gulden et al., 1994).

Before commenting on hexachlorophene's cell viability curves and their associated EC_{50} values, a review of other *in vitro* studies on the chemical is necessary. Hexachlorophene is one of the MEIC chemicals and thus has been numerously investigated *in vitro* using mammalian cell lines and primary cultures (Halle, 2003). In summary, the main players found to significantly affect the cytotoxicity of HCP *in vitro* are: solubility of the compound, serum concentration in the test system, lipid content of the cells being studied, and the absolute cell density used.

Gulden et al. (2001) investigated the effect of cell density on the *in vitro* cytotoxicity of selected chemicals using a bovine sperm cytotoxicity assay. The sperm cells were exposed for 1h to varying concentrations of each chemical and then viability was assayed using ATP content. The greatest change in sensitivity was for *p,p'*-dichlorodiphenyl dichloroethylene (*p,p'*-DDE). Its EC_{50} jumped from 7.4±1 µM when at a density of $15x10^6$ cells/mL to >146±64 µM when at $120x10^6$ cells/mL, a decrease in sensitivity by ~20 times. Comparatively, hexachlorophene's EC_{50} jumped from 0.094±0.03 µM to 0.39±0.2 µM, a decrease in sensitivity by a factor of ~4 times; note the large standard deviation associated with the EC_{50} values, which was also observed in our results. The authors attributed the rather large standard deviation to solubility problems.

An extensive study by Gulden & Seibert (2005) looked into the impact of FBS and cellular lipid content on the cytotoxicity of hydrophobic chemicals and their relation to predict *in vivo* acute fish toxicity. The study's *in vitro* system used the fibroblast-like embryonic mouse Balb/c 3T3 cells and assayed for protein content (a measure of cell growth) 72h after exposing the cells to varying concentrations of each chemical in DMEM medium supplemented with 5% FBS (equivalent to 1.2g/L or 18 µM). They calculated EC_{50}s for each

of their chemicals that were based on 1) nominal concentrations and 2) the bioavailable (free) chemical concentrations as determined by a well-defined mathematical model. They illustrated how some hydrophobic chemicals have a strong tendency to bind to serum albumin proteins. For instance, more than 95% of pentachlorophenol (PCP) was found to bind to serum albumins, whereas 2,4,5-trichlorophenol and nonylphenol bound at 68 and 44%, respectively. In the case of hexachlorophene, its fraction bound to serum albumins was 62%. And its nominal-EC_{50} was 4.06 µM, whereas it dropped to only 0.027 µM when corrected for chemical loss, a drop of ~150 times. Importantly, based on the 22 chemicals tested, the authors concluded that their *in vitro* system (based on nominal concentrations) was 17.5 and 5.6 times less sensitive than the acute fish toxicity assays previously done using rainbow trout and fathead minnow, respectively. The same parameter, however, is reduced to 4.2 and 1.8, respectively, if *in vivo* LC_{50}s are compared to those EC_{50}s corrected for chemical loss.

Another study by Gulden et al. (2005) investigated the impact of the exposure duration on the cytotoxicity of hydrophobic chemicals also using Balb/c 3T3 cells but instead assaying for protein content (measure of cell growth) after 24 and 72h. They found that many of the compounds exhibited significant differences in viability between the two incubation periods tested. For instance, the EC_{50} for PCP was dropped from 133 µM after 24h of incubation to 39 µM after 72h, an increase in sensitivity by a factor of ~3.5. As for hexachlorophene, its EC_{50} dropped from 55 µM after 24h to 4.1 µM after 72h, a factor of ~13 times. This observation suggests that the cytotoxicity of hexachlorophene requires multiple days to fully manifest; in other words, 24h may not be long enough.

Another important study by Shrivastava et al. (1992) compared the cytotoxicity of hexachlorophene using McCoy (human epithelial synovial cells) and MDBK (bovine kidney cells) cell lines with that of rat primary hepatocytes. EC_{50}s were 18 and 11 µM for McCoy and MDBK cells, respectively, but only 2 µM by the hepatocytes. The authors attributed this difference to the fact that hepatocytes are more fragile to pressure forces than the cell lines or because of their increased metabolic capacity.

Although the studies listed in the Halle database for HCP *in vitro* toxicity have an average EC_{50} of 7.94 µM, the individual values range from 0.93 to 25 µM, which is a reflection of the various factors noted above. Comparatively, calculated AB and NR EC_{50}s from the current thesis for both cell lines are in good agreement with FHM's *in vivo* LC_{50}. They are closer to it than that documented in the Halle database (Table 3-4), but the associated standard deviation is large. Although care was taken to fully replicate the experimental trials, many factors could have acted in an additive manner to lead to the SD observed. These include 1) the variation in the absolute number of cells seeded to each well (within a plate and between trials), which would affect the cell density and total lipid content, and 2) the variable loss of the chemical due to adsorption to the various plastics used such as pipette tips and microplate surfaces. Although these factors are usually not considered significant, hexachlorophene's high hydrophobicity makes them more relevant.

Although much of this contaminant was likely partitioning into lipid phases as in cells' lipid bilayers, note that CFDA-AM's dose-response curves hovered around the 50% viability mark after the 24h of exposure (Table 3-4), which indicates that, relative to cell metabolism and lysosomal activity, the integrity of cell membranes stays intact, at least for 24h post exposure. This observation was verified morphologically as seen in Figure 3-4,

panels C & D, suggesting that hexachlorophene toxicity is manifested from the inside-out, so that much of the chemical that freely diffuses to the inside of the cells impairs certain intracellular functions before proceeding to act on the enclosing cell membrane.

4.5 4-fluoroaniline

The cytotoxicity of 4-fluoroaniline to both cell lines also followed a dose-dependent fashion, but there were many issues to consider. As referred from Table 3-5, the obtained EC_{50} values are 16-40 times higher than that of FHM's expected LC_{50} value. But one should keep in mind, however, that 4-fluoroaniline became more and more insoluble in its solvent (DMSO) as its tested concentrations were increased; in fact, crystals of 4-fluoroaniline became visible with the naked eye beginning at the second lowest concentration, but were more pronounced at the second highest concentration of 13,195 µM as indicated by arrows on the graphs and their representative diagrams (Figure 3-5). This observation would clearly decrease the bioavailable fraction of 4-fluoroaniline to the cells, further contributing to the cells' apparent insensitivity to this haloaniline contaminant.

The story of 4-fluoroaniline toxicity does not end here. Although, until now, the *in vitro* cytotoxicity of 4-fluoroaniline has not been studied using any cell line system (neither have other ubiquitous haloaniline pollutants like chloroaniline and 3,4-dichloroaniline), the parent compound aniline has been investigated rather extensively both *in vitro* and *in vivo* (Bhunia et al., 2003; Cravedi et al., 1999; Dady et al., 1991; Hong et al., 2000; Kalsch et al., 1991; Leguen et al., 2000; Ramos et al., 2002). Perplexing conclusions from those aniline studies help shed some light on the clear ineffectiveness of 4-fluoroaniline toxicity *in vitro* using our system.

The *in vivo* intraspecies sensitivity to aniline has been well-documented (Bhunia et al., 2003; Ramos et al., 2002). For instance, aniline's 96h-LC_{50} for rainbow trout is 10.60 mg/L, for goldfish 187 mg/L, and for fathead minnow 134 mg/L (Bhunia et al., 2003; Nielsen et al., 1993). Accordingly, rainbow trout is ~13 times more sensitive to aniline toxicity than the fathead minnow, which is a rather troubling finding since *in vitro* cytotoxicity studies do not usually take intraspecies differences into account.

Assuming that aniline's mode of toxic action is comparable to 4-fluoroaniline, our approach ends up underestimating the cytotoxicity of 4-fluoroaniline to rainbow trout by ~56 times, whereas it would underestimate its toxicity to fathead minnow by ~5 times. But since we investigated the cytotoxicity of 4-fluoroaniline using two rainbow trout cell lines, it would be more appropriate to compare our *in vitro* results with rainbow trout's *in vivo* value as opposed to fathead minnow's. Regardless, looking at it from our original perspective, our exposure of rainbow trout cell lines to 4-fluoroaniline underestimated the chemical's toxicity by ~36 times when compared to FHM's 4-fluoroaniline-induced LC_{50} (Table 3-5).

In any case, the dominant story here is that there is a poor correlation between *in vivo-in vitro* tests of haloanilines. It is true that insolubility is a contributing factor, but a more biological explanation stems from previous work by Cravedi et al. (1999) that looked into *in vivo-in vitro* correlations in aniline toxicity using primary cultures of rainbow trout hepatocytes. Their big conclusion is that the hepatocytes, unlike the intact fish, were unable to biotransform aniline all the way to its hydroxylated metabolites, which are thought to be the key players in aniline toxicity (Hong et al., 2000; Rankin et al., 1995; Okazaki et al., 2003). Accordingly, we can conclude that the insensitivity of our cell lines may be further

attributed to their inability (or relatively weak ability) to bioactivate 4-fluoroaniline to its reactive hydroxylated metabolites.

4.6 Allyl alcohol

It is clear from the dose-response curves in Figure 3-6 that the two cell lines differ in their sensitivity to allyl alcohol toxicity. RTgill-W1's complete lack of sensitivity supports previous findings that allyl alcohol exhibits organ-selective toxicity, which we now know to be the liver (Koerker et al., 1976). RTL-W1's response further supports those earlier findings that allyl alcohol first requires its metabolism by a liver-specific alcohol dehydrogenase into the more toxic form acrolein in order to express toxicity (Jaeschke et al., 1987; Koerker et al., 1976; Maddox et al., 2003; Maddox et al., 2004; Rikans & Moore, 1987; Serafini-Cessi, 1972). Those studies have excluded the involvement of other hepatic enzymes (such as CYP450s) in the bioactivation of allyl alcohol by their additions of ADH inhibitors that effectively rendered their cell systems insensitive to allyl alcohol. To achieve the same conclusion for RTL-W1, it would be necessary to conduct further experiments using ADH inhibitors.

Although RTL-W1 was responsive to allyl alcohol toxicity, calculated EC_{50} values were higher than that of FHM's LC_{50} by 365-1500 times (Table 3-6). This observation can be attributed to two phenomena acting in parallel. 1) It's true that RTL-W1 is a liver cell line (Lee et al., 1993), but these liver cells seem to exhibit low and weak ADH activity, which would effectively reduce their production of the real toxic culprit acrolein throughout the 24h exposure period. 2) A logHLC of -5.25atm-m^3/mol and a density of 0.854g/mL (at 25°C) make allyl alcohol highly volatile and its liquid lighter than water, thereby allowing it to evaporate out of solution in a multitude of steps: A) preparation of its dilution series, B)

dosing into the cells, and C) throughout the 24h incubation period. This additive loss greatly reduced the chemical's bioavailable fraction to our cell systems, which then led to the relatively high EC_{50} values that underestimated the chemical's toxicity *in vitro*.

Gill cells are not expected to carry the hepatic form of alcohol dehydrogenase, which explains their insensitivity to the alcohol after 24h. Nonetheless, the story with RTgill-W1 and allyl alcohol was further investigated. Doubling the time of allyl alcohol exposure for the gill cells to 48h seemed to make a difference at the highest concentration possible, that of pure allyl alcohol (Figure 3-7, Panel A). While the involvement of an ADH cannot be ruled out, it is unlikely that it did because a decrease in viability due to allyl alcohol exposure was not observed after 24h and because gill cells would not be expected to carry the hepatic form of ADH. Hence, the decrease in viability can be attributed to the direct action of allyl alcohol. After all, it is a severe irritant and has the ability to disrupt cellular membranes (Belinsky et al., 1986; Jaeschke et al., 1987; Miccadei et al., 1988). Our exposure system, however, would still be subject to chemical loss through volatility. Taking the story further, a 5-day (120h) exposure of the gill cells was also conducted (Figure 3-7, Panel D). Even though the decrease in viability was more pronounced than after 48h, most of the drop can be attributed to cumulative adverse effects from the absence of growth factors, changes in pH and osmolarity, and the buildup of metabolic waste over the 120h of incubation. This observation was supported by the morphological changes where even the control cells ended up fighting for their lives after the 120h (Figure 3-7, Panels E & F). A similar 5-day experiment where the plate was sealed did not make a difference on cell viability (data not shown).

Until currently, the *in vitro* cytotoxicity of allyl alcohol has not been investigated using fish cell lines. Most *in vitro* systems have used either mammalian cell lines or primary cultures of hepatocytes. Of those, a considerable number have focused on mechanistic studies underlying the conversion of allyl alcohol to acrolein and subsequent liver pathogenesis (Koerker et al., 1976; Reid, 1972; Serafini-Cessi, 1972). The lowest *in vitro* EC_{50} reported in the Halle database on allyl alcohol cytotoxicity is 690 µM, which is still much higher (by ~125 times) than FHM's reported LC_{50} of ~5.5 µM. The authors in that study exposed mouse 3T3 cells to allyl alcohol and used protein content after 24h as their toxicity endpoint (Halle, 2003; Spielmann et al., 1992, unpublished results).

Another study that also exposed 3T3 cells to allyl alcohol combined the use of neutral red and kenacid blue (protein content) as their toxicity endpoint after sealing their plates for 24h, after which they determined an EC_{50} of 860 µM (Spielmann et al., 1991). Yet another study used a related 3T3-L1 cell line to investigate the effect of sealing a plate's wells after allyl alcohol exposure using paraffin oil versus those left unsealed (Smith et al., 1992). The unsealed trials yielded an EC_{50} of 9470 µM, whereas the EC_{50} from those sealed dropped by 50% to 4650 µM, both of which are still much higher than FHM's LC_{50}. These relatively high *in vitro* EC_{50}s can be attributed to the absence (or weak) ADH activity in the chosen cell lines. Support for this claim comes from a study that used rat hepatocytes to investigate allyl alcohol's ability to oxidize pyridine nucleotides when at a concentration of 500 µM (Rikans et al., 1996). Cytotoxicity as measured by lactate dehydrogenase leakage was evident after a 60min incubation of the hepatocytes with the chemical. In fact, relative to controls, LDH leakage reached 40% of total activity after 90mins and 80% after 120mins. Interestingly, the addition of 200 µM of an ADH inhibitor, dithiothreitol, to the reaction mixture 30mins after

allyl alcohol administration was enough to fully protect the cells against viability loss, which acts as a testament to how important acrolein generation is to full allyl alcohol toxicity.

4.7 Acrolein

A simple comparison of logHLC values between acrolein and the reference chemical SDS shows that acrolein is ~200 times more volatile than SDS. Acrolein's volatility coupled with its potency has led to rather interesting results. Although Figure 3-8, panel A is a reflection of RTL-W1's response to acrolein, the trend seen can be disregarded. It became clear that when most wells of a plate are dosed with acrolein concentrations of ~40 µM and above, enough of the chemical would evaporate out of solution where it would eventually land in other wells, including those of the control, causing widespread cell impairment and death after 24h of exposure (compare Figure 3-8, Panels D & E). In fact, there was no significant difference in raw fluorescent units (RFUs) when comparing those given by control cells and those exposed to the highest concentration of acrolein at 82.69 µM as based on a visual observation. This phenomenon whereby cytotoxicity is observed in control cells due to a chemical's volatility is known as cross-contamination and is well-documented in the literature (Blein et al., 1991; Smith et al., 1992). Several alternative experimental conditions for a number of volatile chemicals have been explored with various degrees of success. Some of these alternatives have been investigated in this thesis.

One experimental way of minimizing cross-contamination by a chemical's volatility is by covering the plate with a specialized sealant right after dosing. The sealant used (Corning Inc.) is meant to close all gaps that can potentially transport a chemical from one well to another. In the case of acrolein, the sealing of wells proved effective. Acrolein became cytotoxic to RTL-W1 cells in a dose-dependent fashion, whereas the control cells

remained viable as measured by all three endpoints (Figure 3-8, Panel B). Although the associated EC_{50}s are lower than FHM's LC_{50}, they are still much more comparable to it than that recorded in the Halle database (Table 3-7).

The effect of plate sealing on acrolein toxicity has not been investigated before. However, Smith et al. (1992) have investigated the effect of sealing wells of a plate using paraffin oil on a number of volatile chemicals using 3T3-L1 cells and assayed by the Kenacid Blue method (total protein content of cells after 72h). The study concluded that the cytotoxicity of many of the volatile chemicals greatly increased by sealing the plate. For instance, acetaldehyde's EC_{50} dropped from 270 µg/mL (unsealed plate) to 36 µg/mL (sealed), a drop of 87%. Similarly, acetone's cytotoxicity increased by 68%, allyl alcohol by 51%, and ethyl acetate by 29%.

Another study by Riddell et al. (1986b) investigated the effect of exposure period (24h vs. 72h) on the cytotoxicity of various chemicals. Acrolein's 24h-EC_{50} dropped from 660 µM to <0.178 µM after 72h, which reflects an increase in sensitivity by >3700 times. The difference in sensitivity was even more pronounced for 6-mercaptopurine that reported an increase in sensitivity by >100,000 times. Hence, the study's conclusion stressed the importance of exposure period when investigating the cytotoxicity of chemicals *in vitro*. Yet another study using NBP_2 cells (clone of C1300 mouse neuroblastoma cells) has reported an acrolein-induced EC_{50} of 1 µM based on the sloughing of cells from the flask, but the resulting EC_{50} was found to increase to 30 µM if attachment of the cells to the underlying substratum was used as an endpoint (Koerker et al., 1976).

Another way of protecting control cells from cross-contamination is by using a separate plate for each chemical concentration. By doing so in this thesis, the control cells did remain viable, and those exposed to various toxicant concentrations also showed toxicity in a dose-dependent fashion (Figure 3-8, Panel C), but their resulting EC_{50} values are about twice as high as those obtained by sealing the plate. Nonetheless, both conditions led to EC_{50}values that are much closer to FHM's LC_{50} than that obtained from the Halle database (Table 3-7).

4.8 Acetaldehyde

Although acetaldehyde's cell viability curves indicate that the chemical is nontoxic to both cell lines after 48h of exposure in sealed plates (Figure 3-9, Panels A & B), many practical problems were faced in our handling of the chemical. The major limitation can be attributed to the chemical's highly volatile nature. It has a boiling point of 21°C (or room temperature), a density of 0.785g/mL (at 25°C), and based on its logHLC is ~400 times more volatile than the control chemical SDS. Thus, loss of the chemical throughout its preparation and subsequent exposure in multiwell plates may help explain the eventual nontoxicity of the chemical.

Although acetaldehyde is highly soluble in water ($1x10^6$ mg/L at 25°C), a pre-stock solution of only 100 mg/L was first prepared from the pure form of the chemical in an attempt to contain its loss due to its rapid volatilization. But because of the chemical's relatively weak cohesive and adhesive forces (when compared to water), an unknown volume kept dripping back out (lost) from the pipette's tip during its initial preparatory step. The best way of minimizing the volume of chemical loss was by working with relatively small volumes. Specifically, 25.48 µL of acetaldehyde was added to 200 µL of distilled water to

form the desired 100 mg/L solution of acetaldehyde. This act of diluting the pure form of the chemical is similar to the idea behind the preparation of formaldehyde solutions at 37% (v/v). The pre-stock solution was further diluted by 1000 times to reach the desired range of chemical concentrations for the cytotoxicity assays (70.9-2270.3 µM) and left overnight to make sure the chemical is well mixed in the water. Acetaldehyde's LC_{50} to fathead minnow is 691.83 µM; thus, RTgill-W1's and RTL-W1's complete insensitivity to acetaldehyde after 48h of exposure is likely due to significant loss of the chemical throughout its preparation and subsequent exposure in multiwell plates.

Acetaldehyde's average EC_{50} as reported in the Halle database is 2454.71 µM, ranging from as low as 540 µM to as high as 9000 µM. Interestingly, these low and high EC_{50}s were obtained from the same set of experiments that differ only in the nature of toxicity endpoint used (Koerker et al., 1976): NBP_2 cells were exposed to acetaldehyde for 24h where cell viability was then measured by either the sloughing of cells into the medium (lower EC_{50}) or the attachment of cells to their substratum (higher EC_{50}). From this outcome one can conclude that the toxicity of acetaldehyde *in vitro* causes significant monolayer breaks and subsequent cell detachment. The study's outcome, however, is contradictory to what we observed in the current thesis. Not only were we unable to establish acetaldehyde EC_{50}s, but we were also unable to observe any morphological changes even in cells exposed to 2270 µM (Figure 3-9, Panels C & D).

In another study, Smith et al. (1992) exposed 3T3-L1 cells to acetaldehyde and other volatile chemicals in order to investigate the effect of sealing a plate's wells with paraffin oil versus those left sealed. The toxicity endpoint was protein content after a 72h exposure of the cells to acetaldehyde. Plates unsealed led to an acetaldehyde-induced EC_{50} of 6130 µM,

whereas those sealed led to an EC_{50} of 820 µM, which is ~13% of the former condition. Although plates were sealed in the current thesis (albeit in a different way), no toxicity was observed after 48h of exposure using any of the three indicator dyes nor were there any morphological signs of toxicity (Figure 3-9). Having said that, a study by Künstler & Bartnik (1987) did use the neutral red uptake assay as their toxicity endpoint after exposing BALB/c-3T3 cells to acetaldehyde for 24h and were able to determine an EC_{50} of 3650 µM (See also Halle, 2003). Accordingly, one could conclude that our highest acetaldehyde concentration of 2270 µM is simply nontoxic to both RTgill-W1 and RTL-W1. In any case, the insensitivity of our cell lines seems to be due to the combination of 1) acetaldehyde loss through volatility, 2) the use of insufficiently toxic levels of the chemical, and 3) the use of highly resistant cell types to acetaldehyde toxicity, though the latter in unlikely. The downside to using a higher range of acetaldehyde concentrations is the associated acceleration in the chemical's volatility.

4.9 2,3-dimethyl-1,3-butadiene

Similar to the case of acetaldehyde, 2,3-dimethyl-1,3-butadiene's cell viability curves indicated that the chemical is nontoxic to both cell lines after 72h of exposure in sealed plates (Figure 3-10), but likewise many practical problems were faced in our handling of this chemical. Initial attempts to solubilize DMBD in each of L-15/ex, DMSO, and ethanol proved unsuccessful as the chemical was observed to be completely immiscible in each of the solvents; it formed two layers and eventually settled on top. Subsequent vigorous vortexing did not emulsify the solution mixture either. At a density of 0.726g/mL (at 25°C) and a logHLC of -0.72atm-m^3/mol, the chemical is more than $1x10^6$ times more volatile than SDS; hence, the major limitation in the cytotoxicity of DMBD can also be attributed to its highly

volatile nature. Loss of the chemical throughout its preparation and subsequent exposure in multiwell plates may help explain the eventual nontoxicity of the chemical.

It is worth noting that the cytotoxicity of 2,3-dimethyl-1,3-butadiene has never been tested *in vitro* before. In our attempt to deal with the solubility and volatility of DMBD, a solution was prepared in L-15/ex that took into account the chemical's known solubility of 300 mg/L (~3612 µM) in water and was then left overnight to make sure the chemical is well mixed in the water. The problem with this set up is that once added to a well's exposure medium as part of the cytotoxicity assay (the working solutions were 200x concentrated), the highest tested concentration would be ~18 µM, which is much less than FHM's LC_{50} of 83.2 µM. In fact, cells exposed to this high concentration of 18 µM remained healthy without signs of adverse effects after 24h of exposure. The same was observed when cells were later exposed to concentrations up to 109 µM for 72h (Figure 3-10, Panels C & D). Therefore, RTgill-W1's and RTL-W1's complete insensitivity to 2,3-dimethyl-1,3-butadiene after 72h of exposure is likely due to the combination of 1) significant loss of the chemical throughout its preparation and subsequent exposure in multiwell plates, 2) the use of insufficiently toxic levels of the chemical, and 3) the use of highly resistant cell types to DMBD toxicity, though the latter is unlikely.

In contrast to 2,3-dimethyl-1,3-butadiene, the cytotoxicity of 1,3-butadiene has been evaluated using smog chamber-cell *in vitro* assays (Doyle et al., 2007). Smog chambers are commonly used in air quality research to investigate the toxicity of photochemically-active contaminants under ambient environmental conditions. A549 cells, a human pulmonary type II epithelial-like cell line, were exposed to 1,3-butadiene at concentrations that resemble those in the atmosphere in a serum-free F12K medium (Doyle et al., 2007). After 9 hours of

exposure, cytotoxicity as measured by lactate dehydrogenase release was ~6-fold higher in exposed cells than those exposed to clean air (control condition). Since 2,3-dimethyl-1,3-butadiene is also an atmospheric toxicant, it may be more appropriate to evaluate its toxicity using similar chamber-cell *in vitro* assays.

Chapter 5
CONCLUSION

It has become clear after a close examination of each of the reactive chemicals that many of them have not been investigated for their ecotoxicological effects, which is rather unacceptable since we know that the environment acts as the ultimate sink for contaminants. Additionally, all eight contaminants have been studied for their toxicity using model mammalian systems, namely rats and mice, but the effect of these xenobiotics on aquatic species is more important because of their increased likelihood of coming into contact with the chemicals. Aquatic organisms are an integral part of nature and so the impact of these chemicals on the aquatic environment is a serious issue that deserves more attention from both scientists and environmentalists.

The realization that toxicity is first manifested at the cellular level, coupled with other important factors such as the large number of chemicals in commerce, time, and the associated cost of screening these chemicals for toxicity has helped pave the way for the use of animal cells in culture as alternatives to the use of whole animals for such purposes. The use of animals cells is rapid, inexpensive, and thus much more economical. But the use of mammalian primary cultures and cell lines for the cytotoxicity testing of anthropogenic chemicals is simply insufficient to allow us to accurately depict or extrapolate the findings to fish species. Fish cell lines are an obvious alternative to the use of mammalian cells in culture for our understanding of the toxicity of these chemicals to aquatic organisms, or even the aquatic environment as a whole.

It is true that the use of cells in culture for toxicological purposes has not been perfected as of yet. After all, both RTgill-W1 and RTL-W1 failed to accurately predict the cytotoxicity of four of the studied reactive chemicals of concern. But a closer look would blame the nature of the chemicals themselves as opposed to the choice of cell line or even cell viability endpoint. Whereas most (if not all) inert chemicals can be reliably tested *in vitro* for their toxicity, each reactive chemical seems to present its own challenge, namely that of volatility or solubility. But that should not detract our attention from further improving the use of cells in culture. After all, ~80% of xenobiotics belong to the inert class of chemicals. And many studies have reported on good correlations between their *in vitro* EC_{50} values and those done *in vivo* using intact animals. For instance, Borenfreund and Borerro (1984) investigated the cytotoxicity of 34 toxicants on many cell types, such as 2nd to 9th passages of primary explants of rabbit corneal cells and various established cell lines. Irrespective of the cell type used, their results correlated well with eye irritancy as measured by the Draize test. In fact, the Draize test has now been replaced with cell lines in many European countries. And so it is only a matter of time before the full potential of the use of cells in culture is fully understood.

One must always keep in mind the three Rs that guide the discipline of *in vitro* toxicology: Replace, Reduce, and Refine the use of animals whenever possible (Gad, 1990). The use of *in vivo* fish lethality tests will always be limited by staff, cost, and available space, but the successful application of cell lines within the realm of toxicology would eventually allow for designing automated ways for the high-throughput screening of chemicals. Such automation should serve as further incentive to invest in more ways to improve our *in vitro* screening of chemicals. The successful application of cell lines will also help improve and

contribute more to our understanding of the ecology and biochemistry of the many environmental contaminants.

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