Toxicity Assessment of Oil Sands Process-Affected Water Using Fish Cell Lines

by

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Authors 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

Toxicity assessment of large numbers of oil sands process-affected waters (OSPW) are needed in order to reclaim mined oil sands aquatic reclamation scenarios, such as End Pit Lakes (EPLs). Conventional toxicity testing using whole animals can make this process extremely costly, thus alternatives are being sought. A non-lethal bioassay is being developed and validated to aid in supporting reclamation planning. This study employed six fish cell-lines (WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHML, FHMT) in 24h viability assays for rapid fluorometric assessment of cellular integrity and functionality. Eight ml from forty-nine OSPW samples received from Syncrude Canada Ltd. were mixed with 2 ml of 5X concentrated L-15/ex minimal media solution and used to expose cells. After 24h exposure to OSPW samples, significant decreases in cell viability as measured by Alamar blue (AB) were seen in all cell lines for a number of samples. Bioassays were done in blind, but when OSPW chemical composition was revealed there was a consistent correlation between decreasing cell viability and increasing naphthenic acid (NA) concentrations present in the samples. Regression analysis yielded correlation coefficients^2 as high as 0.6171 (WF-2 cell line, AB; $p<0.0001$). NAs have been identified as the chief toxicants in OSPW. Therefore, a fish-cell line bioassay sensitive to fluctuations in NA concentration could be a tool integral to the safe implementation and biomonitoring of wet reclamation landscapes in the Athabasca oil sands region, such as EPLs.

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List of Abbreviations

AB	Alamar Blue
AB_{50}	EC_{50} value as measured by AB
CF	carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
CFDA-AM_{50}	EC_{50} value as measured by CFDA-AM
OSPW	Oil sands process-affected water
FBS	Fetal bovine serum
FHML-W1	Fathead minnow liver cell line
FHMT-W1	Fathead minnow testis cell line
GFSk-S1	Goldfish skin cell line
L-15	Liebovitz L-15 growth medium
L-15/ex	Simple exposure media for experiments (Appendix A)
NA	Naphthenic acid
NR	Neutral red
NR_{50}	EC_{50} value as measured by NR
RFUs	Relative fluorescence units
RTgill-W1	Rainbow trout gill cell line
RTL-W1	Rainbow trout liver cell line
SD	Standard deviation
SDS	Sodium dodecyl sulphate
WF-2	Bluegill fry cell line

1 Introduction

The ecological impact of the oil sands industry has been significant (Giesy et al., 2010; Holowenko et al., 2000), and with many oil sands projects approved or pending approval this impact is likely to grow in the near future. In order to obtain reclamation certification, that is, return post-mining areas to a land capability similar to that which existed prior to mining, a self-sustaining, self-propagating ecosystem must be established (ERCB, 2009). Part of this ecosystem development will rely heavily on sound testing processes likely to include a battery of toxicological tests leading to an overarching evaluation of the potential success of the ecosystem as a whole. Fish will be an integral part of a reclaimed ecosystem. Through the use of established fish cell-lines, as well as new cell-lines derived from fish native to the oil sands region, the development and validation of a non-lethal bioassay to aid in supporting reclamation planning and implementation was explored in this thesis. An overview on the Athabasca oil sands is introduced for the general audience before the details of the project are presented in the remainder of this thesis.

1.1 Athabasca Oil Sands

The Athabasca Oil Sands, located in north-eastern Alberta, is an expansive reserve of fossil fuels consisting of crude bitumen, silica sand, clay minerals and water (Schramm *et al*, 2000). Conservative estimates of conventional and non-conventional oil reserves in Canada are a combined 180-185 billion barrels (AEUB, 2007; Chastko, 2004).

Subsequent to mining, oil sand undergoes the Clarke hot water extraction process to separate the bitumen from silica sand, and clay minerals (Schramm *et al*, 2000) using large volumes of water (FTFC, 1995a). This process promotes the solubilisation of NAs due to the alkalinity

(pH=8) due to the presence of NaOH during extraction, thereby concentrating them as mixtures of sodium salts in the aqueous tailings (Rogers et al., 2002). The oil sands process-affected water (OSPW) produced by this method is composed mainly of sand, clay, and unrecoverable bitumen and hydrocarbons (FTFC, 1995b). The volume of oil sand and water being processed is so great that up to 119 million litres of hydrocarbon-containing process-affected material is produced daily (AOSIU, 2010).

However, the Canadian Energy Research Institute estimates the processing and development of the oil sands will generate an overwhelming $800 billion surge in the Canadian economy (Righton, 2006), a decidedly strong, positive aspect of oil sands development. This places critical accountability on both the government and oil sands companies working in Alberta. The responsibilities of the latter are two-fold: A zero-discharge policy states that all oil sands process affected material be contained on approved oil sand lease sites (Grant et al. 2008); and all processed land must be reclaimed, meaning mining areas must be returned to a land capability equivalent to that which existed prior to mining (Carey, 2008). The evident paradox forces companies to effectively reclaim the disturbed land on which they are storing process-affected material containing adverse chemical components such as naphthenic acids (NAs), polycyclic aromatic hydrocarbons (PAHs), trace metals, and elevated salinity (Westcott, 2007).

The main reclamation initiative aimed at satisfying zero-discharge and reclamation responsibilities is called an End Pit Lake (EPL). EPLs (Figure 1; Westcott, 2007) are an aquatic reclamation system in the form of an engineered body of water located below grade in oil sands post-mining pits resulting from the strip mining process (Westcott and Watson, 2007). An EPL will be a meromictic, or permanently stratified body of water with a well defined top, or cap (mixolimnion), and a bottom layer containing mature fine tailing, composite tailings, lean oil

sand, overburden, and waste water. (monimolimnion; Grant et al. 2008). The intention is this stratification may allow a permanent, self-sustaining and biologically productive ecosystem to exist in the of fresh water cap above dense, process-affected material effectively fulfilling both the zero-discharge and reclamation policies.

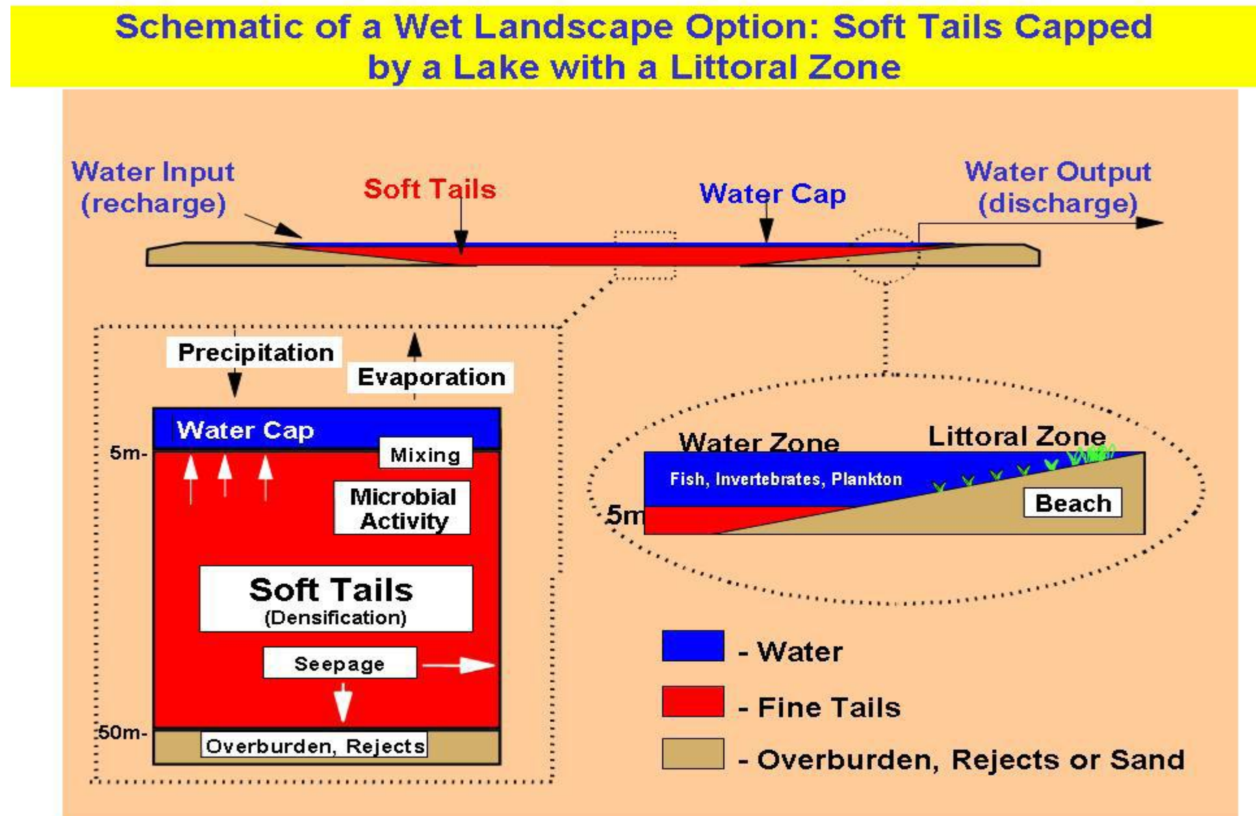

Figure 1.1 – Schematic of proposed End Pit Lake. End pit lakes are aquatic reclamation scenarios proposed for the reclamation of the Athabasca oil sands lease sites in Alberta. They will exist in post-mining pits and contain dense, process-affected material at the bottom and a layer of fresh water on top. This layer of fresh water is proposed to support an self-sustaining ecosystem in time. (Adapted from Westcott and Watson, 2007)

In order for EPLs to support healthy, functioning and sustainable aquatic ecosystems the potential acute and chronic toxicity of an EPL must be assessed and minimized; a major biophysical issue is the toxicity of EPL-water and -sediment to aquatic life. As EPLs are developed and implemented there will be growing demand for established ecotoxicity and

biomonitoring models to ensure that levels of toxicants remain within acceptable ranges in and around reclamation systems.

For every barrel of crude oil extracted, it is estimated that as much as four barrels of water are used in the extraction process (Holowenko et al., 2002). This water must be cleaned as per Directive 74 of the Energy Resources Conservation Board (ERCB, 2009), and remediation estimates run up to $2 million dollars/week (Sassoon, 2010). These costs could wipe out any earnings for the oil extraction companies involved, thus alternatives are being sought at all levels of the remediation process, including the biomonitoring of EPL waters and toxicity assessment steps that must be done on a routine basis. Testing of such samples on whole organisms can be extremely expensive, especially when the water samples need to be carted to testing facilities miles away from the sites. Furthermore, because relevant organisms in aquatic ecosystems are often fish, large sample volumes would be required, which means tanker loads of test waters would be needed. Additionally, the impact on indigenous fish species would be deemed most relevant, and testing representative native fish species would be cost prohibitive. A synopsis on general environmental monitoring techniques is presented below as well as alternative methodologies, including the topic of this thesis on the application of non-lethal bioassays based on indigenous fish cell lines for assessing toxicity of oil sands process affected waters.

1.2 OSPW samples and chemicals

This study looked at the cytotoxicity of 49 whole oil sands process-affected water (OSPW) samples from the Syncrude Canada Ltd. lease site in north-eastern Alberta. Industrial effluents from mining operations often contain heavy metals and synthetic detergents or surfactants liberated during the extraction, production or refinement processes (Feng et al., 2005).

The chief toxicants in OSPW are thought to be naphthenic acids (NA) (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986). NAs are natural constituents of petroleum evolved from the oxidation of naphthenes (cycloalkanes). NAs are an eclectic mixture of mono- and poly-saturated carboxylic acids accounting for up to 4% of raw petroleum by weight (Rogers et al., 2002). NAs have the general formula $C_nH_{2n+z}O_2$ (where z is zero or a negative, even integer whose absolute value divided by two gives the number of rings in a compound) (Young et al., 2002). Hence, in addition to metals and other processing byproducts, NAs represent an important component of waste generated during oil sands processing and will likely be ubiquitous in EPLs where fish will be readily exposed.

NAs likely behave as surfactants as they consist of a hydrophilic head and a hydrophobic tail giving them unique solubility properties (Ivankovic and Hrenovic, 2010). These compounds are commonly found in detergents or cleaning products used in mining, oil, food and textile industries (Sandbacka et al, 2000). Untreated industrial effluents often contain surfactants or surfactant-like compounds in concentrations sufficient to elicit acute toxicity in aquatic organisms (Ankley and Burkhard, 1992). Surfactants can induce emulsification of phospholipid bilayers leading to extensive cellular damage, and subsequent cytolytic release of proteins, and lysosomal and cytoplasmic enzymes (Effendy and Maibach, 1995; Lee et al, 2000). Surfactants are generally classified as anionic, cationic, amphoteric, and non-ionic depending on the charge of their head group. Anionic surfactants are the most common and have applications as detergents or common soaps, and even biotechnology and other industrial processes, such as cosmetics (Ivankovic and Hrenovic, 2010).

The anionic surfactant, sodium dodecyl sulphate (SDS) was chosen for this study because it exerts a low log *P* (octanol/water partition coefficient) and log *HCL* (Henry's law coefficient)

value and is miscible in water. For these reasons, it is easy to handle and sorption to the exposure vessel and evaporation will not likely confound the derivation of effective concentrations (Schirmer et al., 2008). Also, SDS is classified as membrane damaging, thus, toxicity should be easily measured by any non-specific bioassay. Lastly, SDS represents the group of anionic surfactants used in industrial processes that can be of environmental concern (Cserhati et al., 2002).

1.3 Tissue Culture and Applications in Toxicology

Tissue culture originated in the early twentieth century (Harrison, 1907) in an effort to study individual cells free of systemic variation that may affect the way we understand basic cellular biology. Subsequent development of cell culture was facilitated by research into viruses and the production of antiviral vaccines, and the need for a better understanding of neoplasia (Freshney, 2007). Scientists are provided the unique ability to monitor and control the physicochemical environment and the physiological conditions of cells in culture, which has led to prevalent use of tissue culture techniques in research areas such as cancer, immunology, tissue engineering, and toxicology (Atala and Lanza, 2002).

Of particular interest in this study are the applications of tissue culture in the area of toxicology, specifically environmental aquatic toxicology: the study of the interactions of natural and anthropogenic toxicants with aquatic biological systems and their subsequent impacts on structure and function (Landis and Yu, 1995). This practice is fundamental to the risk assessment paradigm that functions to identify hazardous substances and conditions of their exposure to predict adverse effects to humans or the environment (Derelanko, 2002). Currently,

risk assessment is largely achieved through the elucidation of gross toxic effects such as acute lethality, organ-related toxicity, birth defects, and cancer. To this end, animal models are used as human surrogates or representatives of the same or similar animals in the wild. These animals are used to evaluate the likelihood or nature of a response (i.e., death, tumour induction, reproductive impairment) to a certain chemical (Bengtson and Henshel, 1996).

1.4 Current methods in aquatic toxicology

Fish are the largest and most diverse group of vertebrates, making them important models in a number of research areas including environmental biology (Powers, 1989). Fish cell lines and associated bioassays are important *in vitro* models often used to ascertain relevant physiological data quickly, inexpensively, and with a high degree of reproducibility, both within and between laboratories (Lorenzen et al., 1999). Aquatic regulatory testing with fish is often done measuring endpoints such as mortality in the static acute fish toxicity assay, the evaluation of bioconcentration factors in a flow-through system (Halder and Worth, 2003), or effects on specific stages of development (Schirmer, 2006).

The most widely used is the fish acute lethality test (OECD, 2009 test guideline 203). The test involves the exposure of fish to effluent samples for up to 96h, using at least 7 fish per concentration at a minimum of 5 concentrations plus controls, in search of the concentration causing 50% of the fish to die (LC_{50}). However, fish acute lethality tests are costly, fail to meet societal pressure to reduce animal testing, and reflect an integrative endpoint, which makes it difficult or impossible to differentiate routes of toxic mechanism (Fent, 2001). In addition to these points, the European Commission encourages the development and application of animal test alternatives in order to ethically and economically facilitate the new European legislation of

the Registration, Evaluation and Authorisation of Chemicals (REACh) which will likely require the accurate testing and characterization of thousands of compounds (European Commission, 2006). Whole-animal alternatives in toxicology will be desirable for use in projects of similar magnitude, such as oil sands reclamation, where vast numbers of samples need to be tested or constant biomonitoring necessary to aid in the safe facilitation of viable reclamation scenarios in a time- and cost-effective manner.

1.5 Whole-animal alternatives in aquatic toxicology

In recent years, the ‘3Rs’ principle of replacement, reduction and refinement (Russell and Burch, 1959) has gained a higher profile in toxicology due to a convergence of scientific, ethical/animal welfare, financial and legislative imperatives. The adoption of *in vitro* alternatives reduces the cost and time of toxicity assays as well as the number of animals necessary to safely evaluate potential toxicants. As such, there has been a movement for the application and refinement of existing laboratory animal test-alternatives in toxicology and an impetus for the development and implementation of new alternative methods (Bruner et al., 1996).

In order to establish an effective *in vitro* alternative that will reduce or eliminate the use of animals in toxicity testing, it must be shown that *in vitro* results accurately predict *in vivo* results across a range of similar chemical concentrations. Thus, we must develop a prediction model that marries *in vitro* results with *in vivo* predictions (Figure 1.2). Such a prediction model is very complicated and well beyond the scope of this project, however, preliminary steps can be made to improve *in vitro* methods such that they more accurately predict *in vivo* results.

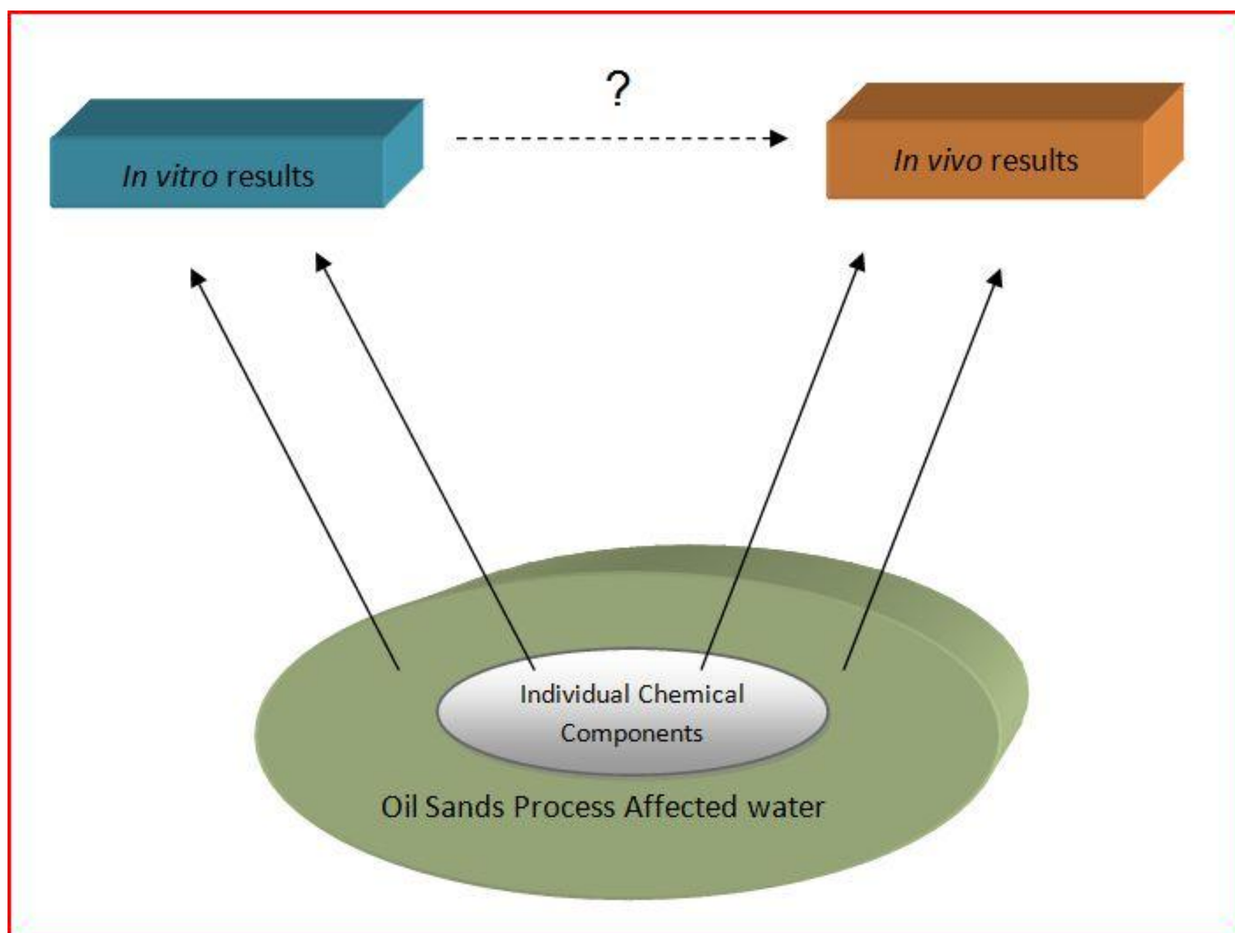

Figure 1.2 – Prediction model schematic**.** Illustration of the current gap between *in vitro* and *in vivo* data. *In vitro* improvements may help to bridge this gap and eventually reduce or eliminate the use of animals in regulatory toxicity testing.

Current alternative toxicity tests include mathematical models and experimental, whole-animal alternatives. Mathematically derived quantitative structure activity relationships (QSARs) are computer models designed to make *in vivo* predictions about toxicity based on physico-chemical properties of test chemicals. However, these models largely rely on existing data and future data compiled by animal tests.

Experimental alternatives include single-cell organisms, fish embryos, and vertebrate cell cultures (Schirmer, 2006). Single-celled prokaryotes, such as *Vibrio fischeri* (Microtox assay;

Frank et al., 2008), eukaryotes like the algae, *Selenastrum capricornutum* (Ke et al., 2010), and even protozoans like *Tetrahymena* (Dayeh et al., 2004) are commonly used as alternatives in toxicity testing but do not necessarily reflect the impact on vertebrate species (Lee et al., 2008). The zebrafish embryo test (*Dar*T) is typically a 48h exposure at fertilization followed by microscopic observation of development and vitality (Nagel, 2002). However, this method does require the maintenance of a breeding stock of non-treated, mature zebrafish and subsequent collection of delicate fertilized eggs. In 2005, *Dar*T was implemented to substitute fish tests in national regulatory testing of waste water in Germany. Such regulatory implementation of fish and mammalian cell cultures as alternatives to fish tests have yet to be established (Segner, 2004).

1.6 Vertebrate cell cultures as whole-animal alternatives

Vertebrate cell cultures have been recognized in toxicology research since the 1960’s. In 1985, Ahne proposed fish cell lines be used as alternatives to the fish lethality test in order to reduce the use of animals in ecotoxicological testing (Schirmer, 2006). The philosophy underlying the application of vertebrate cells for predicting the toxicity of chemicals in whole animals is that chemical interaction with an organism is initiated at the cellular level (Lee et al. 2008; Schirmer, 2006). Cells’ reactions may be useful in extrapolating and predicting subsequent tissue, organ and entire organism reactions to similar concentrations of those chemicals.

There are two types of vertebrate cell cultures that can be used to study animal cells *in vitro*: primary cultures and cell lines (Dayeh et al., 2005; Freshney 2005). The initiation of primary cultures involves the mechanical or enzymatic dissociation of tissue or organ pieces and

subsequent growth on plastic or glass surfaces (Bols and Lee, 1991; Freshney 2005). Successful subculturing of a primary culture results in a cell line (Bols and Lee, 1991; Dayeh et al. 2005).

Disaggregation of cells explanted from an organism followed by plating dispersed cells was first demonstrated in 1916 (Rous and Jones, 1916). Once cells are explanted from their *in vivo* environment, cell viability becomes fundamental, particularly with regard to experimental manipulations (Freshney, 2005). That is, cells can be used experimentally *in vitro* to elucidate toxicity. Primary cultures are useful because cells retain their differentiated function, however, primary cultures can be disadvantageous as they: 1) are often employed while recovering from their traumatic initiation; 2) may become heterogeneous over time; 3) may be harbouring resident pathogens; 4) are short-lived; 5) offer little ease of interlab reproducibility (Bols and Lee, 1991). Vertebrate cell lines, notably those of fish, are advantageous and particularly useful in aquatic toxicology due to their physiologically relevant storage and testing temperatures, ease of maintenance (Bols et al., 2005), and tolerance of simple culture media (Schirmer, 2006).

Vertebrate cell lines compare well with fish lethality tests in their relative sensitivity toward toxicants (Schirmer, 2006). However, cell monocultures employing a single cell line often show decreased absolute sensitivity when compared to *in vivo* studies (Segner, 2004; Magwood and George, 1996; Saito et al., 1994); most likely due in part to the invariable reduction in target sites compared to a whole organism (Schirmer, 2006). Reduced absolute sensitivity observed in vertebrate cell lines may also be due to decreased exposure duration (typically 24 h), the specific tissue origin of the cell line being used (cells derived from tissues of varying characteristics may be affected differently by toxicants of varying physico-chemical characteristics), and even the viability endpoint being measured (cell line bioassays may appear

less sensitive if the chosen endpoint is monitoring a cell viability criteria not directly, or initially affected by the toxicant).

Using a multitude of cell lines from varying species and tissue origin may ameliorate this limitation rendering the target site diversity of the *in vitro* assay more akin to that of a whole organism. Six cell lines of varying tissue and species origin were chosen for this study (Table 1.1) based on one or more of the following criteria: economic relevance of origin species, direct OSPW exposure of origin tissue, origin tissue function, and indigenity of origin species.

The use of cell lines derived from fish indigenous to the Athabasca region in Alberta was important because these species were more likely to be representative of the receiving environment (Giulio and Hinton, 2008) in which aquatic reclamation scenarios (i.e., EPLs) will be implemented. As shown in Table 1.1, the cell lines used are derived from tissues from rainbow trout, fathead minnow, goldfish, and bluegill. Nelson and Paetz (1993) state that rainbow trout and fathead minnow are indigenous to the Athabasca region, while goldfish can be found as well but primarily due to illegal release. It remains unclear whether bluegill are found in the Athabasca region specifically, although they are found in lakes in North America, such as the Great Lakes (Page and Burr, 1991).

The WF-2, FHML-W1, and FHMT-W1 cell lines were also from tissues that may provide physiologically relevant data regarding oil- or mining-derived chemical impact on whole-fish fry, liver tissue, and reproduction, respectively. Similarly, the respective liver- and gill epithelium-derived RTL-W1 and RTgill-W1 cell-lines may provide data important for predicting liver and gill function in whole-fish exposed to similar chemical compounds. The Rainbow trout and other Goldfish cell-lines have also been used extensively in ecotoxicology studies in the past

(Reeves et al., 2007, Dodd and Jha, 2009, Lee et al., 1997, Kuhnel et al., 2009, Dayeh et al., 2009, Woelz et al., 2009, Schnell et al., 2009).

Table 1.1 – List of cell lines used in this study, media supplementation, and rationale
*WF-2 cells were originally thought to have been derived from Walleye. However, this cell line turned out to be of Bluegill origin after subsequent molecular authentication.

Cell line	Common name	Species	Tissue	Source	FBS	Rationale
WF-2	Walleye* Bluegill	Sander vitreus* Lepomis macrochirus	Fry	B.W. Calnek (Wilensky and Bowser, 2005)	10%	-Economic relevance -Direct OSPW exposure
RTgill-W1	Rainbow Trout	Onchorynchus mykiss	Gill	Bols et al. (1994)	10%	-Direct OSPW exposure -Indigenous species -Economic relevance
RTL-W1	Rainbow Trout	Oncorhynchus mykiss	Liver	Lee et al. (1993)	5%	-Detoxifying organ -Indigenous species -Economic relevance
FHML	Fathead Minnow	Pimephales promelas	Liver	Lee et al. (2009a)	5%	-Indigenous species -Detoxifying organ
FHMT	Fathead Minnow	Pimephales promelas	Testes	Vo et al. (2010)	10%	-Indigenous species -Reproductive implications
GFSk-S1	Goldfish	Carassius auratus	Skin	Lee et al. (1997)	10%	-Direct exposure to OSPW

1.7 Assays used to detect acute toxicity

Effective whole-animal alternatives also require rapid and sensitive cell viability assays. Past studies have used fish cell lines to measure cytotoxicity, but have done so by looking at acute cytotoxicity as opposed to damage specific to one or more cellular viability criteria (Segner, 1998). This methodology is supported by Ekwalls (1995) theory of basal cytotoxicity stating that rapidly developing cell death due to chemical insult will likely be seen for similar chemical concentrations, regardless of the cell system applied. However, this concept is limited by the fact that every chemical can cause acute cytotoxicity at sufficient concentrations. This makes it difficult to learn anything about the nature of a toxicant and its mode of toxic action. Therefore, testing at appropriate chemical concentrations on numerous cell lines, using multiple viability assays can give valuable insight into the mechanism of cell death.

Numerous assays for cell viability have been developed, but the best are those that measure impairment to the integrity of the plasma membrane and metabolism using fluorescent indicator dyes because they can be used after only short exposures (Dayeh et al., 2005). Also, the development of multiwell fluorometric plate readers has made the use of these fluorescent indicator dyes quite easy and rapid (24h) (O'Connor et al., 1991). Microwell plates are later read by a fluorescent microwell plate reader, and relative fluorescence units are assigned to each well as they compare to control wells. Fluctuations in observed fluorescence units (FU) indicate changes in the cell viability criteria being measured. This combination of microwell plate and reader also allows for large numbers of replicates while conserve resources and cells, it helps

facilitate interlab reproducibility of results, and managing data on the computer is very convenient.

By using multiple fluorescent indicator dyes measuring slightly different cell viability criteria it is possible to deduce a compounds mode of toxic action and simply interpret results with greater strength (Schirmer, 2006). This study used three different indicator dyes measuring membrane integrity, metabolic impairment, and lysosomal activity.

1.7.1 Membrane integrity (CFDA-AM)

Membrane integrity is critical for cell viability as many cellular processes depend on effective compartmentalism. Membrane integrity has traditionally been measured by a cells ability to exclude large bulky dyes, such as Trypan blue. However, this method is tedious and requires observation and quantification of cells under the microscope (Dayeh et al., 2005).

The fluorescent indicator dye to measure membrane integrity in this study was 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). CFDA-AM diffuses into cells rapidly where non-specific esterases in viable cells convert it into a polar, fluorescent product, 5-carboxyfluorescein (CF) which is largely retained by cells. Decreases in FU indicating impaired esterase function could be due to declines in membrane integrity or decreases esterase activity (Dayeh et al., 2005). Loss of esterase function could be achieved through loss of cell membrane integrity causing eseterases to readily leave the cell during toxicant exposure where they are subsequently evacuated from the microwell or are denatured by the extracellular milieu. In some cases, the toxicant being tested may directly affect esterase activity. This would be rare and is a good reason why more than one fluorometric indicator dye should be used.

1.7.2 Metabolic impairment (Alamar blue)

Reduction of the dye resazurin (commercially available as Alamar blue, AB) in both cytoplasmic and mitochondrial locations by enzymes, such as diaphorases, is thought to be indicative of cellular metabolic integrity. Thus, decreasing FU as measured by AB suggests impairment of cellular metabolism. Metabolism can also be measured by ATP content or by a cells ability to reduce 3-(4,5-dimethylthizol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (Segner, 1998), but AB is advantageous because it can be measured fluorometrically or spectrophotometrically and can even be used repeatedly over time on the same culture (Ganassin et al., 2000).

1.7.3 Lysosomal activity (Neutral red)

Neutral red (NR; 3-amino-7-dimethylamino-2-methylphanzine hydrochloride) can detect cell damage specific to the lysosomes (Dayeh et al., 2005) as only viable cells will accumulate NR in the lysosomes (Borenfreund and Puerner, 1984). However, it should be noted that NR accumulation in the lysosomes is likely dependent on intact cell membranes, sufficient metabolic integrity, as well as a functioning lysosomes making NR a detector of all three cellular viability criteria (Dayeh et al., 2005).

1.8 Thesis goals and organisation

The goal of this thesis is to compare the sensitivity of six fish cell-lines to OSPW samples from the Athabasca Oil Sands, and apply them as rapid, inexpensive alternatives to the use of whole fish in ecotoxicology testing. This will be achieved through the use of cytotoxicity or cell

viability assays monitoring different cellular processes or endpoints to determine the live/dead status of cells.

2 Materials and Methods

2.1 Fish cell cultures and maintenance

Six fish cell-lines of varying tissue and species origin were chosen for this study: WF-2 (Wilensky and Bowser, 2005) originally reported as being derived from Walleye (Sander vitreus) but subsequent genetic barcode testing identified the cell line as Bluegill (Lepomis macrochirus); two rainbow trout (*Oncorhynchus mykiss*) cell lines derived from liver (RTL-W1; Lee et al., 1993) and gill (RTgill-W1, Bols et al., 1994; ATCC Accession No. CRL-2523) tissue; two fathead minnow (*Pimephales promelas*) cell-lines derived from testis (FHMT; Vo et al., 2009) and liver (FHML; Lee et al., 2009) tissue; and GFSk-S1 cells (Lee et al., 1997), derived from Goldfish skin (*Carassius auratus*).

Cells were routinely cultured in 75 cm^2 tissue culture flasks at ambient room temperature (20 ± 2°C) in Leibovitz's L-15 culture medium (Gibco BRL, Bulrington, ON, Canada) supplemented with fetal bovine serum (FBS, Sigma; 10% for WF-2, Rtgill-W1, GFSK-S1, FHMT and 5% for FHML, RTL-W1) and 2% penicillin-streptomycin (100µg/ml, 100 IU/ml penicillin; Gibco, BRL). Culture supplies and subcultivation procedures were as previously described (Bols and Lee, 1994; Schirmer et al., 1994). Prior to toxicant exposures, 100µl of cell suspension was plated in 96-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NY) at a cell density ranging from 2 X 10^4 to 9 X 10^4 (specific optimal cell density was not determined, but cells were always plated within an appropriate range as determined by standard curves) cells per 100µl L-15/ex and allowed to adhere for 24h. L-15/ex is a simple exposure medium originally developed by Schirmer et al. (1997) to study polycyclic aromatic

hydrocarbon toxicity in the RTgill-W1 cell line. This simple media formulation (Appendix A) supports cell viability but lacks supplements that may interact with toxicants in some way during experiments resulting in potential errors in derived effective concentrations. L-15/ex is also quite inexpensive and can be used to assess whole-water samples, such as produced water and effluents (Dayeh et al., 2005).

2.2 Species of origin authentication of cell lines

All sample preparation was performed in a sterile flow hood. Powder-free nitrile gloves were worn to limit the possibility of human DNA contamination. Confluent flasks of cells were rinsed in Hank's Buffered Salt Solution (HBSS) and the cells were removed from the flask using TrypLE, a recombinant form of trypsin, trademarked by InVitrogen, or scraped using cell scrapers (Falcon). Enzymatic activity was diluted after 5 min by adding 8 ml of HBSS to a 2ml of TrypLE cell mixture. This cell mixture was centrifuged at 1000g for 5 min on a bench top centrifuge. The supernatant was removed and the cell pellet was resuspended in 200 to 1000 µl of sterile, HBSS to have a cell density of approximately 10^4 cells/µl. A 50 µl sample of the cell suspension was then blotted onto FTA cards (Whatman), allowed to dry within the flow hood and stored before being taken to the DNA barcoding laboratory at the University of Guelph. FTA cards are trademarked filter papers that contain a proprietary formulation from Whatman that lyses cells and denatures proteins upon contact. The nucleic acids (DNA & RNA) are retained in the filters and are protected from UV damage and from bacterial or fungal attack.

2.3 Chemical Preparation

Preliminary testing of all cell lines was done using known toxicants of varying physicochemical characteristics using all fluorometric dyes in order to validate sufficient bioassay sensitivity. A stock solution (10,000 µg/ml) of the model toxicant $CuSO_4$ (Castaño et al., 1995; Ryan and Hightower, 1994; Segner et al., 1994) was prepared by weighing 0.001 g of CuSO4 (Sigma) on an analytical balance and dissolving in 10 ml L-15/ex. Stock solution was then filter sterilized using a 10 ml syringe and a 0.2 µm pore-size syringe filter. Sterile stock solution then made into serial dilutions (0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100 µg/ml) again using L-15/ex.

A commercial (Acros) naphthenic acid preparation (CNA – 0.1, 1, 10, 20, 25, 30, 40, 50, 100, 1000 µg/ml), and a crude naphthenic acid extract (Cr.NA – 0.1, 1, 7, 15, 30, 60, 120, 250, 500, 1000 µg/ml; prepared as per Frank et al., 2006) were also assayed. The CNA stock solution (100,000µg/ml) was made by dissolving solid CNA in a solution of 70% tissue-culture grade ethanol (Commercial Alcohols Inc.) made with e-pure water. The stock solution was not filter sterilized because the sterilization filters clogged at such a high NA concentration. The stock CNA solution was diluted to the highest experimental concentration (1000 µg), then filter sterilized similar and serial dilutions made in L-15/ex.

Sodium dodecyl (lauryl) sulphate (SDS, CAS 151-21-3), an anionic surfactant, was weighed, dissolved in L-15/ex and filter sterilized as was the $CuSO_4$ solution. Leibovitz's-15/ex was used again to prepare serial dilutions of SDS (0.1, 1, 10, 20, 25, 30, 40, 50, 100, 1000 µg/ml).

2.4 OSPW-Sample Preparation

Since direct testing of water samples is not feasible with cell cultures, OSPW samples were tested at 80% concentration after mixing with a 5x concentrate of L-15/ex. This is the exposure media that contains basic salts and is physiologically compatible with fish cells in culture. Forty-nine OSPW samples were shipped in 100 ml vials from Syncrude Canada, Ltd. and subsequently stored in the dark at 4°C. Only OSPW sample numbers were disclosed prior to toxicity testing, not chemical composition. Leibovitz's – 15/ex media was prepared as described by Schirmer et al. (1997) but solutes were dissolved in 1/5 the suggested volume of ePure water resulting in a 5X concentrated version of L-15/ex media. Eight ml of each OSPW sample was then added to 2 ml of the 5X concentrated L-15/ex solution to adjust the osmolality of each OSPW sample. Each sample was filter sterilized using 10 ml syringe (BD 309604) and 0.2µm syringe filter (VWR 28145-501). Adding OSPW samples to a concentrated solution of L-15/ex resulted in 80% OSPW-sample concentration that greatly minimized necessary sample preparation. More importantly, this method allowed the direct exposure of cell lines to iso-osmotic, chemically unmodified OSPW samples. Furthermore, this method reduced labour intensive sample preparation and helped facilitate rapid sample assessment.

Osmolality and pH of raw and L-15/ex-containing samples were measured using a vapour pressure osmometer (Westcor 5001B) and a pH meter, respectively. Cell viability assays were done on the WF-2 cell line to verify that the measured fluctuations in pH were not cytotoxic.

2.5 Chemical and OSPW sample exposures

After a 24h incubation period allowing cells to adhere to the bottom of plate wells, L-15/ex was removed from the plate wells by inverting over paper towel. This plate-inversion method helped eliminate the risk of unwanted cell aspiration and effectively reducing the overall cell manipulation during the bioassay resulting in more consistent results and smaller standard deviations between wells. Cells were then exposed to 100µl/well of serial dilutions of the aforementioned chemicals in replicates of 6-8 wells per sample or chemical concentration. Each 96-well plate also contained 6-8 no-treatment wells containing only cells and fluorescent dye and 6-8 no-cell wells containing only fluorescent dye from which background fluorescence could be calculated and subtracted from experimental wells after viability assays. Treated plates were then incubated at their routine culture temperature of 20°C for 24h. It should be noted that previous experiments have confirmed fish cell lines to survive for at least 48h in L-15/ex made up in both cell culture water (Schirmer et al., 1997) and industrial effluents (Dayeh, 2004).

2.6 Fluorometric indicator dyes

Three fluorometric dyes were used to measure cell viability; Alamar Blue (AB; Biosource International DAL1100), 5'-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Sigma), and Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (NR; Sigma). Dye preparation was followed as described in Dayeh et al. (2003) (Appendix C).

2.7 Cell line viability assays

Step-by-step protocols for alamar blue and CFDA-AM can be found in Ganassin et al. (2000), and in Dayeh et al. (2003) for neutral red (adapted in Appendix C). After the 24h incubation period chemical compounds were removed from plate wells by inversion over paper. Microwell plates were treated with the fluorometric dye solutions and then incubated for 1h at 20°C after which fluorescence was quantified using the SpectraMax Gemini XS microplate reader (Molecular Devices 02518) at respective excitation and emission wavelengths of 485 and 530 nm for CFDA-AM, 530 and 595 nm for AB, and 530 and 645 nm for NR.

2.8 Data analysis

The no-cell control wells were treated the same as the experimental wells during the experiment. The subsequent designation of these wells as *blanks* during fluorometric measurements automatically subtracted their raw relative fluorescence units (RFUs) from that of the no-treatment control wells and all experimental wells in order to eliminate background fluorescence. The 6-8 wells per plate for each chemical concentration were averaged and expressed as a percentage of the no-treatment control wells (L-15/ex control). Decreasing cell viability indicating toxicity was marked by decreasing RFUs as compared to the L-15/ex control. Results were plotted and means and standard deviations were calculated in Microsoft Excel (Microsoft Corporation, Redmond, WA). EC_{50} values were calculated using GraphPad Prism 5.02 (Appendix D). Regression and correlation analyses were done using GraphPad InStat 3.06. Unpaired *t*-test was used to compare two EC_{50} values, and an ANOVA was used for comparing three or more.

For OSPW samples, significant deviation of cell viability from control was measured by one-way analysis of variance, followed by Dunnett's test (α=0.05). Regression analysis was done through the generation of a Pearson correlation matrix to identify simple correlations between cell viability and OSPW sample components. Multiple-regression analysis was also done to look for multicollinearity within data for the individual components of the OSPW samples.

3 Results

3.1 Authentication of cell lines

DNA barcoding performed at the University of Guelph, through the Barcode of Life Database (BOLD), with universal primers for cytochrome c oxydase gene I, used for fish species identification as reported by Ivanova et al., 2007, confirmed the origin of species for 5 of the 6 tested fish cell lines. WF-2 were the only cells that did not come back as originating from Walleye. The cells were attributed to belong to Bluegill (Lepomis macrochirus), but since Bluegills are commonly occurring species in North America, the WF-2 cells were still used as representative indigenous fish species.

3.2 Standard curve generation for cell viability assays

Standard curves were generated for the three fluorometric indicator dyes using all six cell lines. The purpose of these standard curves was to demonstrate that increases in measured RFUs correspond to increases in the presence of viable cells. Serial dilutions of a high-concentration cell suspension were exposed to AB, CFDA-AM and NR as per the standard protocol for these indicator dyes. For all cell lines tested, linear correlation of increasing cell numbers to increasing fluorescence units could be established routinely for all three fluorescence assays. A representative graph for FHML is presented in Figure 3.0. The R^2 values close to 1 indicate a strong correlation between increasing number of viable cells and increasing RFUs. Therefore, in an experiment where cells are exposed to putative toxicants, cytotoxicity can be quantified by decreases in measured RFUs.

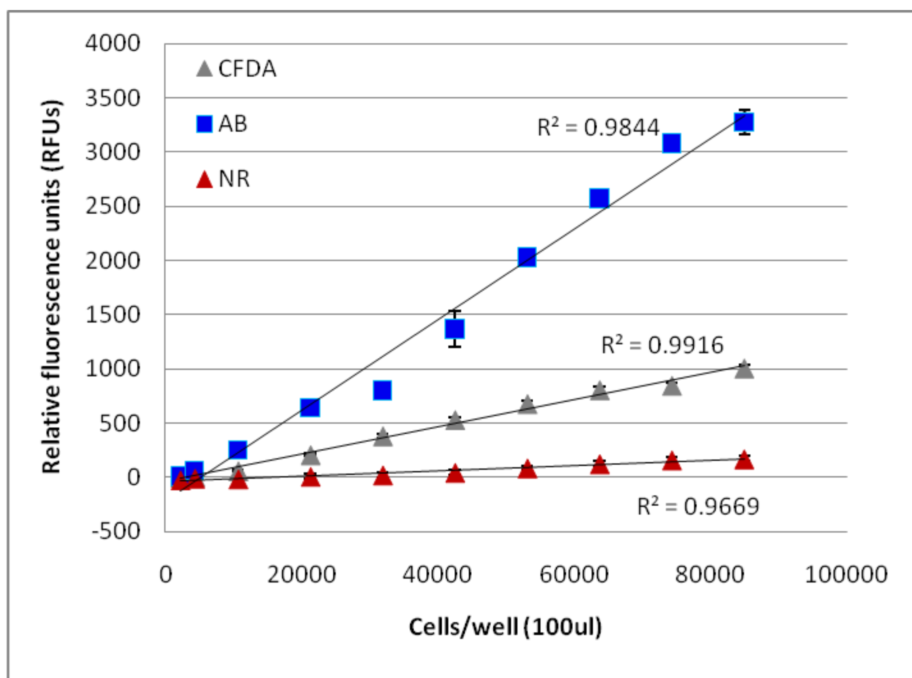

Figure 3.0 - Fluorescence response of FHML-W1 cells to AB, CFDA-AM, NR. A standard curve was generated to determine the correlation of increasing cell number and relative fluorescence units as measured by these indicator dyes. Cells were plated in a 96-well microplate, incubated for 24 hours at 18°C. Cells then underwent standard exposures to AB, CFDA-AM, and NR. Six-well replicates (n=6) were used for each cell concentration. Data points are shown as mean RFUs of the six wells with error bars representing standard deviations. R^2 values close to 1 indicate a strong correlation between increasing number of viable cells and increasing RFUs.

3.3 Cell line exposure to chemicals

Cell lines were used to evaluate the toxicity of four putative toxicants as measured by the suite of three viability assays; CFDA-AM, alamar blue (AB), and neutral red (NR).

The four chemical compounds evaluated were $CuSO_4$, sodium dodecyl sulphate (SDS), a commercial naphthenic acid (CNA), and a crude naphthenic acid extract (Cr.NA). Cell exposure to all four chemical compounds caused an overall dose-dependent decline in cell viability as measured by at least two fluorometric indicator dyes (graphs shown in Appendix E). EC_{50} values (concentrations causing a 50% decline in cell viability) calculated for individual cell lines (Table 3.1) for $CuSO_4$ using AB and CFDA-AM ranged from 3.5 ± 1.6 to 8.32 ± 0.9 µg/ml and

were not statistically different ($p>0.05$) from one another for any cell lines except for FHMT (38.7 ± 3.49 µg/ml).

NR was not used to assay $CuSO_4$ toxicity as it has yielded confounding results in a previous study (Dayeh et al., 2005). This finding was validated by exposing RTgill-W1, FHML, and FHMT cells to $CuSO_4$ ranging from 0.01 – 100µg/ml for 24h in L-15/ex and assaying with NR. Results showed a similar biphasic curve with decreases in viability observed until 10µg/ml at which point cell viability appeared to increase again (Appendix F – Figure 5.7).

Viability data for SDS, CNA, and Cr.NA as measured by CFDA-AM was problematic. As cell viability measured with AB and NR dropped, CFDA-AM measurements appeared to spike, showing erratic increases in relative fluorescence units. In the event that the relative viability of cells did not drop below 50% of the control, EC_{50} values were not calculated; this was often the case for CFDA-AM when testing CNA and is simply indicated by a dash in Table 3.1. Where EC_{50} values could be calculated for these chemicals, the values were quite erratic ranging from 34.09 ± 7.73 µg/ml to 2168 ± 152 µg/ml, often much greater than EC_{50} values calculated using AB or NR, and always significantly greater than reported LC_{50} values obtained *in vivo*.

For these latter three chemical compounds, there was a relatively similar dose-dependent decrease in cell viability as measured by both AB and NR. In most cases, the EC_{50} values obtained for both fluorometric dyes were not statistically different from one another ($p>0.05$). Where values were significantly different they were still relatively close and well within the same order of magnitude. The only exception were the values obtained for Cr.NA using the WF-2 cell line ($p=0.0466$).

Reported lethal concentrations (LC_{50} - the concentration lethal to 50% of the test organisms *in vivo*) for each of the test compounds determined *in vivo* are listed in Table 3.1. Where multiple values were found, the mean was calculated and standard deviation indicated. Correlations between calculated EC_{50} (μg/ml) values for all test compounds and the reported LC_{50} (μg/ml) values reported in the literature are shown in Figure 3.1. Individual test compounds are not labelled for visual clarity but can be inferred from Table 3.1. There is a relatively good correlation observed for all three fluorometric dyes (AB $R^2 = 0.7395$, $p<0.0001$; CFDA-AM $R^2 = 0.5358$, $p<0.001$; NR $R^2 = 0.6142$, $p<0.0001$) indicating relative sensitivity of the cell line bioassay comparable to the *in vivo* LC_{50} data. The dashed line on each graph indicates a correlation of 1.0. Although there appear to be discrepancies between the absolute sensitivity of the *in vitro* and *in vivo* data it should be noted that they do show considerable relative agreement with each other.

Appearance of cells was also monitored during experiments via phase contrast microscopy. All cell lines showed very similar morphological differences pre- and post-exposure. Figures 3.2 – 3.3 show only the WF-2 cell line before and after chemical exposure. In all cases, changes in morphology were present at the highest chemical concentrations as compared to pre-exposure morphology. Exposure to 100μg/ml $CuSO_4$ resulted in cell shrinkage accompanied by a dark cell contrast. Similarly, exposure to Cr.NA resulted in cell shrinkage but cells appeared quite rounded and not as dark as those seen after $CuSO_4$ exposure. Lastly, the cells observed after exposure to SDS and CNA were completely disrupted, showing absolutely no morphological similarity to their pre-exposure counterparts. These last two compounds appear to have totally deteriorated the integrity of the cellular membranes.

Table 3.1 ***In vivo*** **and** ***in vitro*** **effective concentrations after exposure to CuSO4, SDS, CNA, and Cr.NA**

Fish/Cell line	96h LC_{50} or 24h EC_{50} (µg/ml) ± STD (n)			
	$CuSO_4$	SDS	CNA	Cr.NA
Bluegill*	3.2±4.3(3)[5,3]	4.5 (1)[1]	30.24 ± 22.57(12)[1¥]	64.9 ± 14.5(3)[11]
WF-2 cell line				
AB	8.13 ± 1.5(4)	31.35 ± 5.37(3)	42.91 ± 4.2(3)	66.64 ± 9.08(3)
CFDA-AM	8.32 ± 0.9(4)	141.3 ± 58.5(3)	-- (3)	402.1 ± 149.3(3)
NR	§	18.1 ± 5.09(3)	13.44 ± 2.1(3)	120.9 ± 19(3)
Rainbow Trout*	0.55±0.48(2)[6,7]	14.4 ± 15.1 (13)[1]	30.24 ± 22.57(12)[1¥]	64.9 ± 14.5(3)[11]
RTL-W1 cell line				
AB	4.04 ± 0.143 (4)	11.51 ± 0.45 (3)	15.48 ± 3.6(3)	45.26 ± 2.44(3)
CFDA-AM	3.92 ± 1.1(4)	34.09 ± 7.73 (3)	-- (4)	158.8 ± 24.2(3)
NR	§	16.26 ± 6.3 (3)	18.22 ± 5.36(3)	174.23 ± 26.1(3)
RTgill-W1 cell line				
AB	6.06 ± 1.25(3)	5.89 ± 1.23(3)	6.84 ± 1.95 (3)	76.35 ± 12.63 (3)
CFDA-AM	7.08 ± 0.67(3)	398.93 ± 104.5(3)	81.48 ± 52.83 (3)	682.7 ± 163.4 (3)
NR	*27.0 ± 7.87(3)*	4.11 ± 0.16(3)	4.23 ± 0.73 (3)	126.07 ± 72.4 (3)
Fathead minnow*	0.67±0.37(3)[4,8,9]	7.7 ± 3.1 (12)[1]	30.24 ± 22.57(12)[1¥]	64.9 ± 14.5(3)[11]
FHMT cell line				
AB	3.47 ± 0.84(4)	21.75 ± 0.67(3)	24.7 ± 14.22 (3)	146.3 ± 46.61 (3)
CFDA-AM	38.7 ± 3.49(3)	2168 ± 152(3)	-- (4)	536.7 ± 128.2 (3)
NR	*37.8 ± 17.1(3)*	27.18 ± 0.87(3)	12.67 ± 4.39 (3)	319.9 ± 159.95 (3)
FHML cell line				
AB	7.83 ± 3.6(3)	14.0 ± 1.43(3)	14.15 ± 8.36 (5)	74.49 ± 15.27(3)
CFDA-AM	4.9 ± 2.6(3)	122.7 ± 61.56(3)	-- (4)	370.2 ± 58.36 (3)
NR	*67.69 ± 19.2(3)*	26.2 ± 3.8(3)	13.98 ± 10.51 (3)	103.67 ± 51.7(3)
Goldfish*	2.5±1.9(3)[3,4]	28.4 (1)[1]	30.24 ± 22.57(12)[1¥]	64.9 ± 14.5(3)[11]
GFSK-S1 cell line				
AB	5.086 ± 0.29 (4)	9.08 ± 0.47 (3)	24.31 ± 2.65(3)	101.6 ± 8.2(3)
CFDA-AM	3.5 ± 1.6 (3)	180.14 ± 36.8 (3)	-- (3)	390.97 ± 185.7 (3)
NR	§	20.09 ± 2.67 (3)	26.44 ± 5.13 (3)	163.8 ± 28.6(3)

* 96hLC50 reported in literature

"—"= EC_{50} value not calculated because relative fluorescence units did not fall below 50%

§ - Neutral red assay not done for $CuSO_4$.

¥ - Data retrieved from pesticideinfo.org. CNA (CAS # 61790-13-4) data specific to these fish were scarce, but 96h LC_{50} data from 5 fish were averaged (Russian sturgeon, *Acipenser gueldenstaediti*; Common goby, *Neogobius melanostomus*; Chum salmon, *Oncorhynchus keta*; Kutum, *Rutilus frisii kutum*; Caspian roach, *Rutilus rutulius caspicus*)

Underlined EC_{50} values *within a cell line (row) and for a single toxicant* were found not to be statistically different from one another (p>0.05)

Superscripts correspond to numbered references – (11) refers to Microtox assay done by Frank et al., (2006).

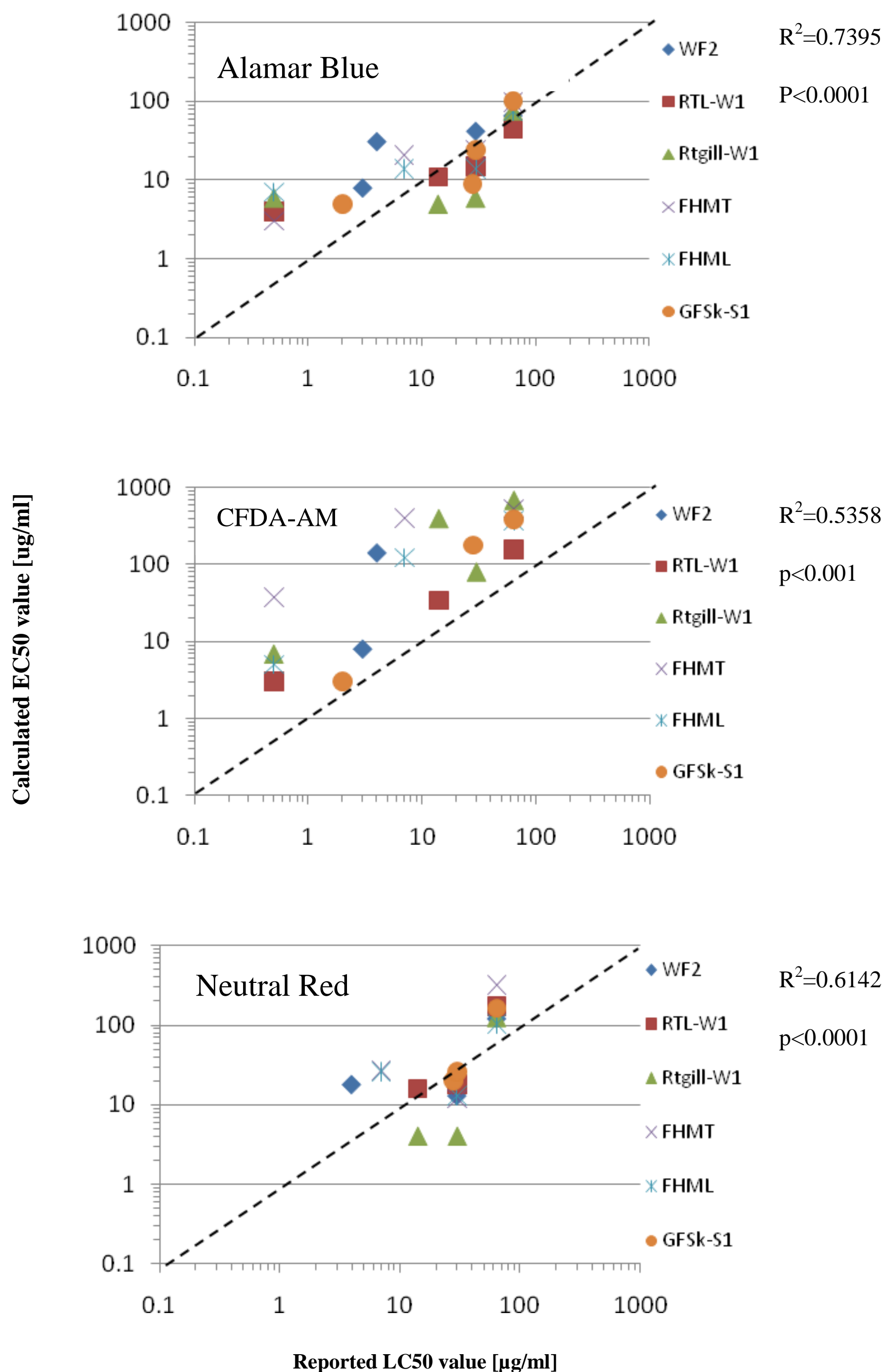

Figure 3.1 - Correlation between AB_{50}, CFDA-AM_{50}, and NR_{50} values and reported LC_{50} values found *in vivo*. WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHMT, and FHML cell lines were exposed to varying concentrations of $CuSO_4$, SDS, CNA, and Cr.NA. Graphs illustrate the correlation between the calculated EC_{50} value and the reported LC_{50} values in the literature for a given test compound (values taken from Table 2.1). Linear regression analysis was done to generate correlation coefficients and p-values. Individual chemical compounds not labelled for visual clarity. Data points represent the average of three EC_{50} values calculated from separate experimental trials. Dashed line indicates *in vitro/in vivo* correlation of 1.0.

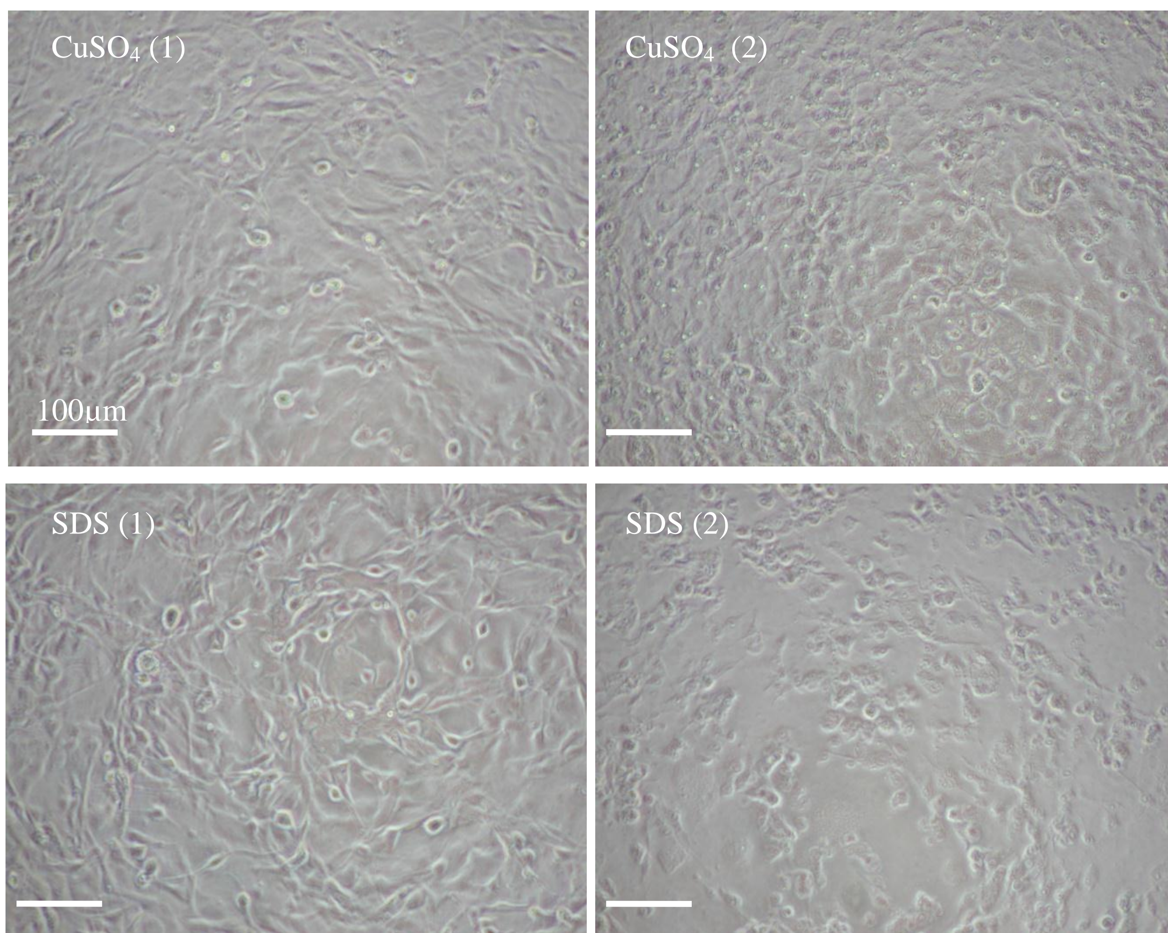

Figure 3.2. Phase contrast micrographs of WF-2 cells before and after 24 h exposure to CuSO4 and SDS. WF-2 cells were exposed to varying concentrations of $CuSO_4$ and SDS in L-15/ex media for 24 h. Phase contrast images were taken before (1) and after cells exposed to 100µg/ml $CuSO_4$ or SDS (2). Cells exhibit typical morphology pre-exposure but appear dark and shrunken after $CuSO_4$ exposure. WF-2 cells appear disrupted and globular after exposure to SDS. In the latter case, this is likely due to membrane damage from exposure to a surfactant.

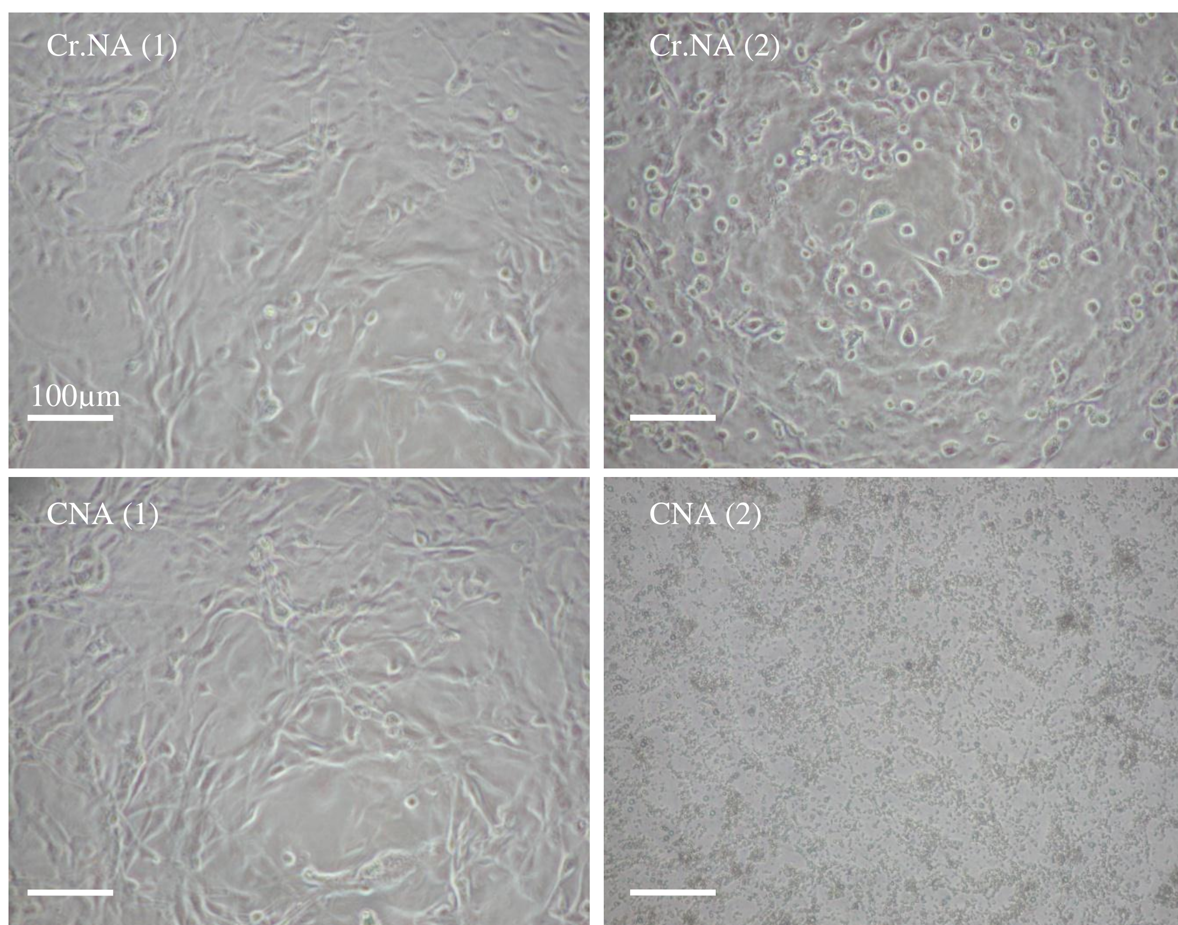

Figure 3.3. Phase contrast micrographs of WF-2 cells before and after 24 h exposure to Cr.NA and CNA. WF-2 cells were exposed to varying concentrations of Cr.NA and CNA in L-15/ex media for 24 h. Phase contrast images were taken before (1) and after cell exposure to 1000µg/ml µg/ml Cr.NA or 50µg/ml CNA (2). Cells exhibit typical morphology and appear very similar pre-exposure but appear quite rounded and some have detached from the plate after Cr.NA exposure. WF-2 cells have been totally disrupted and appear as a monolayer of homogenous remains after exposure to CNA indicating severe membrane damage.

3.4 OSPW graphs and correlations

After standard curves and dose-response curves for positive control chemicals were generated, cell lines were exposed to iso-osmotic OSPW samples prepared in the minimal media, L-15/ex. Samples prepared in L-15/ex ranged in osmolality from 270 – 326 mOsm/kg and pH from 6.9 – 9.1. Most cells in culture have a wide tolerance for fluctuations in osmolality (Waymouth, 1970), but anything with the range of 260 – 320 mOsmol/kg is acceptable (Freshney, 2005). In a separate experiment, WF-2 cells were exposed to L-15/ex solution with pH ranging from 6 – 9 (pH adjusted by drop-wise addition of HCl or NaOH) with no significant decreases in viability as measured by AB (data not shown).

Decreases in cell viability were detected using all three fluorometric indicator dyes, dropping below 50% of that of the control for some OSPW samples. Some samples had a slight stimulatory effect on cell viability, and further tests may need to be performed to elucidate the cause of such an effect.

Cell line responses to OSPW samples as measured by AB were the most consistent both from trial to trial for each cell line and between cell-lines (Figure 3.4). One-way analysis of variance (ANOVA) followed by Dunnett's test (α=0.05) identified a number of OSPW samples for which the mean RFUs deviated significantly from the control values (not identified in the graphs for lack of space). OSPW samples (Table 3.2) 8, 12, 13, 16, 19, 42, 43 resulted in a decrease in viability below 50% of the control for all cell lines. Samples 23, 35, 36, 44, 47, 48 showed similar decreases below 50% of the control in all cell lines but one (most often GFSk-S1). And, samples 7, 17, 18, 20, 24 and 37 showed a similar decrease in 3 or more cell lines. All

samples showing such decreases below 50% were found to be significantly different than the control ($p<0.01$), with the exception of samples 47 and 48 using the GFSk-S1 cell line.

Data was extremely inconsistent for CFDA-AM and NR assays showing high variability and low consistency between trials or across cell lines (Appendix G – Figures 5.17 & 5.18). Based on the erratic results and the high degree of inconsistency of the data generated by CFDA-AM and NR when testing the crude NA samples most of the analysis in this section was done using AB.

The chemical composition of the OSPW samples was unknown throughout the testing period so as to not bias reporting. When the composition of the samples was revealed (Table 3.2), data analysis was done to identify correlations between the viability data and the relative concentrations of the OSPW sample components. A correlation was found between the concentration of NAs present in a given sample and the measured viability of cells exposed to that sample. Specifically, with increasing concentrations of NAs, decreases in cellular viability were measured. Table 3.3 summarizes the degree of correlation between cell viability and NA concentration for each OSPW sample. Again, AB was the most consistent and also showed the highest correlation between decreasing viability and increasing NA concentration with R^2 values as high as 0.6171 ($p<0.0001$). The cell line responses to OSPW-sample exposure were not nearly as consistent when measured by CFDA-AM or NR. The highest correlation coefficient produced by linear regression analysis using CFDA-AM was 0.4352 ($p<0.0001$), while none of the data generated by the NR assay was considered significant.

Regression analysis was also done on the other components of the OSPW samples revealing a high degree of correlation between the viability of cells and the concentration of

OSPW sample-components other than NA, specifically, Na, HCO_3, and sample conductivity. Table 3.4 shows correlation between these sample components and cell viability after 24 h exposures. AB generally had the highest correlation coefficients ranging from 0.4025 – 0.8757 ($p<0.0001$). CFDA-AM was much lower (R^2=0.09398 – 0.6317), and NR almost never generated a significant correlation (with the exception of the RTgill-W1 cell line).

Multiple regression analysis of the OSPW component data revealed a high degree of multicollinearity indicating concomitant increases between a number of the OSPW sample components. The R^2 values for multicollinearity were high (>0.8) for all components listed in Table 3.2 except pH, and above 0.9 for conductivity, bicarbonate, and sodium. A Pearson correlation matrix (Table 3.5) between select OSPW sample components and RFUs (WF-2, AB) shows significant correlations between rising levels of NA, HCO_3, and between sample conductivity and major ions (Na, Cl, and K).

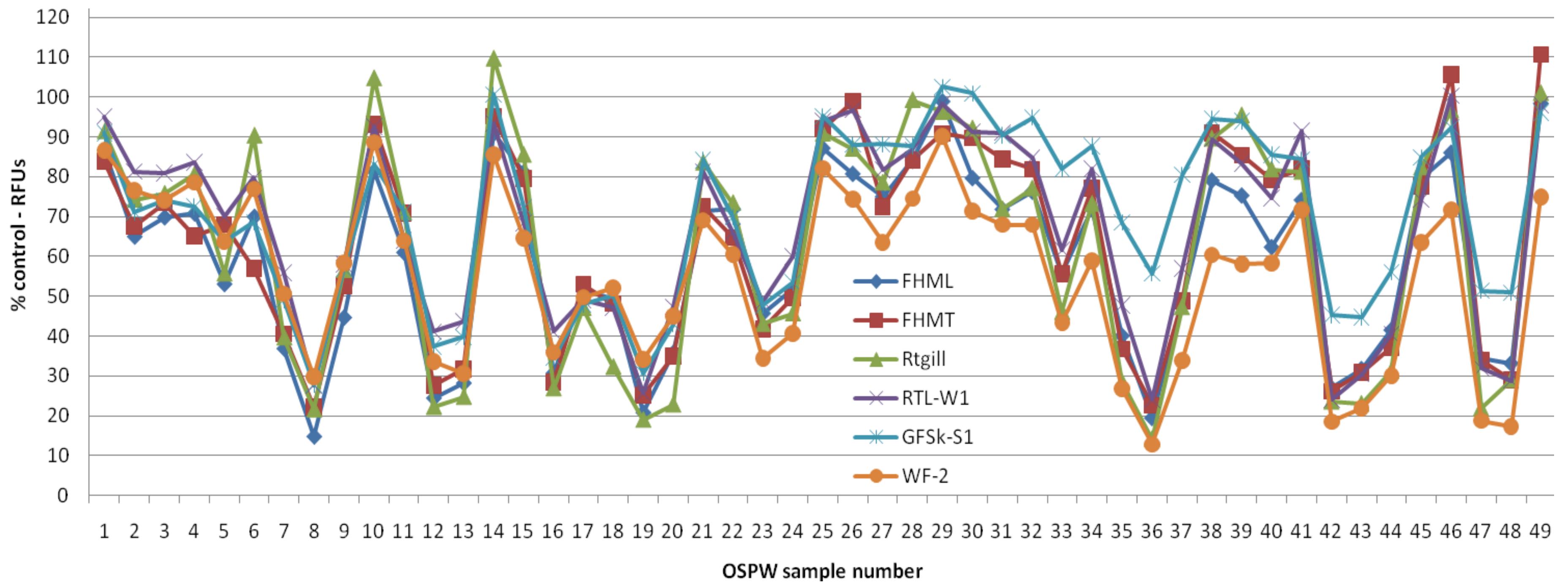

Figure 3.4 – Cell line response to 24 h exposure to OSPW samples as measured by AB. FHML, FHMT, RTgill-W1, RTL-W1, GFSk-S1, and WF-2 cells were exposed to iso-osmotic OSPW samples for 24 h at 18°C. Cell viability was then measured by AB. Data points represent the mean of 4 separate experiments (each experiment consisted of 6-well replicates for each OSPW sample). Cells were plated at densities ranging from $3.3X10^4$ – $8.0X10^4$ cells/well.

Table 3.2 – Annotated chemical composition of OSPW samples (µg/ml unless stated otherwise; chemical data obtained from Dr. Mike MacKinnon).

Sample #	Source	pH	NA	Cond. (uS/cm)	HCO_3	Na	SO_3	NH_4 (ppm)	K	Mg	Ca	Cl
1	FE1	7.20	1.35	704	182	76.9	259	0.286	0.5	32.7	57.6	4.6
2	FE2	7.60	3.01	696	367	144	37.6	0.274	0.5	16.9	21.6	35
3	FE3	7.60	2.85	690	348	143	43.8	0.295	0.5	16.5	19.5	31
4	FE4	7.60	3.56	667	322	137	58.4	0.278	0.5	17.6	18	27
5	FE5	7.90	11.20	2340	481	614	777	2.08	8	39	20	140
6	FE6	7.70	2.48	1260	385	273	308	0.209	0.5	30.8	19.7	34
7	TPWPOND	8.10	21.64	2040	664	528	122	0.183	6.16	10.1	8.95	240
8	STORPD	8.20	36.80	2740	959	795	234	0.289	7.21	11.3	10.8	320
9	BPIT	8.00	8.29	1530	575	379	164	0.349	5.13	18	14.2	110
10	SHALWL-Ditch	7.40	0.37	620	223	91.2	125	0.212	0.5	38.1	19.3	14
11	CT POND	7.70	27.65	3750	357	1040	1220	0.01	14.6	33.5	35.9	650
12	MLSP-OP	7.60	44.22	1920	1030	548	71.6	2.31	0.5	11.6	27.8	220
13	BCV-A5	8.00	24.05	2490	821	628	89.2	0.01	0.5	21.6	45.9	480
14	MLAKE	7.40	0.30	340	160	22.9	29.8	0.155	0.5	10.1	35.9	12
15	BCV-B16	7.80	1.94	1280	346	176	229	0.234	0.5	34.6	103	150
16	DD B2506	7.50	65.53	2310	1050	702	295	2.72	8.96	14.2	18.1	240
17	MLSB	7.60	5.50	3200	648	704	424	16.9	15.6	11.9	19.5	440
18	WIP	7.70	15.42	3380	742	844	384	13.4	15	10.7	16	530
19	DDW	7.90	30.23	2740	969	677	300	2.61	8.9	12.7	15.9	250
20	WIP	7.70	21.39	3460	825	793	383	14.9	14.2	10.8	16.3	520
21	SCL_Golden Pond	8.83	3.39	1680	163	225	746	<0.01	1.1	57.6	115	38
22	SUN_High SO4WL	7.64	15.19	2980	239	437	1590	<0.01	15.9	118	200	4.4
23	SUN_4m CT	8.25	22.34	1953	512	326	595	0.22	13.5	58.5	83.3	43
24	SUN_NatWL	9.11	44.12	1242	504	292	204	0.56	11.9	14.1	19.4	17
25	CNRL	9.32	2.37	256	120	22.3	22.2	<0.01	0.6	8.7	23.0	4.7
26	South Beaver	7.57	3.19	345	231	30.8	5.1	<0.01	0.8	10.3	41.3	6.0
27	SCL_NWID Ditch WL	8.19	2.27	663	333	94.4	37.9	0.11	1.6	22.1	39.7	56
28	SUNCTWL_Waste Area 11	8.69	7.03	868	169	112	308	0.18	9.8	32.4	52.4	6.5
29	U-SHAPED POND	8.91	4.64	342	80	37.1	79.4	0.17	1.0	8.0	29.1	25.0
30	FE1	7.69	1.32	729	173	78.2	249	0.12	1.0	30.0	53.9	5.6
31	FE2	8.35	3.18	688	322	148	51.7	0.70	1.0	15.4	14.9	33.0
32	FE3	8.52	2.42	674	308	147	54.8	0.14	1.0	15.0	13.5	29.0
33	FE5	8.96	10.56	2680	403	630	784	0.23	8.7	37.6	15.1	140
34	FE6	9.03	2.53	1252	259	268	341	0.34	1.0	29.3	12.4	37.0
35	TPW POND	9.20	20.01	2080	553	519	119	0.28	1.0	8.7	5.7	230
36	STOR POND	8.81	44.97	3010	896	780	275	<0.01	7.6	11.0	9.7	310
37	BPIT	9.06	12.14	1584	419	378	188	0.15	1.0	15.8	8.9	112
38	DEEP WL	7.83	0.92	547	258	72.2	78.6	<0.01	1.0	23.9	30.9	12.0
39	SHALWL-Ditch	8.61	0.55	748	218	113	174	<0.01	1.0	37.1	19.9	15.0
40	CT POND	8.72	29.02	4730	298	1080	1260	<0.01	15.0	32.3	31.2	690
41	CT PROTO POND	8.93	5.53	540	158	124	28.5	0.10	1.0	3.5	7.4	69
42	MSLB OP	7.56	68.51	2230	1025	570	84.5	2.17	5.5	11.7	28.1	210
43	SCP1	7.96	46.64	2270	914	557	124	0.51	5.6	17.6	40.0	250
44	BCV-A5	8.07	19.81	2280	756	519	91.3	<0.01	1.0	18.2	44.8	340
45	BCV-B16	7.49	5.36	1261	351	159	185	<0.01	1.0	32.0	94.9	130
46	ETB POND	8.94	12.87	535	184	123	29.1	<0.01	1.0	3.0	7.5	55
47	DD B2506	7.59	82.30	2850	1020	706	310	2.81	9.5	14.4	18.9	250
48	DD B2503	7.20	75.84	2950	1040	733	301	2.70	10.5	14.6	24.3	280
49	MLAKE	8.22	0.40	287	138	17.9	31.1	<0.01	1.0	9.8	33.7	8.0

Table 3.3 – Correlation coefficient[2] (r^2) between fish cell line viability and NA concentration of OSPW sample

	Alamar blue	CFDA-AM	NR
WF-2	0.6171***	0.2274**	0.02247
GFSk-S1	0.5048***	0.3797***	0.06599
FHML-W1	0.5086***	0.1131*	0.02942
FHMT-W1	0.4519***	0.4352***	0.01877
RTL-W1	0.5658***	0.3718***	0.006414
RTgill-W1	0.5637***	0.3903***	0.04314

*p<0.05, **p<0.01, ***p<0.0001

Table 3.4 – Correlation coefficient[2] (r^2) between fish cell line viability and sample conductivity, [HCO_3], [Na] (µg/ml)

	Conductivity (uS/cm)			HCO_3			Na		
	AB	CFDA-AM	NR	AB	CFDA-AM	NR	AB	CFDA-AM	NR
WF-2	0.4025***	0.1304*	0.02648	0.6802***	0.1141*	0.05260	0.4381***	0.09398*	0.03153
GFSk-S1	0.4568***	0.2631**	0.0001	0.8150***	0.3200***	0.01674	0.5680***	0.2242**	0.0001
FHML-W1	0.4610***	0.1680**	0.0027	0.7937***	0.1348**	0.04128	0.5643***	0.2055*	0.0056
FHMT-W1	0.4104***	0.2938***	0.02037	0.7222***	0.5968***	0.02107	0.4600***	0.3850***	0.02771
RTL-W1	0.5636***	0.3655***	0.09498*	0.8015***	0.6917***	0.00254	0.6025***	0.4547***	0.08426*
RTgill-W1	0.4785***	0.3495***	0.5150***	0.8757***	0.6003***	0.6991***	0.5822***	0.4290***	0.6327***

*p<0.05, **p<0.01, ***p<0.0001

Table 3.5 – Correlation matrix (Pearson) for OSPW sample chemical components and WF-2 cell line bioassay RFUs (as measured by AB); significant coefficients underlined, (p<0.05)

	pH	NA	Cond.	HCO_3	Na	SO_3	NH_4	K	Mg	Ca	Cl	RFUs
pH	1.0000											
NA	-0.1764	1.0000										
Cond.	-0.1214	0.5774	1.0000									
HCO_3	-0.3067	0.8282	0.6339	1.0000								
Na	-0.1119	0.6500	0.9686	0.7206	1.0000							
SO_3	-0.0091	0.1058	0.6512	-0.0486	0.5215	1.0000						
NH_4	-0.2706	0.1175	0.4461	0.3653	0.4224	0.0820	1.0000					
K	-0.1028	0.4536	0.7885	0.3907	0.7262	0.6841	0.5314	1.0000				
Mg	-0.1034	-0.1754	0.1663	-0.2783	-0.0197	0.7326	-0.1958	0.2695	1.0000			
Ca	-0.2048	-0.1417	0.0477	-0.2431	-0.1513	0.5061	-0.1671	0.1304	0.8256	1.0000		
Cl	-0.1376	0.4514	0.8696	0.5687	0.8952	0.3771	0.4981	0.5923	-0.1721	-0.1733	1.0000	
RFUs	-0.0592	-0.7856	-0.6345	-0.8245	-0.6619	-0.0994	-0.1347	-0.4042	0.0721	0.0978	-0.4616	1.0000

4 Discussion

The present work was carried out to develop whole-animal alternatives or preliminary screening tests for toxicity assessment to aid in current and future remediation operations in the Athabasca oil sands. The use of fish cell lines appear promising as alternative biomonitoring systems and the viability assays used in this study, specifically Alamar Blue, appears to be a sensitive, cost- and time-effective assay for detecting cytotoxic samples. However, the ecotoxicological relevance of these assays still needs to be evaluated as the EC_{50} values obtained for model chemicals were below those reported in the literature for whole organisms. Nevertheless, the general trend was in agreement with those values reported for the respective chemicals and was consistent for all cell lines over numerous replicates. Therefore, the present work sets the path for further work on the evaluation of OSPW using fish cell lines.

4.1 Cell line exposures to CuSO4, SDS, CNA, and Cr.NA

Interactions between organisms and chemical compounds is initiated at the cellular level, hence, vertebrate cell cultures are potentially valuable tools in predicting toxicity in whole-animals (Schirmer, 2006). Furthermore, bioassays utilizing cell lines and various fluorescent indicator dyes allow for the elucidation of toxic mode of action of a chemical compound (Kramer et al., 2009) and can do so in a rapid, inexpensive fashion (Lee et al., 2008).

Cell exposure to all four chemical compounds in this study caused an overall dose-dependent decline in cell viability as measured by at least two of three fluorometric indicator dyes. Although the three fluorometric indicator dyes did not always yield EC_{50} values similar to

one another, two of the assays were usually not significantly different and were within the same order of magnitude as reported LC_{50} values for the same or similar compounds.

Tollefsen et al. (2008) found similar discrepancies between fluorometric dye sensitivity when exposing primary cultures of rainbow trout to a range of alkylphenols and alkylated non-phenolics, subsequently measuring cell viability using AB and CFDA-AM. It was found that EC_{50} values derived AB data were lower than those from CFDA-AM for most chemicals tested. Thus, toxicity resulting in metabolic inhibition was observed at lower concentrations than loss of membrane integrity.

At first glance, the EC_{50} variability across assays undermines their strength. However, each fluorescent indicator dye is indicative of a unique cellular viability criterion. Therefore, just as chemicals of varying physico-chemical properties may affect the cell in different ways we might expect measured viability readings from our three unique indicator dyes to be different as well.

4.2 Fish cell line viability after 24h exposure to $CuSO_4$

AB and CFDA-AM were successful in evaluating the viability of all six cell lines after a 24 h exposure to copper. These fluorometric dyes showed consistent dose-dependent declines in cell viability with increasing concentration of $CuSO_4$ with viability always falling below 50% of the control cells at high concentrations. CFDA-AM_{50} values for $CuSO_4$ ranged from 3.5 ± 1.6µg/ml to 8.32 ± 0.9µg/ml for the six cell lines tested while the respective LC_{50} values reported for the same compound ranged from 0.55 ± 0.48µg/ml to 3.2 ± 4.3µg/ml. Similar AB_{50} results were obtained with effective concentrations ranging from 3.47 ± 0.84µg/ml to 8.13 ± 1.5µg/ml (with the exception of one outlier to be discussed later) while respective LC_{50} values ranged from

0.55 ± 0.48μg/ml to 3.2 ± 4.3μg/ml. Dayeh et al. (2005) also reported comparable EC_{50} values (AB = 3.14 ± 0.28 ml/l; CFDA-AM = 5.67 ± 0.46 ml/l) when exposing the RTgill-W1 cell line to $CuSO_4$ for 24 h.

Evidently, there is a discrepancy between the EC_{50} values generated and the reported LC_{50} values (Figure 3.1). *In vitro* models are typically less sensitive than their *in vivo* counterparts generating effective concentrations much higher, sometimes by orders of magnitude (Castano et al., 2003, Segner, 2004, Sandbacka et al., 2000). That is, greater concentrations of test chemical are required to bring about a similar response in cell lines. Whole animals represent an integrative endpoint as toxicity models because they are comprised of a plethora of tissues and organs often interacting in a dynamic and reciprocal fashion where the failure of one or more of these tissues can have fatal implications for the organism. The corollary here is that cell lines derived from single tissues will invariable have fewer target sites and in most cases will likely appear less sensitive to a given toxicant (Schirmer, 2006). Using cell lines of varying species and/or tissue origin, as well as three different fluorometric indicator dyes indicative of different cell viability criteria may actually yield a solid absolute correlation between EC_{50}s and LC_{50}s; with such a cross-section of cell lines and viability indicators, an analogous indicator of an LC_{50} is likely the lowest observed effective concentration from all cell lines and viability dyes combine. In this case, the lowest observed EC_{50} is seen in the FHMT (3.47 ± 0.84μg/ml) cell line using AB, likely indicating an early breakdown in cell metabolism from copper interactions with cellular enzymes. Furthermore, this EC_{50} falls nicely in the range of reported LC_{50}s found in the literature (0.55 ± 0.48μg/ml to 3.2 ± 4.3μg/ml).

There was a consistent increase in viability in the FHMT cell line at 10μg/ml (Figure 5.1), a concentration where cell viability was well below 50% using AB for the same cell line as

well as for all other cell lines using AB and CFDA-AM in all other $CuSO_4$ trials. An explanation for this could be advanced by considering a study by Von Deimling (1985) showing mammalian testis tissue to be particularly high in non-specific esterases such as carboxylesterases, acetylesterases, and cholinesterases. Because CFDA-AM is converted to the fluorescent, carboxyfluorescein by these non-specific esterases, such an abundance of these enzymes present in the FHMT cell line may explain the spike in the measured viability. Of course, if this were the case we might also expect to see such large readings at other concentrations as well, but 10μg/ml $CuSO_4$ may have offered a unique combination of upregulation of cellular enzymes under stress and otherwise sufficient cell damage induced by chemical exposure to readily allow the leakage of cellular contents into the extracellular space. Provided the extracellular milieu could support enzymatic activity, these esterases would likely come in contact with a larger concentration of CFDA-AM than seen in other concentrations of $CuSO_4$.

The use of NR to quantify copper toxicity was found to be problematic by Dayeh et al (2005). They reported a biphasic trend where decreased viability with increasing concentrations of copper was seen initially followed by an increase in measured viability with subsequent increases in copper concentrations. These results were confirmed in this study by exposing FHMT, FHML, and RTgill-W1 cells to $CuSO_4$ and evaluating cell viability with NR. Similar to data reported by Dayeh et al., cell viability decreased in a dose-response manner but then appeared to increase with higher concentrations (Appendix F – Figure 5.7). Cell death was confirmed by microscopic evaluation of cells.

It is thought that copper toxicity is partly due to the formation of reactive oxygen species (ROS) (Bopp et al., 2007) and non-specific binding of the metal ion Cu^{2+} to biologically important molecules. For example, it binds to histidine-, cysteine- and methionine residues in

proteins with high affinity, which may result in dysfunctional enzymes (Camakaris et al., 1999). Since CFDA-AM and AB rely on the functioning of active enzymes to convert them to polar, fluorescent products that can be fluorometrically quantified, we would expect them to be reasonable indicators of viability but also adversely affected by inhibition of enzymes. On the other hand, NR is simply sequestered in the lysosomes by viable cells. Since this is not entirely dependent on enzymatic processes, enzymatic inhibition via $CuSO_4$ toxicity may have less of an impact on NR fluorescence and be less indicative of cell viability in this case.

4.3 Fish cell line viability after 24h exposure to surfactant compounds

The Clarke hot water extraction process used to separate bitumen from the oil sand promotes the solubilisation of NAs due to the alkalinity (pH=8), thereby concentrating them as mixtures of sodium salts in the aqueous tailings (Rogers et al., 2002). For validation and control purposes this study tested a commercial NA preparation and a crude NA extract prepared as per Frank et al. (2006).

Surfactant-induced adverse effects in fish have been reported in a number of studies (Abel, 1976; Misra et al., 1985; Partearroyo et al., 1991). The primary exposure site for surfactants and many other aquatic toxicants is the gill epithelium, as well as dermal tissue and intestinal epithelium (Sandbacka et al., 2000). Furthermore, linear alkylbenzene sulphonic acid (LAS), an anionic surfactant like SDS, was found to be taken up by fish across the gills (Tolls et al., 2003). Subsequent to exposure, concentrations of a few LAS analogues can be found in the liver and other internal organs of juvenile rainbow trout *in vivo*, suggesting these compounds readily enter systemic circulation (Ivankovic and Hrenovic, 2010) and implies potential damage

to internal organs. Thus, the proposed suite of cell lines should be quite relevant for testing surfactants *in vitro*.

The viability trends for the SDS, CNA and Cr.NA were largely similar for each cell line as measured by AB and NR and compared reasonably well with reported LC_{50} values *in vivo* suggesting successful indication of cell viability after exposure to surfactants. In each case, consistent dose-dependent declines in cell viability were shown with experimental RFUs falling below 50% of the control in each trial and for each cell line. It was often difficult to make direct intraspecies EC_{50}/LC_{50} comparisons for a given surfactant, but in these cases LC_{50} data was obtained for a similar species of fish, or, for the unique Cr.NA extract, data was obtained from the Microtox assay using *Vibrio fischeri* bacteria as reported in Frank et al., 2008.

AB_{50} and NR_{50} values calculated for each cell line were lower than, or comparable, to reported LC_{50} values for SDS. GFSk-S1, RTL-W1 and RTgill-W1 were all more sensitive to SDS than the *in vivo* tests cited. The remaining cell lines generated AB_{50} and NR_{50} values consistently similar to one another and comparable to *in vivo* data as well. It should also be noted that all LC_{50} data listed in Table 3.1 are from 96h exposures unless stated otherwise. This exposure-time discrepancy may account for some of the absolute insensitivity of the cell line bioassays in this study. In a study exposing lung carcinoma cells to docetaxel and paclitaxel (chemotherapeutic agents), median EC_{50} values were 0.48, 0.13, 0.03 and 0.02 muM for exposure times of 3, 24, 72, and 120 h, respectively. Thus, cells were far more sensitive with increasing exposure time (Fujishita et al., 2003).

The AB_{50} and NR_{50} values generated for cell line exposure to CNA were also quite good. In fact, they were lower for all cell lines than the average reported LC_{50} values (30.24 ± 22.57μg/ml) except for the AB_{50} value of 42.91 ± 4.2μg/ml for the WF-2 cell line. That is, the

vast majority of cell lines were more sensitive to 24h CNA exposure than the whole-animal, 96h exposure models. The reasons for this are unclear.

The results for the assessment of the Cr.NA extract were more variable. The Cr.NA extract used in this study were received from Richard Kavanagh and were prepared as per Frank et al. (2008). Briefly, the Cr.NA extract used in this study was prepared by the collection of 2000 L of tailings pond water from Syncrude Canada Ltd. West Endpit settling basing in Fort McMurray, Alberta, Canada. NAs and other organic acids were precipitated by acidifying the tailings water. Precipitate was isolated and re-dissolved in 0.1 N NaOH. Samples were kept in 1L amber bottles at 4°C. Naphthenic acids are a diverse mixture of acidic compounds that vary tremendously depending on the source (Rogers et al., 2002). This particular Cr.NA extract was tested for toxicity using the Microtox assay, from which EC_{50} values (64.9 ± 14.5 μg/ml) are used for comparison in this study. CFDA-AM_{50} and NR_{50} values calculated using fish cells in this study were higher, quite significantly in some cases, ranging from 103.67 ± 51.7 μg/ml to 682.7 ± 163.4 μg/ml. AB_{50} values were much better, ranging from 45.26 ± 2.44μg/ml to 146.3 ± 46.6μg/ml. Current concentrations of NAs in holding ponds owned by Suncor Energy Inc. and Syncrude Canada Ltd. are reported to range between 80 and 110 mg/l (FTFC, 1995). Although the NR_{50} and CFDA-AM_{50} results were elevated by comparison, the AB_{50} values were comparable to those obtained using the Microtox assay. Therefore, AB was sufficiently sensitive to detect toxicity at NA levels currently present in AOS holding ponds. In other words, this assay would provide an accurate, cost- and time-effective evaluation of OSPW while providing *in vitro* toxicity data relevant to whole fish.

It should be noted that the toxicity of CAN appeared greater than the Cr.NA extract, consistently yielding lower EC_{50} values. Similar results were found by Nero et al. (2006) when

exposing young-of-the-year yellow perch to a commercial and an extracted oil sands NA mixture resulting in LC_{100} values of 3.6 and 6.8 mg/L, respectively. This difference in toxicity is likely due to the difference in the relative composition of the C-number and Z-value of the NA compounds. Nero et al. (2006) also found that the addition of 1 g/L of salt (Na_2SO_4) reduced the NA toxicity by 40-50%. The addition of salt to the NA solution may cause precipitation of the NA out of solution making the nominal concentration of NA less than the dissolved concentration available to the organism, effectively reducing the apparent toxicity. Therefore, salinity may be an important factor when measuring OSPW toxicity and could be a valuable area of research in the future.

The trend lines for graphs representing toxicity data for Cr.NA in Figure 5.4 do not show a smooth decline in cell viability as surfactant concentration is increased, rather overall dose-dependent declines with intermittent plateaus that seem to indicate no loss of cell viability across a concentration range. This may have been indicative of up-regulation of specific enzymes or repair mechanisms that happen to effect the reduction of fluorescent indicator dye at certain chemical concentrations (Sandbacka et al., 2000). Another explanation may lie simply in the concentrations tested. It is interesting to note that the plateaus observed in this assay all fall within, but do not span across an order of magnitude of concentration across the *x-axis*. A basic principle of toxicology is that response often varies proportionally to geometric increases in dose, not arithmetic (Stine and Brown, 1996). Although we do test sequential doublings of Cr.NA concentration here, perhaps this extract was not acutely toxic enough to show marked decreases in cell viability across some changes in chemical concentration; Cr.NA was the least toxic of all four compounds, yielding the highest EC_{50} values.

Contrary to the promising AB_{50} and NR_{50} values, CFDA-AM was ineffective in the assessment of SDS, CNA, and Cr.NA, yielding erratic results and variable EC_{50} values both within and between cell lines. Similar erratic results were found by Dayeh et al. (2004) using CFDA-AM when measuring toxic effects of the non-ionic surfactant, Triton X-100 on fish cell lines and *Tetrahymena thermophila*; cell viability appeared to increase while that measured by AB and NR appeared to decrease. As mentioned, CFDA-AM is indicative of toxicity by indirectly measuring cell membrane damage via non-specific esterase activity. This is achieved through the conversion of CFDA-AM to a fluorescent product (carboxyfluorescein) by non-specific esterases in one of two scenarios: Esterases in the cell have been released into the extracellular environment due to extensive membrane damage since surfactants induce cytotoxicity via narcosis (Frank et al., 2008), or esterases from within the cell readily interact with the CFDA-AM when there is cell membrane damage sufficient to allow the indicator dye to pass through the damaged membrane into the cell. In the case of significant membrane damage viability appears to be lost when the necessary enzymes that reduce CFDA-AM are essentially removed from the exposure environment during the assay protocol. That is, fewer esterases remain in the exposure wells when fluorescent dyes are added.

An explanation for the erratic and often dramatic increases in perceived viability may lie in the protocol used. When microwell plates were inverted to evacuate exposure wells prior to adding fluorescent dye solution, a small ring of exposure solution remained at the bottom of each well possibly containing liberated esterases. To a certain extent, greater membrane disruption, as would be expected with increasing surfactant concentrations, may have resulted in greater concentrations of esterases in this residual solution and subsequent viability readings that were misleading.

4.5 Cell line exposure to OSPW samples

The OSPW samples are an eclectic mixture of chemicals produced from oil sand during the bitumen extraction process (Table 3.1). The implementation of successful wet landscape reclamation options, such as EPLs, must involve the evaluation of salt- and naphthenate-containing OSPW (Leung et al., 2003). Analytical chemistry techniques can be helpful in characterizing and quantifying the components of such whole-water mixtures, but lack the ability to accurately predict its potential toxicity on aquatic biological systems, such as fish. To this end, non-animal toxicity models utilizing fish cell lines can be used to assess toxicity of samples in a rapid, inexpensive, and ethical manner.

The evaluation of toxicity of mining effluents can be extremely complex due to the nature of the mixtures, the diverse physico-chemical properties of the constituents, and a multitude of potential modes of interaction with biological systems. However, the cell line bioassay in this study was able to successfully identify toxicity in a number of OSPW samples after 24 h exposures, showing significant decreases in measured viability, sometimes dropping below 50% of that of the control. No one cell line appeared to be the most or least sensitive for every sample, perhaps due to the complex and varying composition of the OSPW samples. However, the AB assay was the most consistent, yielding similar viability data for all trials both within and across cell lines (Figure 3.1). This assay indicated OSPW samples 8, 12, 13, 16, 19, 42, 43 to be particularly toxic to all fish cell lines, yielding viability data dropping below 50% of that of control cells.

A number of studies have identified NAs as the main toxic constituent of OSPW (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986; Alberta Environmental Protection, 1996; Schramm et al., 2000). NAs are naturally found in surface water at

concentrations up to 1-2µg/ml in the Athabasca region as a result of erosion of exposed oil sand (Alberta Environmental Protection, 1996). However, during the bitumen extraction process, NAs are liberated and dissolved in tailings water where concentrations may be in excess of 100µg/ml (Leung et al., 2003).

NAs have been shown to be toxic to a number of organisms including plants (Wort and Patel, 1970), fish, zooplankton, rats, and luminescent bacteria (Clemente and Fedorak, 2005). Dokholyan and Magomedov (1984) studied acute NA toxicity by exposing various fish species to 12-100mg/l NA for 10 days, generating LC_{50} values ranging from 25-75mg/l. Dorn (1992) found fish to be even more sensitive to oil refinery effluents showing significant toxicity in effluents containing NA concentrations as little as 2.5-5 mg/l. The concentration of NA in the OSPW samples in this study ranged from 0.30 – 82.30 µg/ml. However, it should be noted that the aforementioned studies used differing sources of NAs (e.g., commercial preparations, oil refinery effluents, or NAs isolated from oil sands tailings ponds). This is important because the complex nature of NAs makes estimates of effective concentrations variable (Alberta Environmental Protection, 1996), probably because NA samples of similar concentrations from different sources are likely different in composition with regard to molecular weight, C-number, and Z-value (Nero et al., 2006), making sample-to-sample comparisons difficult (Clemente and Fedorak, 1984).

Peters et al. (2007) specifically tested the toxicity of surface water from Mildred Lake settling basin (MLSB) containing OSPW-characteristic elevations in sodium sulphate, NAs, and low level PAHs. Yellow perch and Japanese medaka eggs were fertilized and exposed to serial dilutions of MLSB water for the duration of their development. They found that the threshold concentration of NA found in the MLSB water to be 7.52 mg/L, above which there was a

positive correlation between NA concentration and deformed embryos. The concentration of NA in the OSPW samples in this study ranged from 0.30 – 82.30 μg/ml.

The chemical composition of the OSPW samples was unknown during toxicity testing so as to not bias reporting. When the viability data was compiled and the composition of OSPW samples revealed there appeared to be a positive correlation between increasing concentration of NAs in samples and decreasing cell viability, especially as measured by AB. The correlation coefficient[2] (R^2) between cell viability and concentration of NA was as high as 0.6171 (WF-2 cell line, AB assay; $p<0.0001$), and ranged from 0.4519 to 0.5658 for the remaining cell lines ($p<0.0001$). Because NAs widely reported as one of the main toxicants in oil sands produced waters it may be expected that correlation coefficients be in the 0.80-0.90 range, with little variation among cell lines. An explanation for the varying correlation can be advanced by considering the biodegradation process of NAs in the tailings ponds and water bodies from which the OSPW samples were collected. The acute toxicity of OSPW has been found to decrease with time (MacKinnon and Boerger, 1986). This decrease in toxicity appears to correlated with an increase in the proportion of NAs that contain ≥22 carbons (Holowenko et al., 2002) which implies OSPW sample toxicity is influenced primarily by low molecular weight NAs (Frank et al., 2008). Frank et al. (2008) showed continual decreases in toxicity as measured by the Microtox assay with increases in the proportion of higher-molecular weight NAs. The shift in proportion of high molecular weight NAs is thought to be the result of greater microbial degradation of low-molecular weight NAs over time. Although the sampling date for each sample in this study was disclosed with the chemical composition data, the nature of the sample and its history on the Syncrude lease site was not. As such, two samples that had been stored in tailings ponds for different periods of time may contain similar concentrations of NAs but a

completely different high-to-low molecular weight composition ratio. And, in turn, this may be reflected in the toxicity data.

Naphthenates occur together with other compounds, such as hydrocarbons, sulphate, and salinity (dominated by sodium, sulphate, and chloride), and there is evidence for toxicological effects of salts derived from the extraction process (Leung et al. 2001). The aforementioned complex nature of naphthenates, along with the presence of these additional process-affected substances makes the ecotoxicological evaluation of OSPW difficult. However, a number of studies have found that some of the toxicity induced by OSPW is related to salinity from major ions or some additional factor, such as PAHs (van den Heuvel et al., 1999; Peters, 1999, Leung et al., 2003).

For this reason, multiple-regression analysis was done to identify additional components of the OSPW samples that may contribute to the observed toxicity. Correlations with cell viability similar to those found with NA concentrations were found between concentrations of sodium, and bicarbonate, as well as sample conductivity (Table 3.4) as measured by AB, and with CFDA-AM for HCO_3. Such increases in these OSPW components and concomitant decreases in cell viability imply they may also inducing toxicity. However, a high degree of multicollinearity was found between the concentrations of NA, Na, HCO_3, and sample conductivity (major ions). This suggests that these values are strongly correlated to the concentration of NAs in a given sample. That is, with increases in NA, similar increases are seen in Na, HCO_3, and sample conductivity. The major ions typically responsible for high conductivity (Cl, Na, K, etc.), as well as HCO_3^-, and SO_3^- are all likely present due to the processing procedures during the Clarke hot water extraction process and can be found in OSPW thereafter (Allen, 2008; Brient et al., 1995).

Conductivity is often used as a measure for the common ions dissolved in freshwater (Goodfellow et al., 2000). With regard to toxicity, conductivity can be used as a general screening tool. The conductivity of a freshwater effluent above 2000 µS/cm may indicate a concentration of dissolved solids high enough to induce toxicity in aquatic organisms (American Petroleum Institute, 1998). However, the correlation between increasing conductivity and toxicity may vary with ionic composition of effluent samples and therefore may not be the best predictor of toxicity. That is, cations and anions are not present individually, but instead are associated with other ions making conductivity per se a poor predictor of toxicity. Twenty of the OSPW samples in this study are reported to have a conductivity measurement in excess of 2000 µS/cm. Allen (2008) states that even if salinity concentrations in process water are insufficient to be acutely toxic, it may act as a stressor effectively increasing the toxicity of other compounds present in the effluent. Therefore, it is critical to compare ion concentrations in the effluent to literature or lab-derived toxic effect concentrations (Goodfellow et al., 2000).

Reported 96h LC_{50} values for HCO_3^- for rainbow trout and bluegill were 7700 µg/ml and 7100 µg/ml (OECD, 2002), respectively. OSPW sample 16 had the highest concentration of HCO_3 of the 49 samples tested, containing only 1050 µg/ml making HCO_3 an unlikely source of toxicity on its own. Similarly, studies have shown that Na^+ is not generally a major contributor to freshwater aquatic toxicology; in fact, the absence of Na^+ can be more toxic (Mount et al., 1997). Generally, toxicity with regard to Na^+ is concerned with the associated anion (Goodfellow et al., 2000).

Very little could be learned about the toxicity of these samples using CFDA-AM and NR. The variability seen here (Figures 5.17 & 5.18) was very much like that seen when testing the Cr.NA extract, and likely for the same reasons. However, the purpose of the fish cell line

bioassay used in this study was to accurately detect potential toxicity in a plethora of whole-water samples in a cost- and time-effective manner. Not only was the toxicity data generated for AB much more consistent and correlate better with NA concentration, the assay is much easier than CFDA-AM and NR. As outlined in Appendix C, there are fewer steps involved in the dye preparation and assay execution for AB than for the other two. Schirmer (2006) suggests retaining a suite of all three indicator dyes in order to evaluate a strong cross-section of viability criteria, which may be particularly useful in the assessment of complex OSPW samples.

4.6 Conclusions

For the purpose of evaluating a number of putative toxicants or unknown mixtures in a rapid, inexpensive, and ethical manner, bioassays using fish cell lines and fluorometric indicator dyes could be very useful, especially in the preliminary assessment of a large number of samples. Effective concentrations generated for each cell line for one or more fluorometric indicator dyes compared well to those obtained *in vivo*. These results were generated in only 24 h and may provide valuable insight into potential adverse affects of a toxicant on the tissue, organ, or species from which they were derived.

The easiest fluorometric indicator dye to use was AB, requiring far less time and effort than NR, and gave consistent dose-response curves that were generally comparable to reported LC_{50} values for those compounds. This is important as a hallmark of these *in vitro* assays is time efficiency and ease of use. AB was also successful with all cell lines used in this study for detecting toxicity of OSPW samples, yielding data that were consistent between trials and across cell lines and correlated well with reported NA concentrations in each sample.

Even though NR and CFDA-AM were problematic in different circumstances, Schirmer (2006) recommends using a suite of all three fluorometric dyes because they evaluate a cross-

section of viability criteria. This may be particularly important in studies like this that look at multiple chemicals of varying chemical characteristics or of complex mixtures of unknown composition, or of complex mixtures whose mode of toxicity is unclear.

In closing, this fish cell line bioassay may have the potential to facilitate preliminary screening of large numbers of OSPW samples ameliorating the magnitude of time, money, whole-animals required to safely implement and monitor wet reclamation landscapes, such as EPLs.

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NOTE: numbered superscripts before a reference correspond with the superscripts accompanying LC_{50} values in Table 3.1

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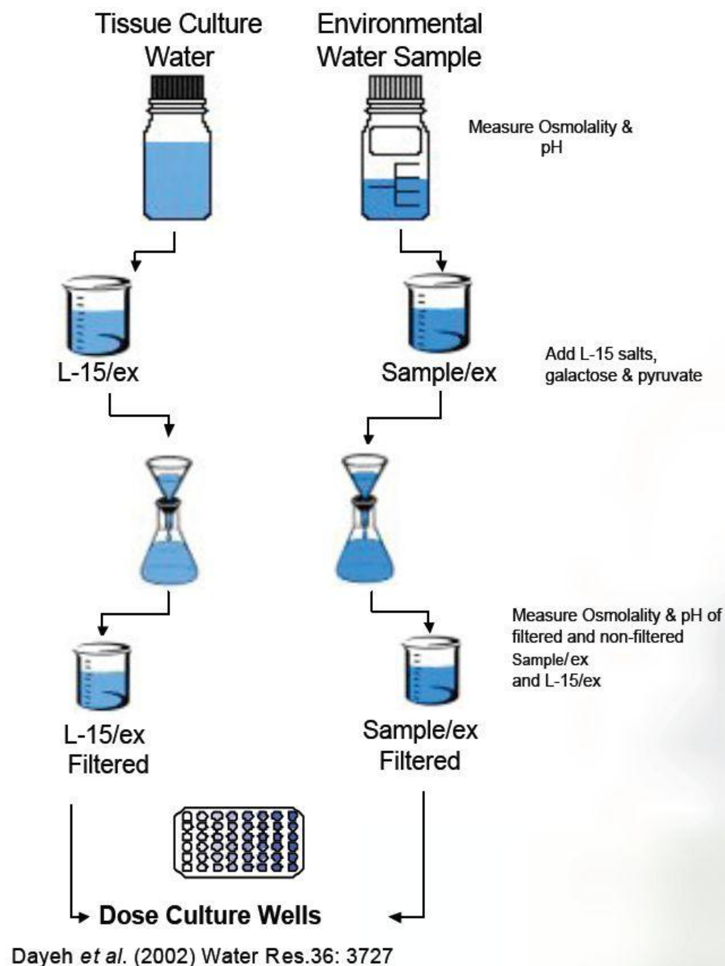
Appendices

Appendix A – Formulation for the preparation of 1L of the minimal media, L-15/ex (exposure)

Inorganic salts	**Supplier**	**Amount (g)**
NaCl	S-5886	8.000
KCl	P-5405	0.400
$MgSO_4.7H_20$	Caledon 4860-1	0.200
$MgCl_2.6H_20$	BDH ACS 474	0.200
$CaCl_2.2H_20$	BDH ACS 186	0.185
Na_2HPO_4 (anhydrous)	Sigma S-5136	0.190
KH_2PO_4 (anhydrous)	BDH ACS 657	0.060
Carbohydrate source		
D-Galactose	Sigma G5388	0.900
Sodium Pyruvate	Alfa Aesar 113-24-6	0.550

Appendix B – Storage, Preparation of OSPW samples

OSPW samples were received from Dr. Mike MacKinnon. The samples were taken from various bodies of water on the Syncrude Canada Ltd. lease site in northeastern Alberta. Samples were stored in a dark cold-room (4°C). Samples were adjusted to physiologically relevant osmolarities by the addition of 8ml OSPW sample to 2ml of 20X concentrated L-15/ex. This means that OSPW samples were actually tested at 80% their original concentration (specific OSPW sample preparation protocol can be found in the Materials and Methods section). Osmolarity of the samples after the addition of L-15/ex was checked via Westcor 5001B vapour pressure osmometer.



Appendix C – Specific assay protocol for Alamar blue, CFDA-AM, Neutral red (protocol adapted from Dayeh et al. 2005)

Alamar blue (AB)

1. Turn on and thoroughly clean laminar flow hood with 70% ethanol solution.
2. Prepare a 5% (v/v) working solution of AB in L-15/ex. This must be done with both the lights in the flow hood and the lab off to prevent photodegradation of the dye.
3. Remove exposure medium from the plates by inverting over paper towel.
4. Add 100µl of the AB solution to each well of the 96-well plate.
5. Incubate plates in the dark for 45 minutes at 18°C.
6. After sufficient incubation time, plates can be read one at a time in a fluorescence multiwell plate reader. The plate reader filters should be set at excitation and emission wavelengths of 530nm and 595nm, respectively. (Lids should be removed from the microwell plate)

CFDA-AM

1. Turn on and thoroughly clean laminar flow hood with 70% ethanol solution.
2. A 4mM stock solution can be prepared by dissolving CFDA-AM in sterile DMSO. Aliquots of 0.5ml can be prepared in order to avoid damage done during repeated freezing and thawing of stock solution. Also, aliquots can be wrapped in aluminum foil to prevent photodegradation of dye.
3. A 4µM solution of CFDA-AM can be prepared by diluting 0.5 mM CFDA-AM stock solution 1:1000 in L-15/ex. Lights in the flow hood and the lab should be out at this point to avoid photodegradation of dye.

4. Remove exposure medium from the plates by inverting over paper towel.
5. Add 100µl of the CFDA-AM solution to each well of the 96-well plate.
6. Incubate plates in the dark for 45 minutes at 18°C.
7. After sufficient incubation time, plates can be read one at a time in a fluorescence multiwell plate reader. The plate reader filters should be set at excitation and emission wavelengths of 485nm and 530nm, respectively. (Lids should be removed from the microwell plate)

Neutral red (NR)

1. Turn on and thoroughly clean laminar flow hood with 70% ethanol solution.
2. Prepare a 3% (v/v) working solution of NR by diluting the NR stock solution 1:100 in L-15/ex. Lights in the flow hood and lab should be off at this point to avoid photodegradation.
3. Remove exposure medium from the plates by inverting over paper towel.
4. Add 100µl of the NR solution to each well of the 96-well plate.
5. Incubate plates in the dark for 60 minutes at 18°C.
6. Remove NR from plates by inverting over paper towel.
7. Add 100µl of NR fixative solution (0.5% (v/v) formaldehyde and 1% (v/v) $CaCl_2$ in deionized water) to each well of the plate.
8. Remove NR fixative solution after 1 minute.
9. Add 100µl of NR extraction solution (1% (v/v) acetic acid and 50% (v/v) ethanol in deionized water) to each well of the plate. Place plates on an orbital shaker (40 rpm) for 10 minutes.

10. Plates can be read one at a time in a fluorescence multiwell plate reader. The plate reader filters should be set at excitation and emission wavelengths of 530nm and 645nm, respectively. (Lids should be removed from the microwell plate)

Appendix D – Calculation of EC_{50} values using GraphPad Prism

1. Open GraphPad Prism program on the computer.
2. Under "Choose a graph", choose "Points only" graph.
3. Under "Sub-column for replicates or error values", leave blank for X error bar and choose either "Enter and plot a single y value for each point" or "Enter and plot error values already calculated elsewhere"
4. Click "Create"
5. Enter data (as a % of Control)
6. Click "Analyze", under "Transform" option double-click "X values using X=log(x)" and then OK
7. Click "Analyze" again, under "XY analysis" option double-click "Non-linear regression (curve fit); under Dose-response-Inhibition select log(inhibitor) vs normalized response
8. Click "OK"
9. A spreadsheet with EC_{50} values, statistical analyses and a sigmoidal dose-response curve will appear.
10. Data and corresponding graphs can be saved under "File" and "Save As"

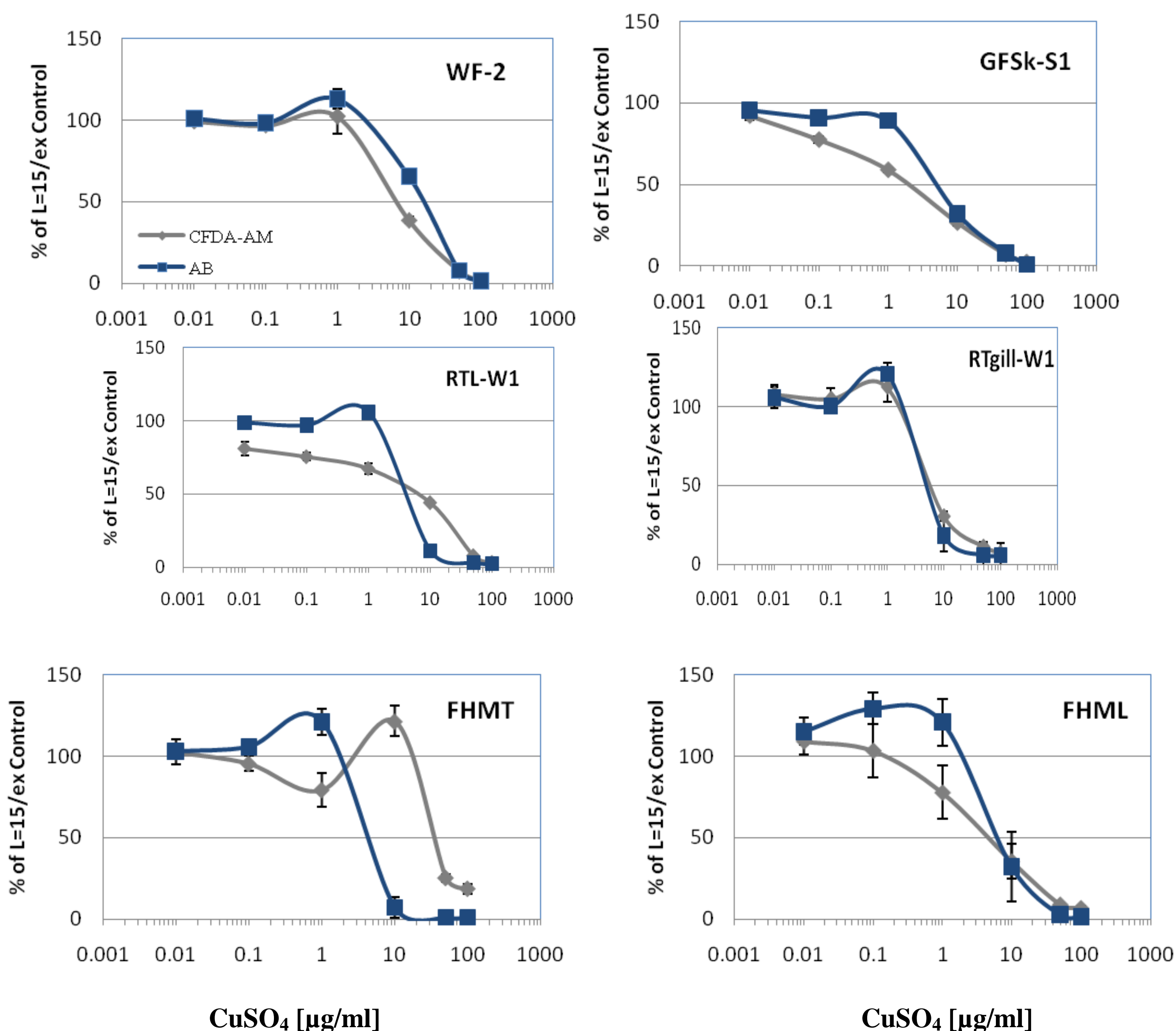


Figure 5.1 - Viability of WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHMT, and FHML cell lines after 24 h exposure to $CuSO_4$. Cell lines were plated between 4.5 and 7.0 x 10^4 cells/well and subsequently exposed to varying concentrations of $CuSO_4$ prepared in L-15/ex media. Chemical exposure lasted 24 h at which point cellular viability was measured using fluorometric indicator dyes: alamar blue and CFDA-AM. Results are expressed as a percentage of the viability of cells not exposed to $CuSO_4$. Data points represent the mean of three separate experimental trials (with standard deviation), each trial consisting of 4-6 wells per chemical concentration.

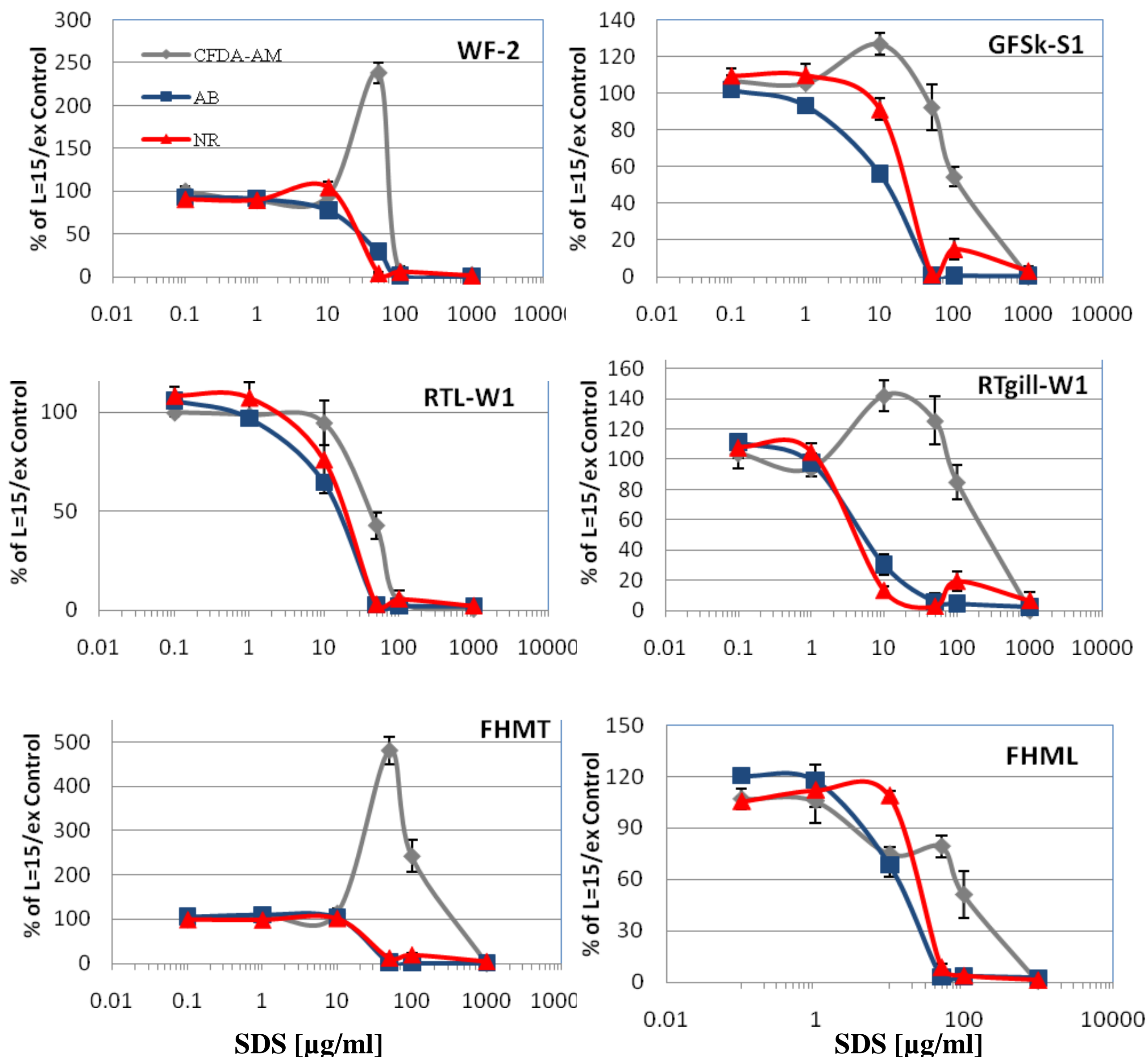


Figure 5.2 - Viability of WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHMT, and FHML cell lines after 24 h exposure to SDS. Cell lines were plated between 3.5 and 6.0 x 10^4 cells/well and subsequently exposed to varying concentrations of SDS prepared in L-15/ex media. Chemical exposure lasted 24 h at which point cellular viability was measured using fluorometric indicator dyes: CFDA-AM, alamar blue, and neutral red. Results are expressed as a percentage of the viability of cells not exposed to SDS. Data points represent the mean of three separate experimental trials (with standard deviation), each trial consisting of 4-6 wells per chemical concentration.

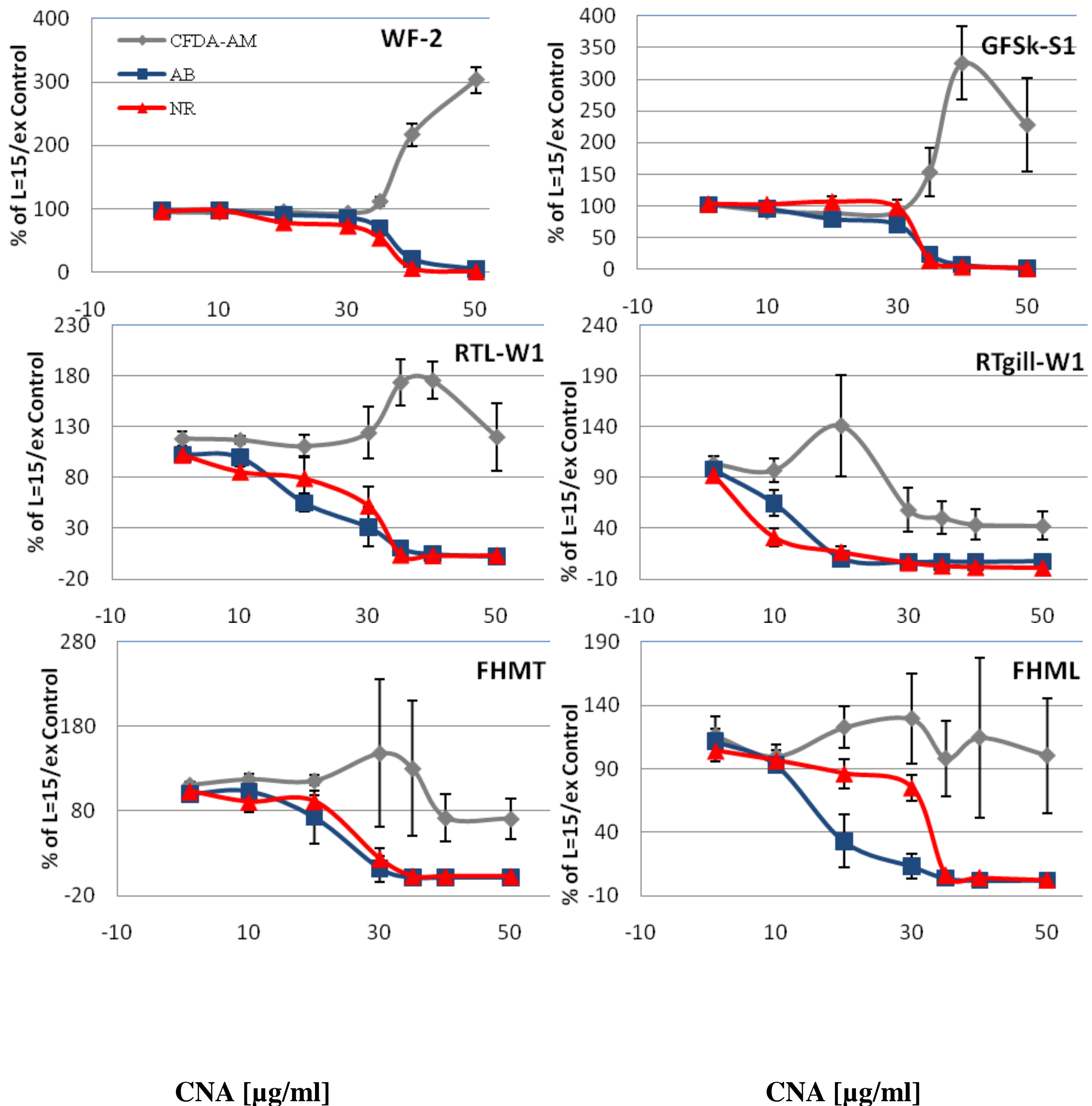


Figure 5.3 - Viability of WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHMT, and FHML cell lines after 24 h exposure to CNA. Cell lines were plated between 5.0 and 8.0 x 10^4 cells/well and subsequently exposed to varying concentrations of CNA prepared in L-15/ex media. Chemical exposure lasted 24 h at which point cellular viability was measured using fluorometric indicator dyes: CFDA-AM, alamar blue, and neutral red. Results are expressed as a percentage of the viability of cells not exposed to CNA. Data points represent the mean of three separate experimental trials (with standard deviation), each trial consisting of 4-6 wells per chemical concentration.

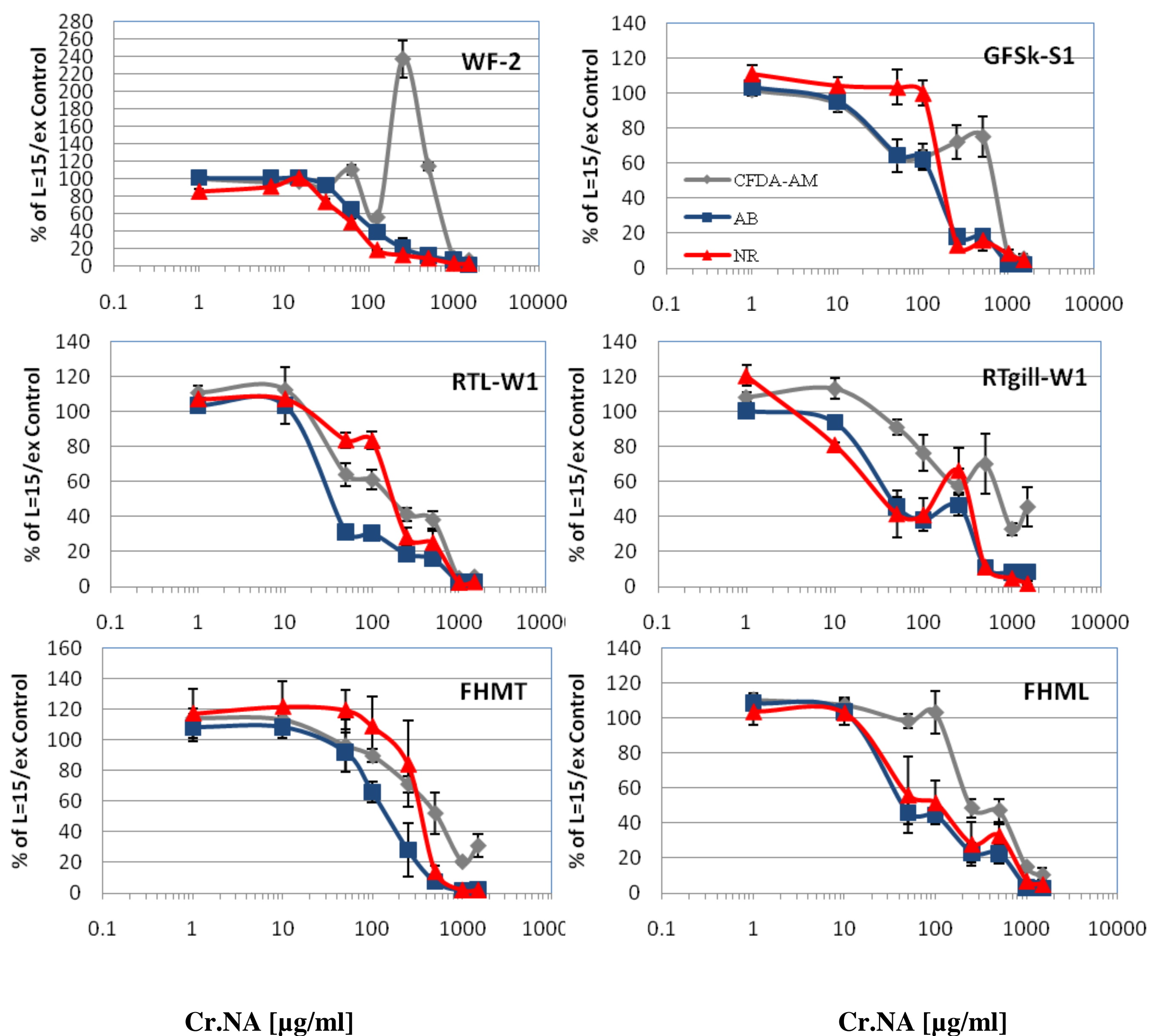


Figure 5.4 - Viability of WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHMT, and FHML cell lines after 24 h exposure to Cr.NA. Cell lines were plated between 4.2 and 7.5 x 10^4 cells/well and subsequently exposed to varying concentrations of Cr.NA prepared in L-15/ex media. Chemical exposure lasted 24 h at which point cellular viability was measured using fluorometric indicator dyes: CFDA-AM, alamar blue, and neutral red. Results are expressed as a percentage of the viability of cells not exposed to Cr.NA. Data points represent the mean of three separate experimental trials (with standard deviation), each trial consisting of 4-6 wells per chemical concentration.

Appendix F – Raw data for individual trials for cell line exposure to $CuSO_4$, SDS, CNA, and Cr.NA

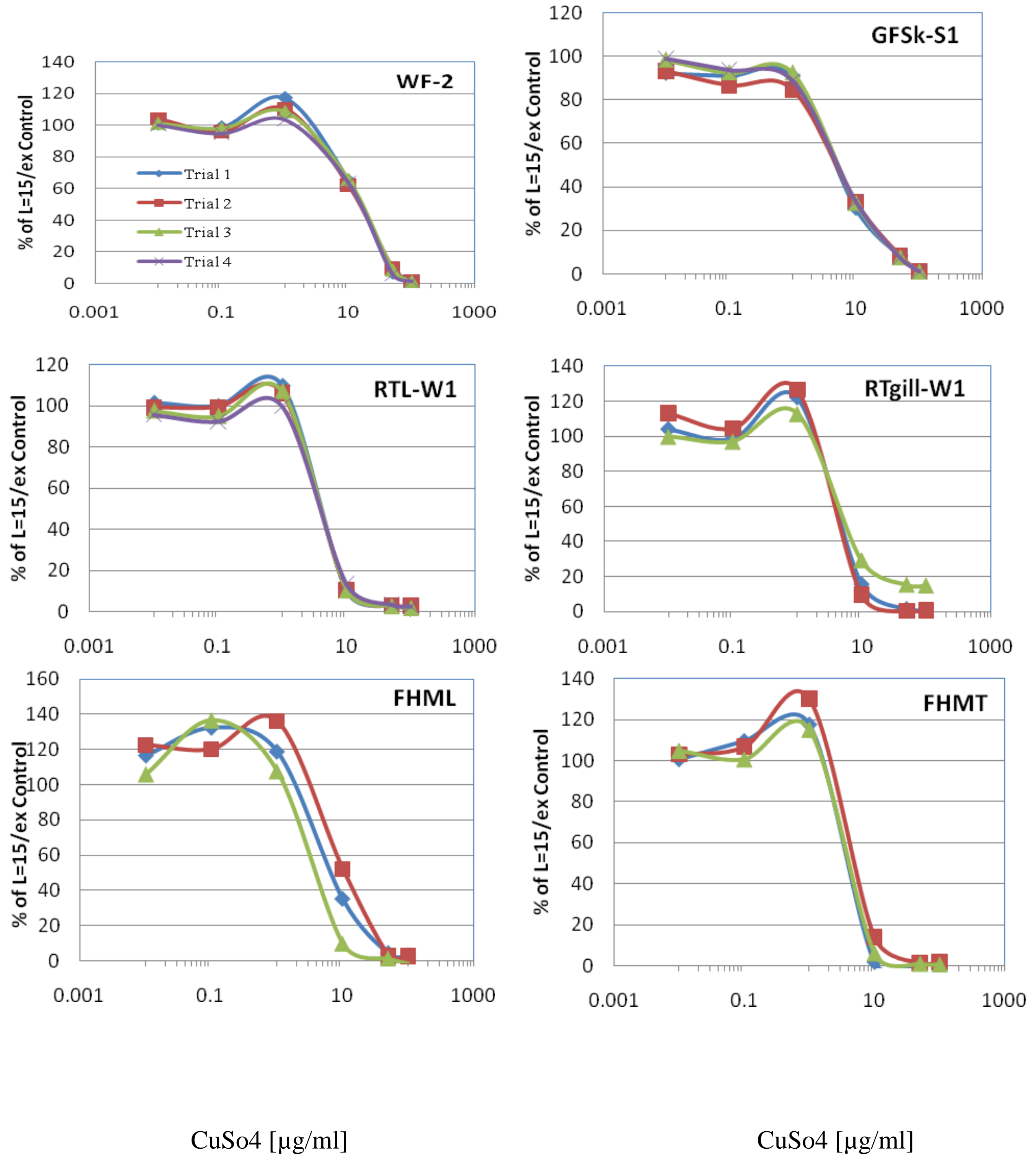


Figure 5.5 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to $CuSO_4$ as measured by alamar blue. Cells were exposed to serial dilutions of $CuSO_4$ (nominal concentrations in µg/ml) prepared in L-15/ex.

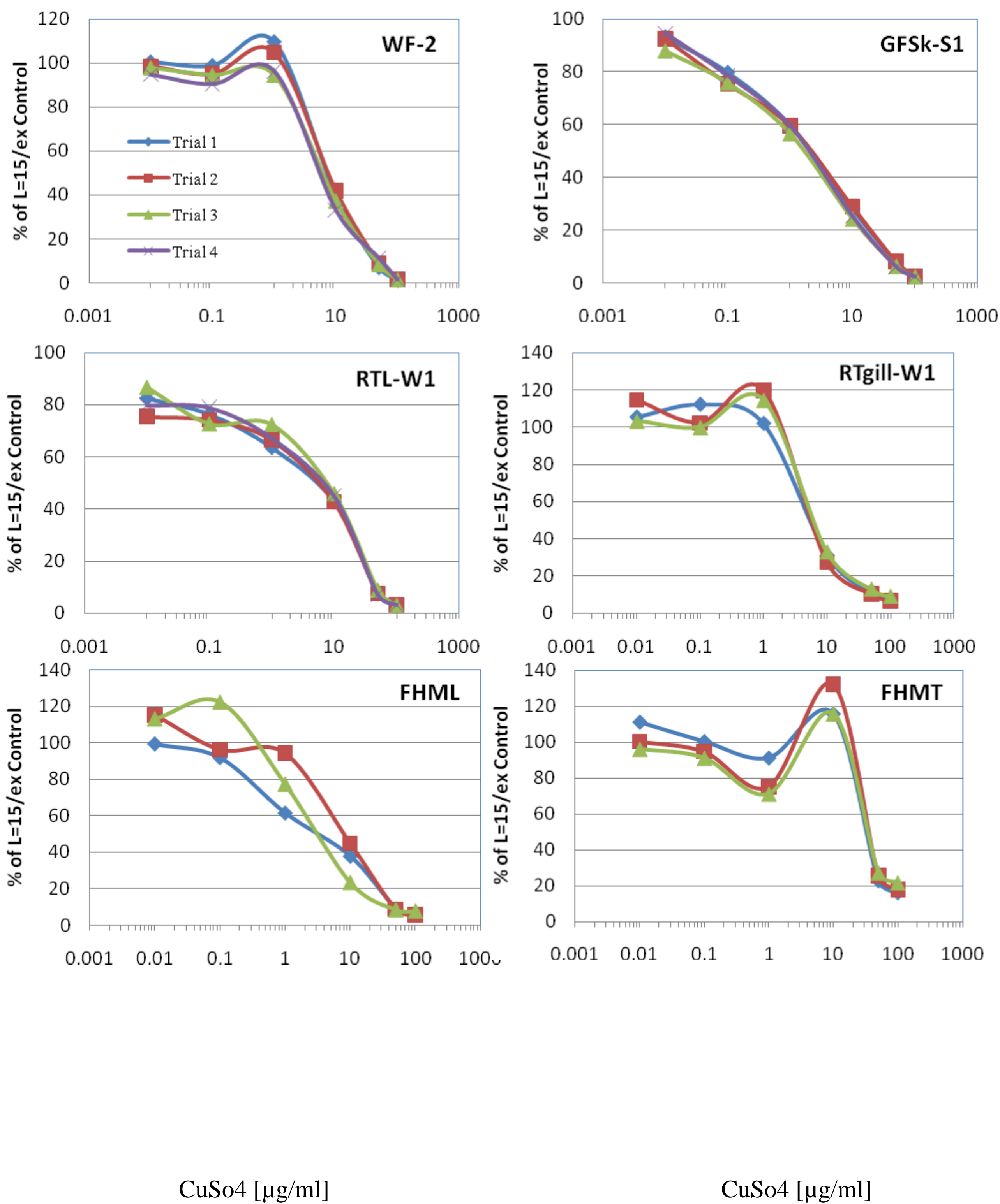


Figure 5.6 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to $CuSO_4$ as measured by CFDA-AM. Cells were exposed to serial dilutions of $CuSO_4$ (nominal concentrations in µg/ml) prepared in L-15/ex.

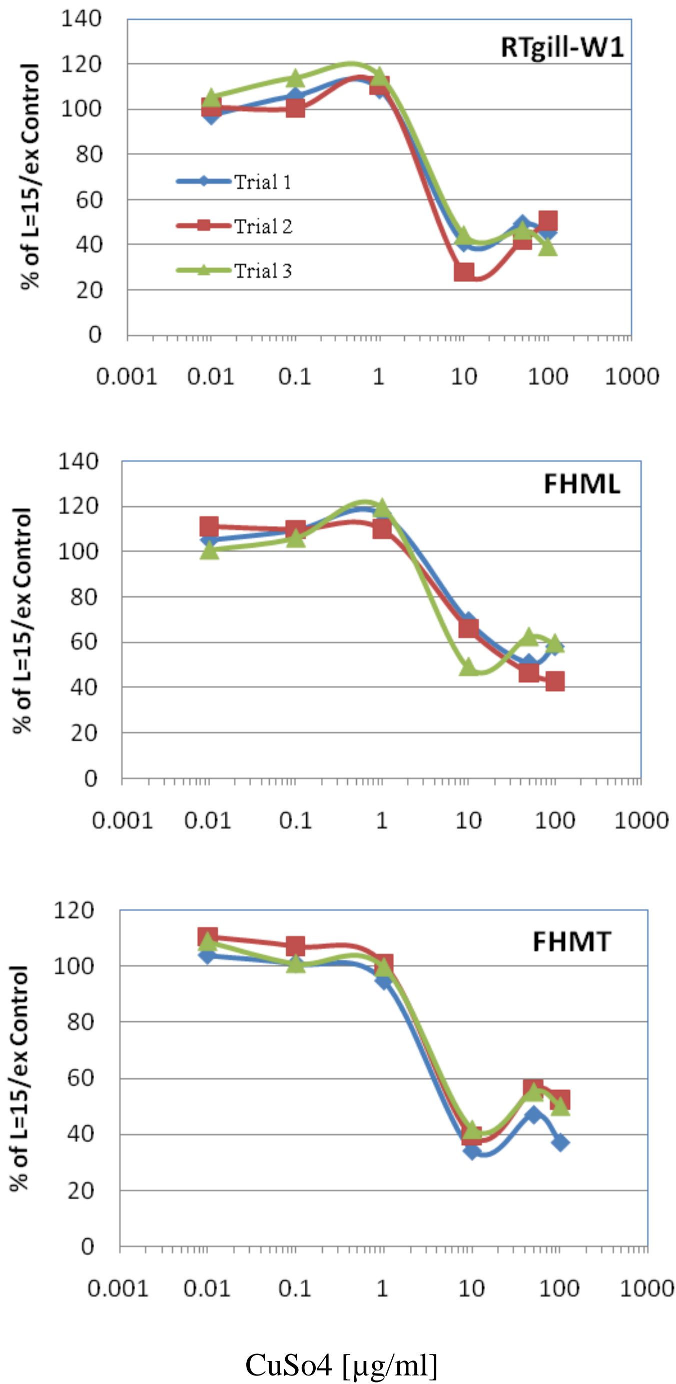


Figure 5.7 - Viability of RTgill-W1, FHML, FHMT cells after 24h exposure to $CuSO_4$ as measured by NR. Cells were exposed to serial dilutions of $CuSO_4$ (nominal concentrations in µg/ml) prepared in L-15/ex.

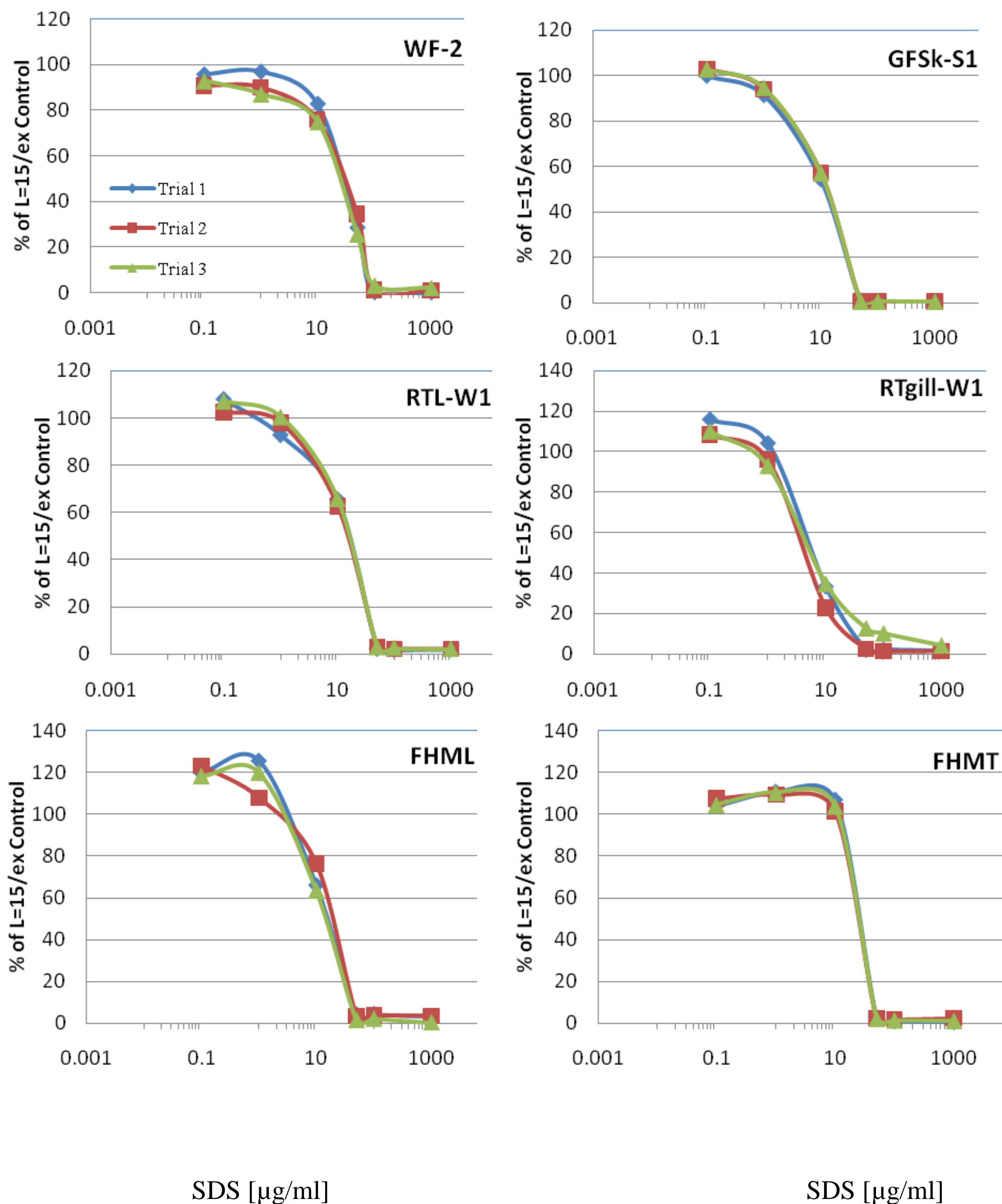


Figure 5.8 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to SDS as measured by alamar blue. Cells were exposed to serial dilutions of SDS (nominal concentrations in µg/ml) prepared in L-15/ex.

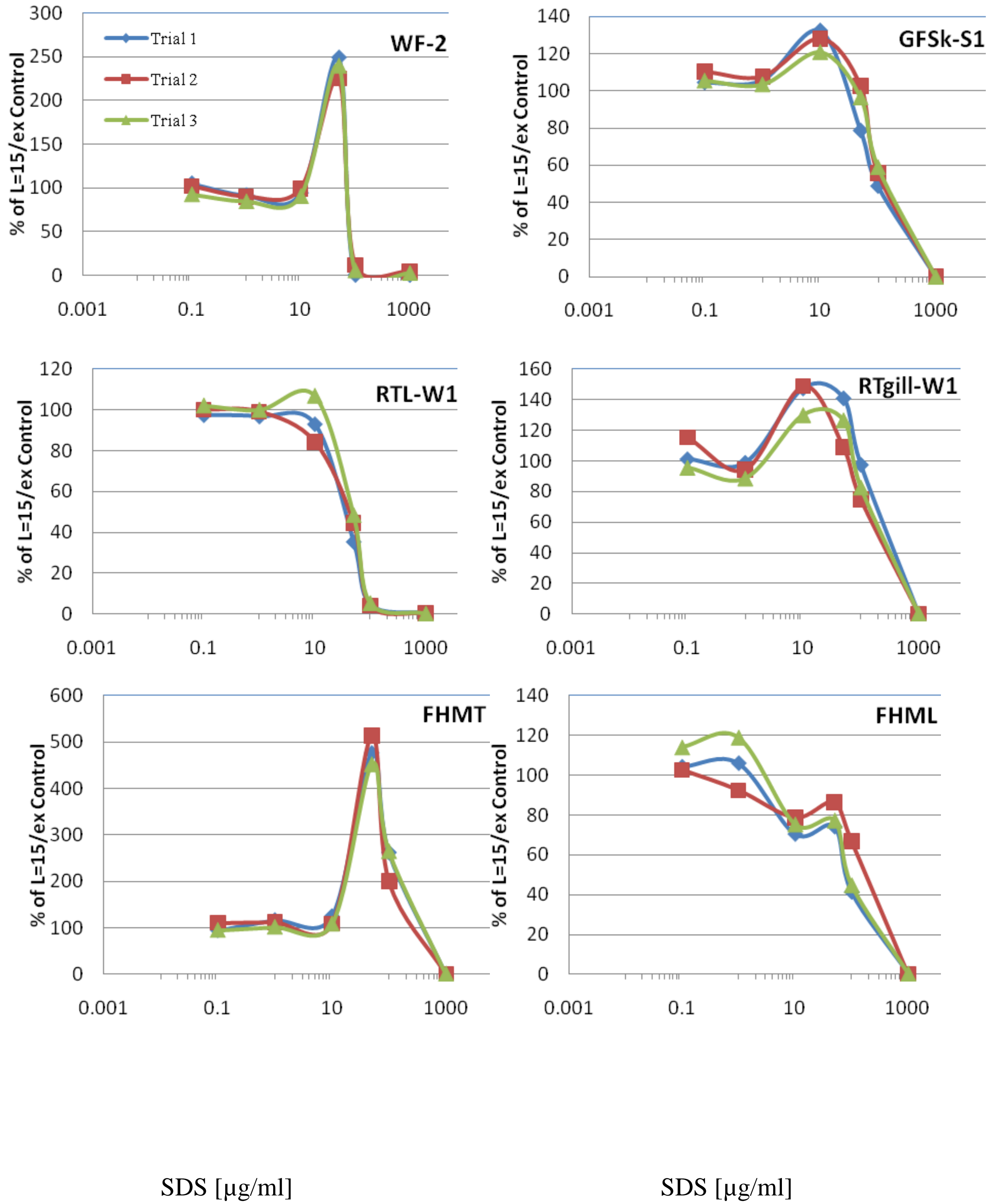


Figure 5.9 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to SDS as measured by CFDA-AM. Cells were exposed to serial dilutions of SDS (nominal concentrations in µg/ml) prepared in L-15/ex.

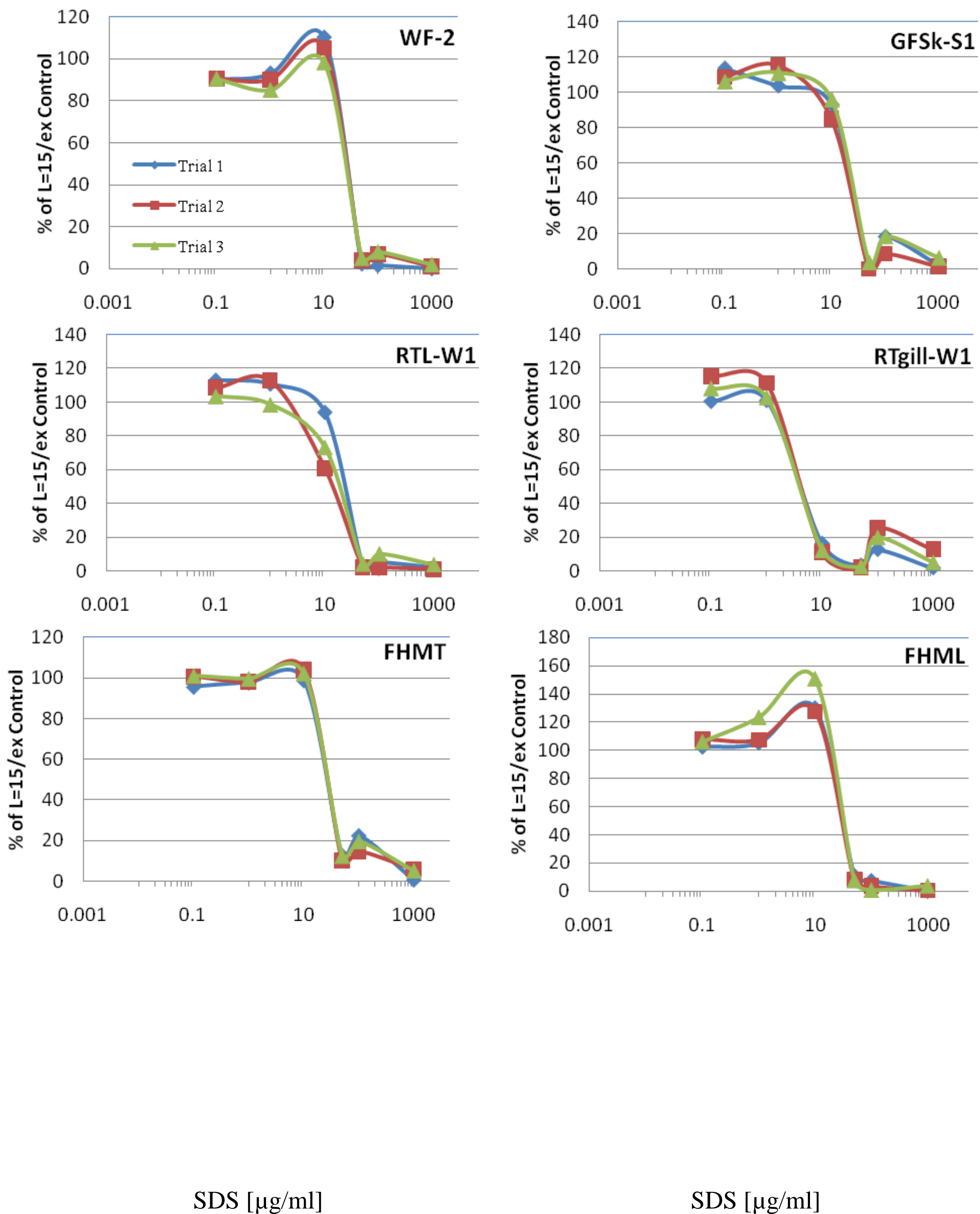


Figure 5.10 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to SDS as measured by NR. Cells were exposed to serial dilutions of SDS (nominal concentrations in µg/ml) prepared in L-15/ex.

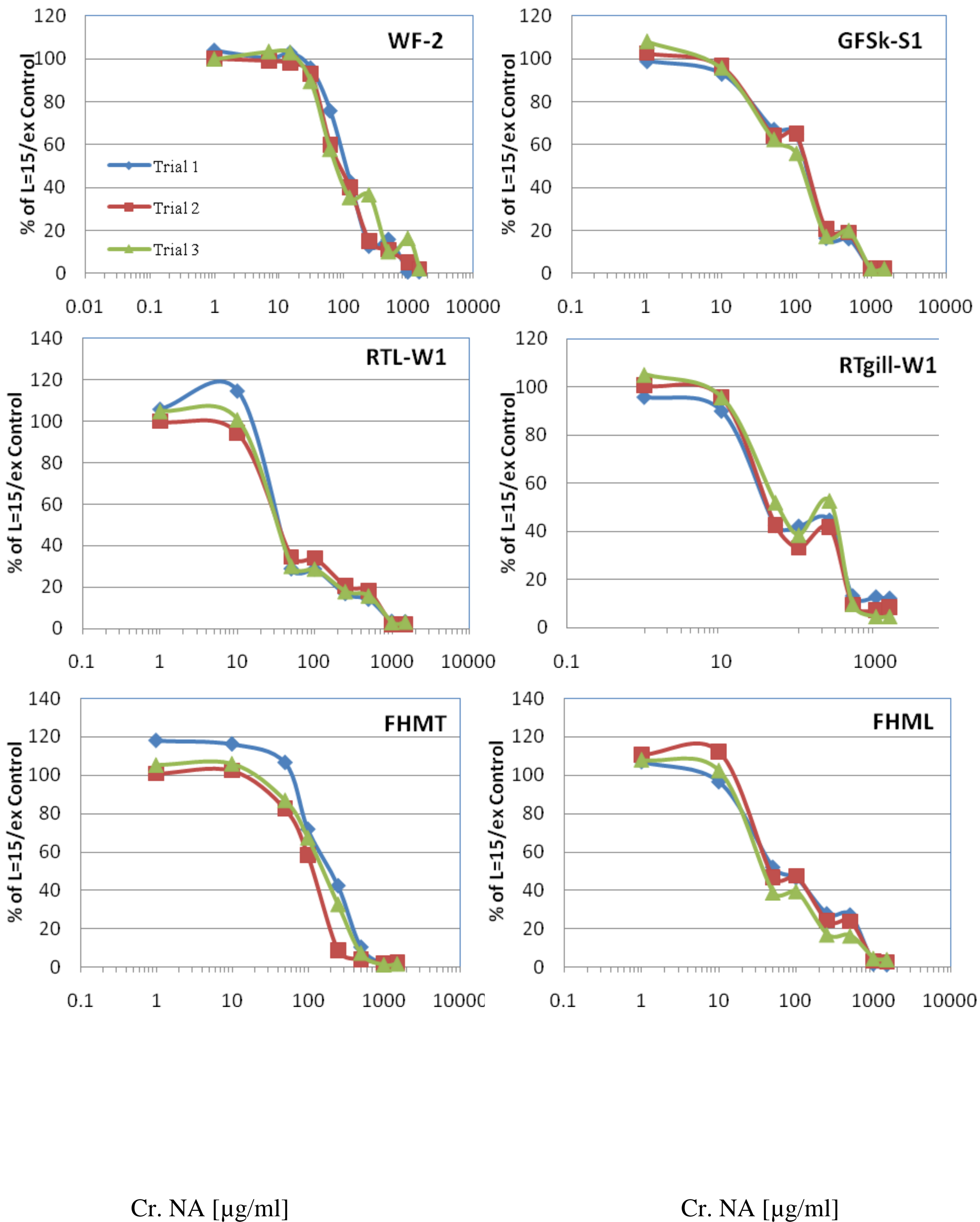


Figure 5.11 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to Cr. NA as measured by AB. Cells were exposed to serial dilutions of Cr. NA (nominal concentrations in µg/ml) prepared in L-15/ex. Results illustrate cell viability measured AB. Data points represent an average of 6 replicate wells.

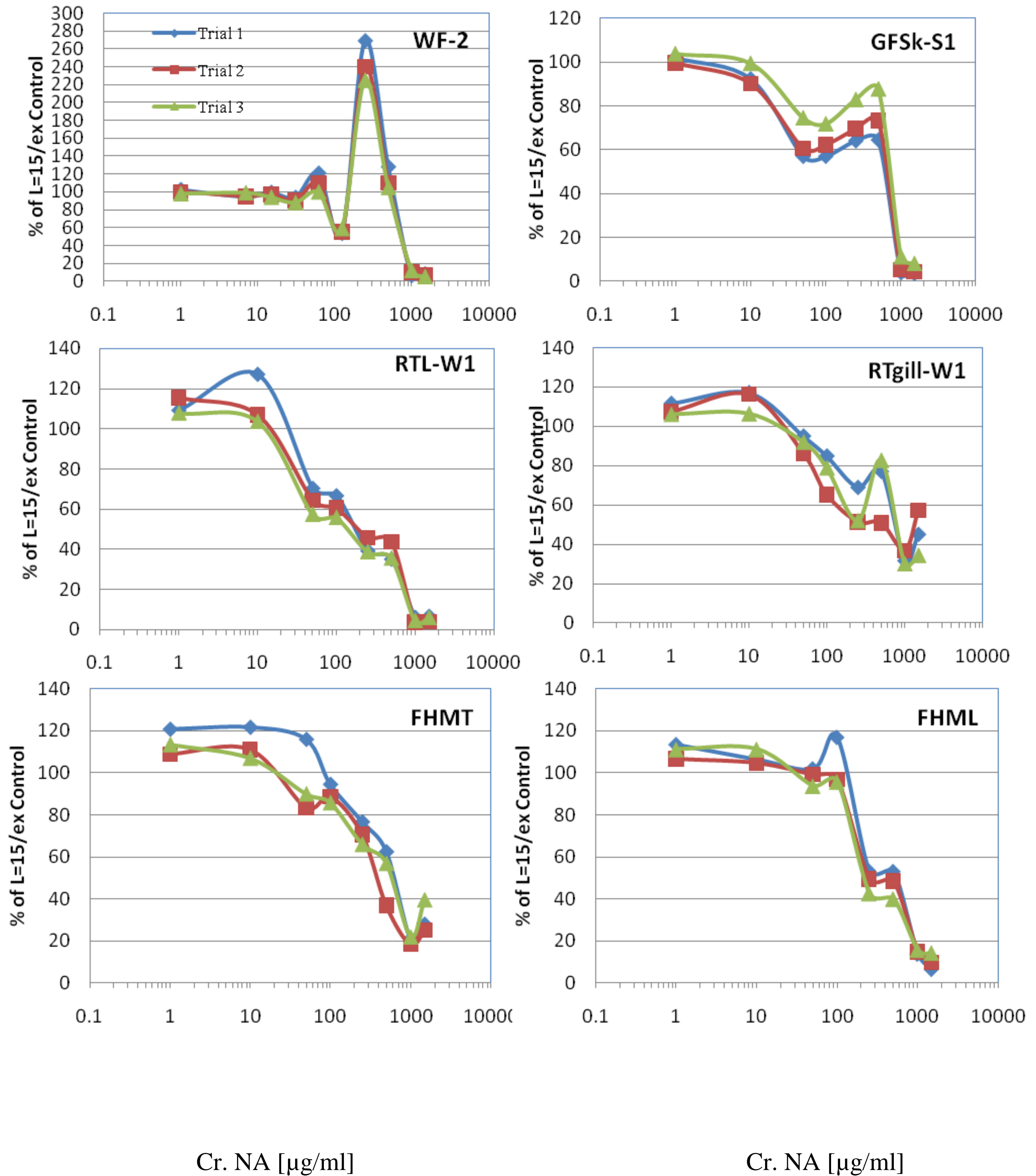
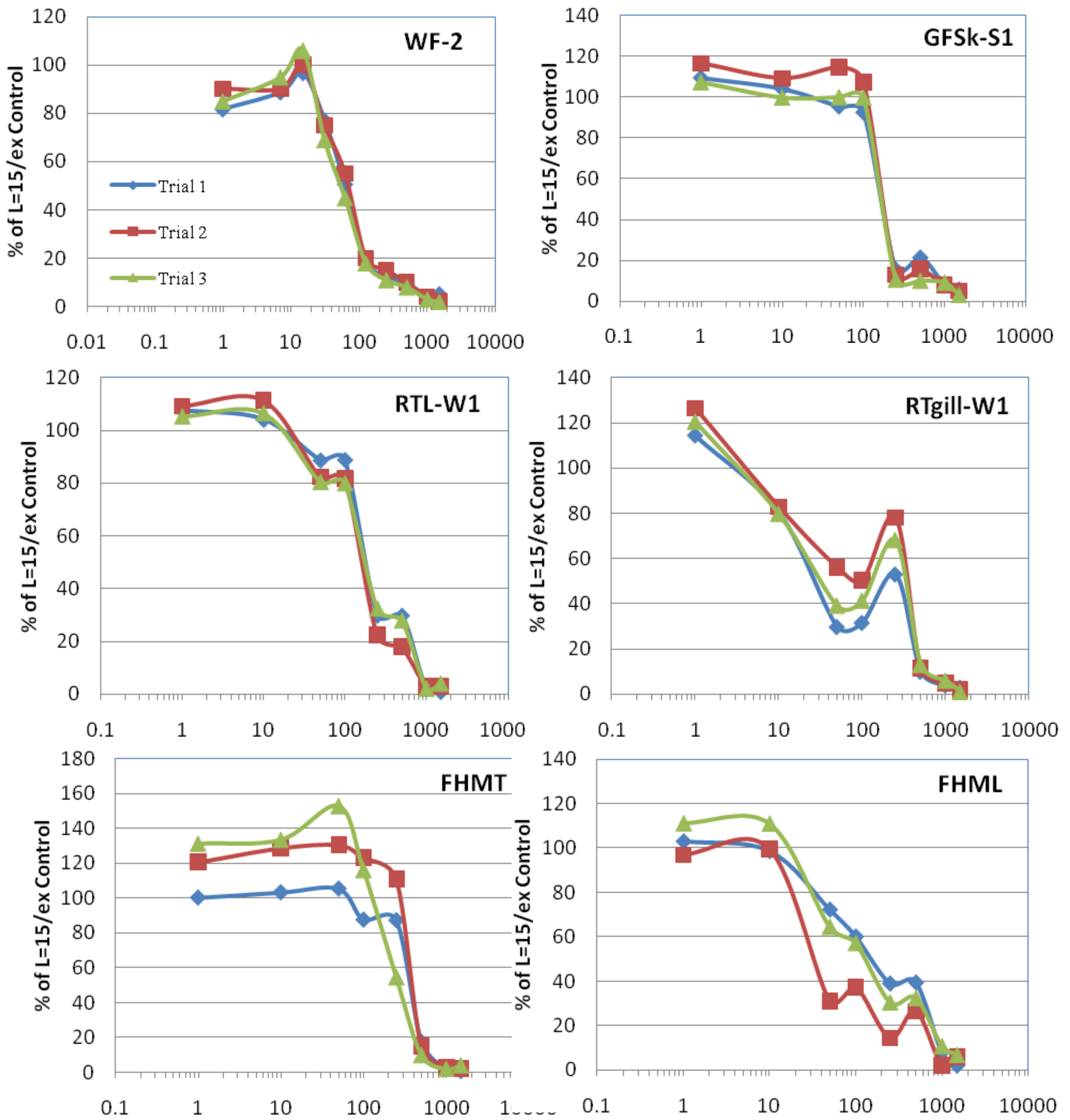


Figure 5.12 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to Cr. NA as measured by CFDA-AM. Cells were exposed to serial dilutions of Cr. NA (nominal concentrations in µg/ml) prepared in L-15/ex.



Cr. NA [µg/ml] Cr. NA [µg/ml]

Figure 5.13 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to Cr. NA as measured by NR. Cells were exposed to serial dilutions of Cr. NA (nominal concentrations in µg/ml) prepared in L-15/ex.

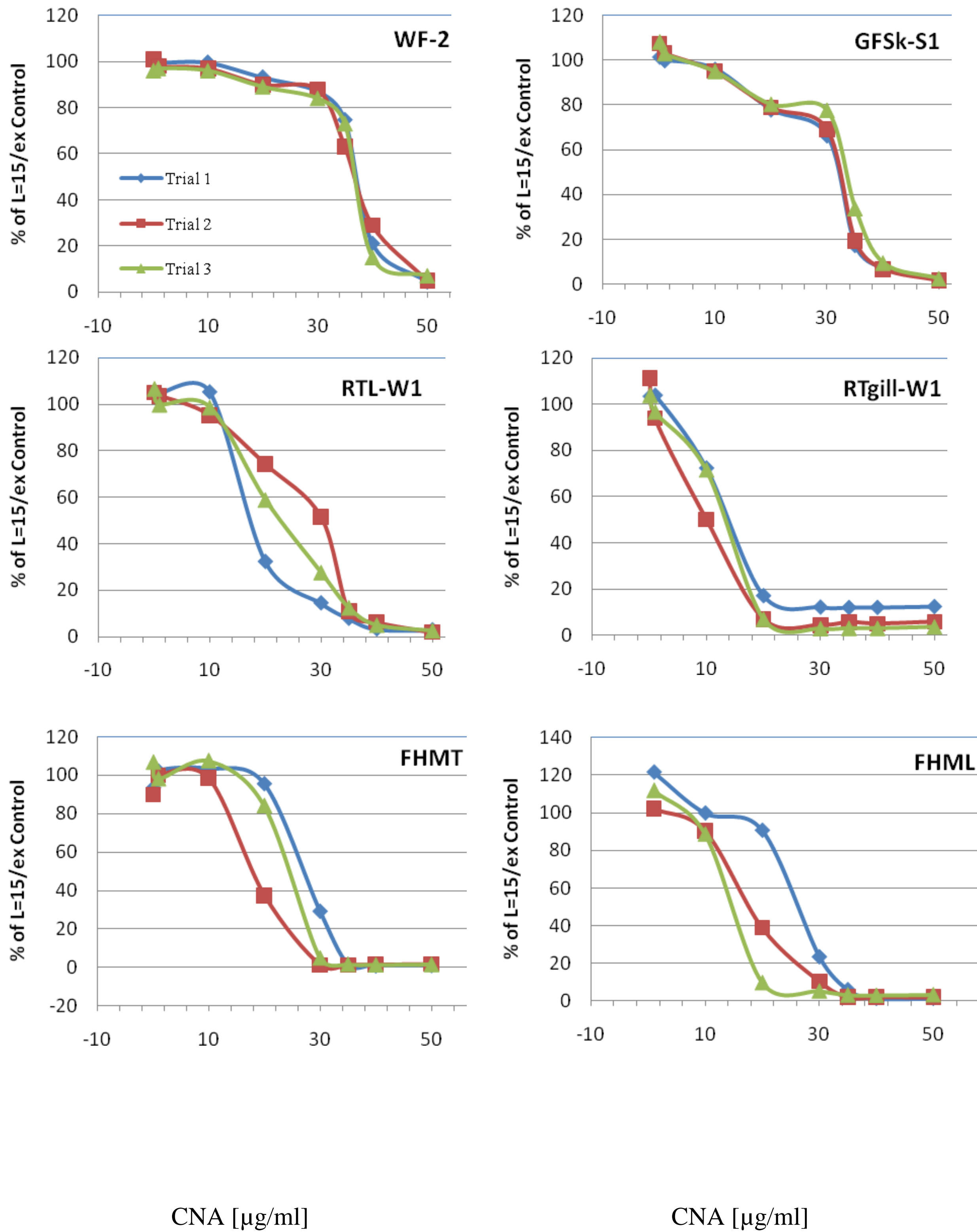


Figure 5.14 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to CNA as measured by AB. Cells were exposed to serial dilutions of CNA (nominal concentrations in µg/ml) prepared in L-15/ex.

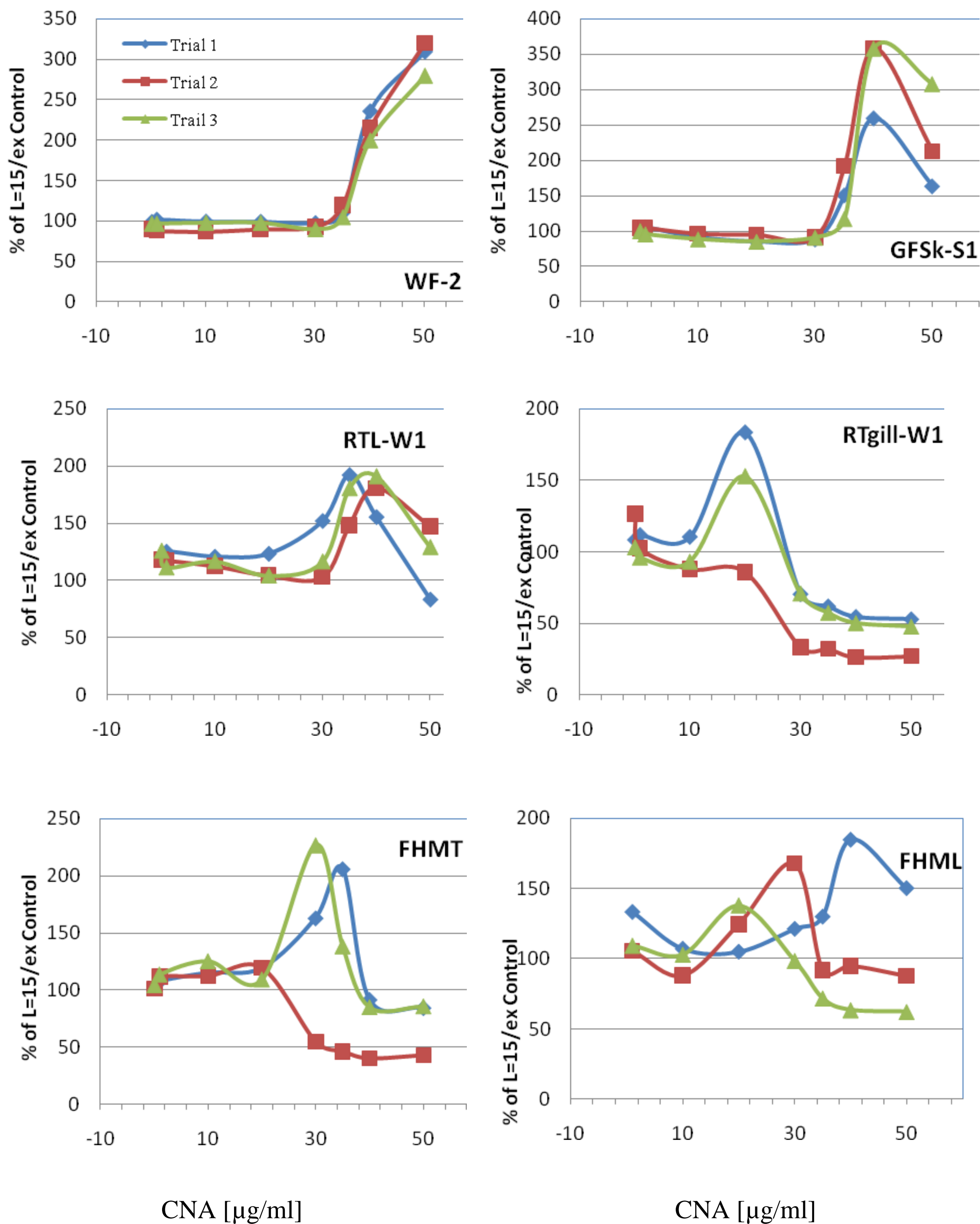


Figure 5.15 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to CNA as measured by CFDA-AM. Cells were exposed to serial dilutions of CNA (nominal concentrations in µg/ml) prepared in L-15/ex.

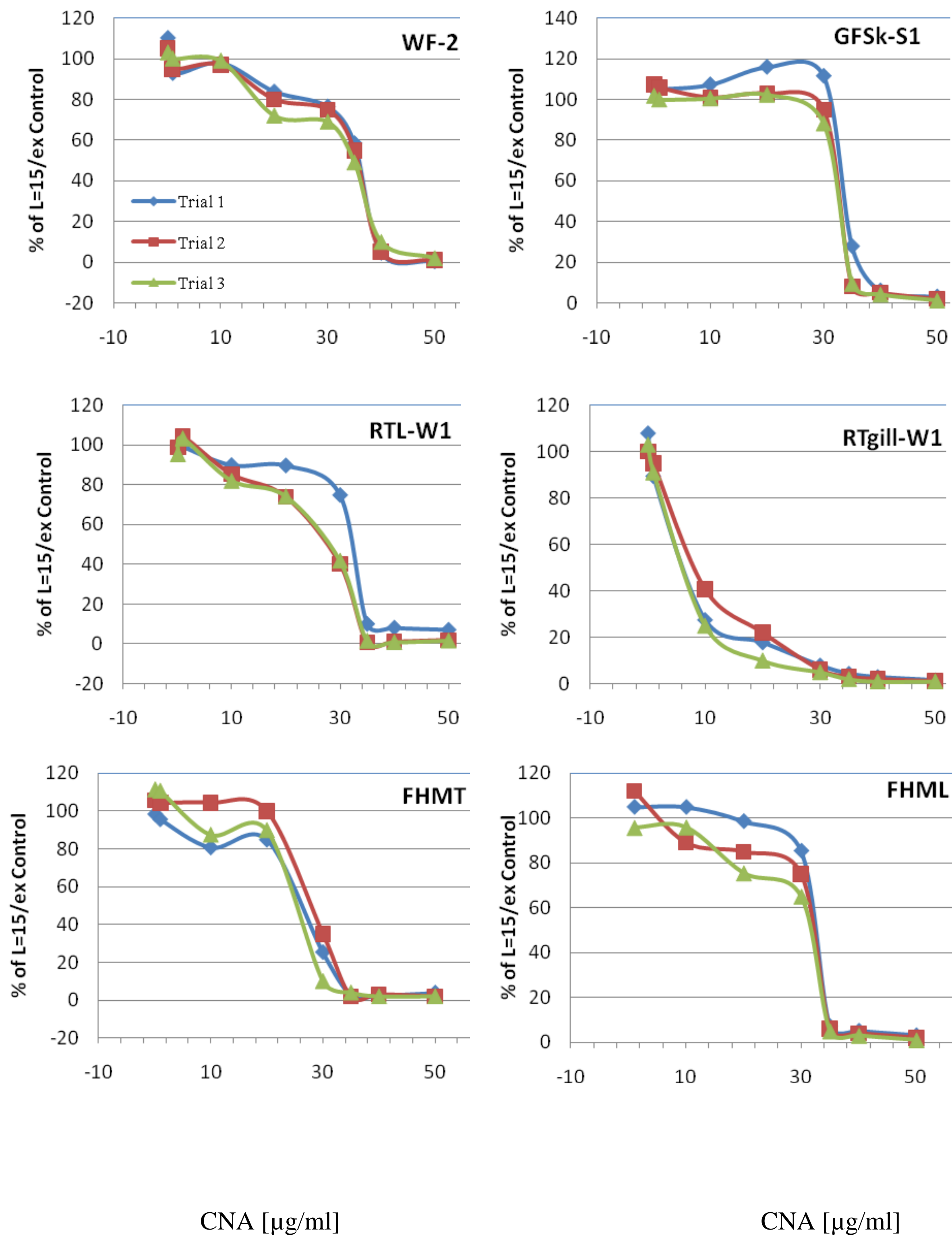


Figure 5.16 - Viability of WF-2, RTgill-W1, FHML, FHMT, GFSK-S1, and RTL-W1 cells after 24h exposure to CNA as measured by NR. Cells were exposed to serial dilutions of CNA (nominal concentrations in µg/ml) prepared in L-15/ex.

Appendix G - Correlation between WF-2 cell-line viability as measured by AB and concentration of NA (ug/ml) in OSPW samples

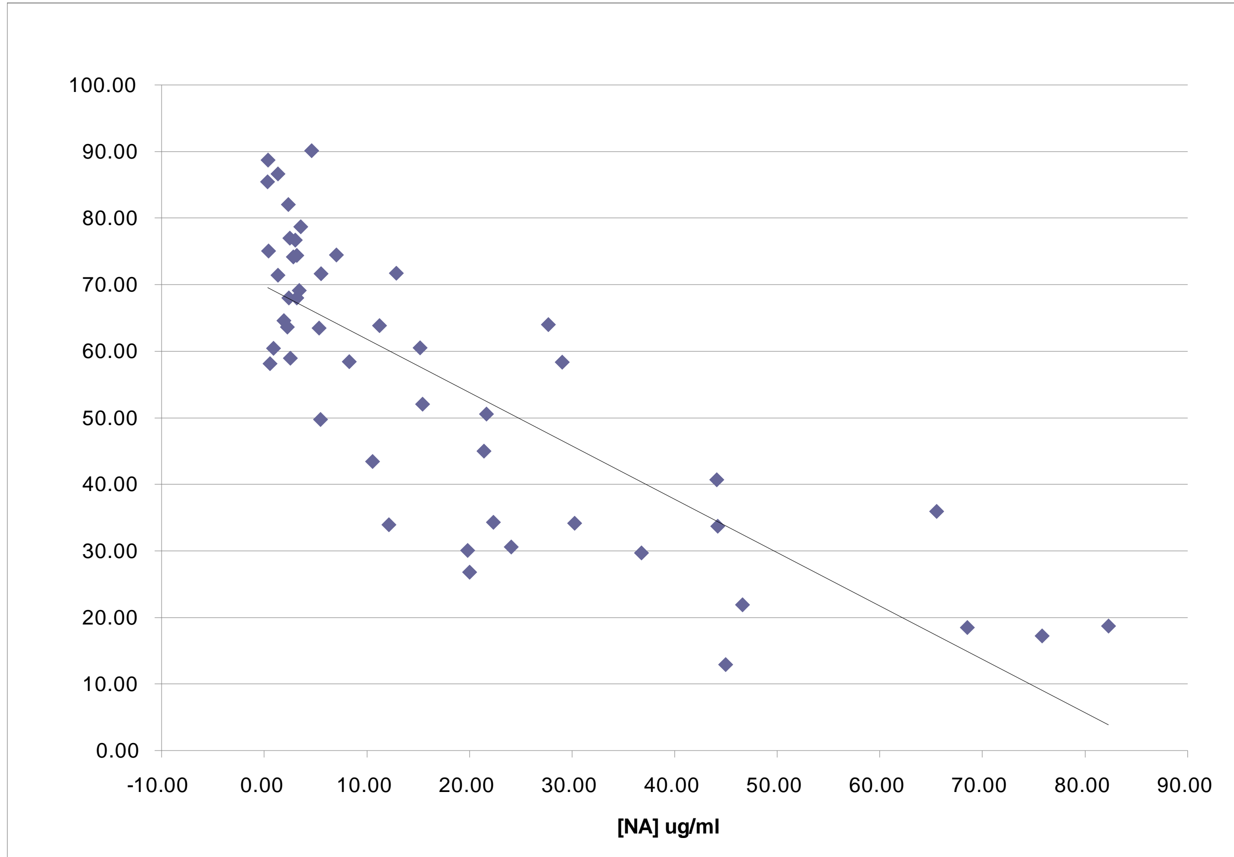


Figure 5.17 – Representative graph showing correlation between cell viability and naphthenic acid concentration of evaluated OSPW samples. WF-2 cells were exposed to OSPW samples for 24h after which cell viability was measured using AB, CFDA-AM, and NR. This was done for all six cell-lines. This graph shows significant correlation between cell viability as measured by AB and concentration of NA present in 49 individual OSPW samples (R^2=0.6171; $p<0.0001$).

Appendix H - Comprehensive OSPW sample composition – Received from Dr. Mike MacKinnon, Syncrude Canada, Ltd.

OSPW Samples 1-20

OSPW #	Site	SCL Pond #	Pond	Date	Tag	N (UTM)	E (UTM)	pH	Cond (uS/cm)	Temp	DO	NA (mg/L)	NH_4 (mg/L)	Na	K	Mg	Ca	F	Cl	SO_4	CO_3	HCO_3	Al	B
1	SCL ts	1	FE1	18-6-07	E44748	6327088	457971	8.24	704	18.1	11.2	1.3	0.29	76.9	0.5	32.7	57.6	*	4.6	259	0.0	182	*	0.15
2	SCL ts	2	FE2	18-6-07	E44749	6327015	457958	8.39	696	17.8	12.5	3.0	0.27	144	0.5	16.9	21.6	*	35.0	36.7	15.9	367	*	0.35
3	SCL ts	3	FE3	18-6-07	E44750	6327034	457942	8.39	690	18.1	10.8	2.8	0.30	143	0.5	16.5	19.5	*	31.0	43.8	17.4	348	*	0.48
4	SCL ts	4	FE4	18-6-07	E44751	6327050	457919	8.53	667	18.5	11.4	3.6	0.28	137	0.5	17.6	18.0	*	27.0	58.4	18.0	322	*	0.43
5	SCL ts	5	FE5	18-6-07	E44752	6327040	457911	8.80	2340	18.1	12.6	11.2	2.1	614	8.0	39.0	20.0	*	140	777	50.1	481	*	1.96
6	SCL ts	6	FE6	18-6-07	E44753	6327023	457932	8.77	1260	18.4	11.8	2.5	0.21	273	0.5	30.8	19.7	*	34.0	308	28.0	385	*	0.74
7	SCL ts	9	TPWPOND	18-6-07	E44756	6326944	458070	8.98	2040	17.9	9.1	21.6	0.18	528	6.2	10.1	9.0	*	240	122	75.6	664	0.6	1.90
8	SCL ts	10	STORPD	18-6-07	E44757	6326913	458076	8.91	2740	18.2	8.6	36.8	0.29	795	7.2	11.3	10.8	*	320	234	121.0	959	0.7	2.83
9	SCL ts	11	BPIT	18-6-07	E44758	6326791	458187	8.90	1530	18.5	10.7	8.3	0.35	379	5.1	18.0	14.2	*	110	164	55.2	575	0.1	1.31
10	SCL ts	13.1	SHALWL-Ditch	18-6-07	E44760	6326603	458112	8.85	620	19.4	14.5	0.4	0.21	91.2	0.5	38.1	19.3	*	14.0	125	25.8	223	*	0.31
11	SCL ts	CTPd	CT POND	18-6-07	E44762	6330006	458808	8.51	3750	19.6	11.1	27.7	0.01	1040	14.6	33.5	35.9	*	650	1220	20.7	357	*	3.34
12	SCL sw	BCV-1	MLSP-OP	18-6-07	E44764	6327922	461466	7.77	1920	11.1	0.8	44.2	2.31	548	0.5	11.6	27.8	*	220	71.6	13.5	1030	*	1.49
13	SCL sw	BCV-4	BCV-A5	18-6-07	E44768	6328325	461811	8.16	2490	17.0	8.2	24.1	0.01	628	0.5	21.6	45.9	*	480	89.2	24.9	821	*	1.17
14	Ref W	ML	MLAKE	18-6-07	E44770	6323413	463560	8.31	340	19.5	9.7	0.3	0.16	22.9	0.5	10.1	35.9	*	12.0	29.8	0.0	160	*	BDL
15	SCL Seep W	BCV-5	BCV-B16	18-6-07	E44771	6330236	462034	7.02	1280	13.1	6.4	1.9	0.23	176	0.5	34.6	103.0	*	150	229	0.0	346	*	0.18
16	SCL Seep W	Dyke Seep	DD B2506	18-6-07	E44772	6325607	463545	7.53	2310	9.5	1.9	65.5	2.72	702	9.0	14.2	18.1	*	240	295	13.5	1050	*	2.41

* Below detectable limits

OSPW Samples 1-16 Continued…

OSPW	Site	SCL Pond #	Pond	Date	Tag	Cr	Cu	Fe	Li	Mn	Mo	Ni	P	Pb	S	Sb	Se	Si	Sr	Ti	V	Zn	Zr
1	SCL Test Site	1	FE1	18-Jun-07	E44748	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	88.3	BDL	BDL	0.2	0.35	BDL	BDL	BDL	BDL
2	SCL Test Site	2	FE2	18-Jun-07	E44749	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	14.6	BDL	BDL	0.2	0.16	BDL	BDL	BDL	BDL
3	SCL Test Site	3	FE3	18-Jun-07	E44750	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	16.8	BDL	BDL	0.2	0.15	BDL	BDL	BDL	BDL
4	SCL Test Site	4	FE4	18-Jun-07	E44751	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	21.7	BDL	BDL	0.3	0.14	BDL	BDL	BDL	BDL
5	SCL Test Site	5	FE5	18-Jun-07	E44752	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	273	BDL	BDL	0.2	0.41	BDL	BDL	BDL	BDL
6	SCL Test Site	6	FE6	18-Jun-07	E44753	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	112	BDL	BDL	0.1	0.25	BDL	BDL	BDL	BDL
7	SCL Test Site	9	TPWPOND	18-Jun-07	E44756	BDL	BDL	0.2	BDL	BDL	BDL	BDL	BDL	BDL	48.1	BDL	BDL	3.5	0.16	BDL	BDL	BDL	BDL
8	SCL Test Site	10	STORPD	18-Jun-07	E44757	BDL	BDL	0.2	BDL	BDL	BDL	BDL	BDL	BDL	85.8	BDL	BDL	4.8	0.24	BDL	BDL	BDL	BDL
9	SCL Test Site	11	BPIT	18-Jun-07	E44758	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	59.4	BDL	BDL	1.8	0.26	BDL	BDL	BDL	BDL
10	SCL Test Site	13.1	SHALWL-Ditch	18-Jun-07	E44760	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	44.8	BDL	BDL	0.2	0.23	BDL	BDL	BDL	BDL
11	SCL Test Site	CTPd	CT POND	18-Jun-07	E44762	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	407	BDL	BDL	0.4	0.74	BDL	BDL	BDL	BDL
12	SCL Seep W	BCV-1	MLSP-OP	18-Jun-07	E44764	BDL	BDL	1.9	BDL	0.2	BDL	BDL	BDL	BDL	27.6	BDL	BDL	5.1	0.24	BDL	BDL	BDL	BDL
13	SCL Seep W	BCV-4	BCV-A5	18-Jun-07	E44768	BDL	BDL	0.5	BDL	BDL	BDL	BDL	BDL	BDL	32.8	BDL	BDL	3.0	0.18	BDL	BDL	BDL	BDL
14	Ref W	ML	MLAKE	18-Jun-07	E44770	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	10.9	BDL	BDL	1.5	0.25	BDL	BDL	BDL	BDL
15	SCL Seep W	BCV-5	BCV-B16	18-Jun-07	E44771	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	81.9	BDL	BDL	2.3	0.27	BDL	BDL	BDL	BDL
16	SCL Seep W	DykeSeep	DD B2506	18-Jun-07	E44772	BDL	BDL	0.2	BDL	BDL	BDL	BDL	BDL	BDL	106	BDL	BDL	5.5	0.54	BDL	BDL	BDL	BDL

OSPW Samples 17-20

	Sample	Water Source	Carbon (Coke) Source	Date	Coke Content (wt%)	Tag	pH	Cond (uS/cm)	Nap Acids (mg/L)	NH_4 (ppm)	Na	K	Mg	Ca	F	Cl	SO_4	CO_3	HCO_3	Al	B
		Coke Slurry Water																			
17	WIP_HC*-1 Slurry	MLSB	Coke 8-1	12-Jun-07	6.7	E44727	7.93	3200	5.5	16.9	704	15.6	11.9	19.5	BDL	440	424	0.0	648	0.33	2.16
		Coke Adsorption Experiment Water																			
18	WIP_Ju 12_HC8-1	WIP	Coke 8-1_Jun 11	13-Jun-2007	20	E44775	8.36	3380	15.4	13.4	844	15.0	10.7	16.0	BDL	530	384	30.9	742	BDL	2.60
19	DDW_May15_HC8-1	DDW	Coke 8-1_Jun 11	13-Jun-2007	20	E44776	8.25	2740	30.2	2.6	677	8.9	12.7	15.9	BDL	250	300	37.2	969	BDL	2.39
20	WIP_Jun12_HC8-1 _CO2	WIP	Coke 8-1_Jun 11	13-Jun-2007	20	E44777	7.34	3460	21.4	14.9	793	14.2	10.8	16.3	BDL	520	383	0.0	825	BDL	2.49

OSPW Samples 17-20 Continued…

	Sample	Water Source	Carbon (Coke) Source	Date	Coke Content (wt%)	Tag	Ba	Cd	Co	Cr	Cu	Fe	Mn	Mo	Ni	P	Pb	S	Se	Si	Sr	V	Zn	Zr
		Coke Slurry Water																						
17	WIP_HC*-1 Slurry	MLSB	Coke 8-1	12-Jun-07	6.7	E44727	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.5	BDL	BDL	BDL	146	BDL	3.0	0.6	3.04	BDL	BDL
		Coke Adsorption Experiment Water																						
18	WIP_Ju 12_HC8-1	WIP	Coke 8-1_Jun 11	13-Jun-2007	20	E44775	0.2	BDL	BDL	BDL	BDL	BDL	BDL	0.2	BDL	BDL	BDL	137	BDL	2.6	0.6	1.47	BDL	BDL
19	DDW_May15_ HC8-1	DDW	Coke 8-1_Jun 11	13-Jun-2007	20	E44776	0.1	BDL	BDL	BDL	BDL	BDL	BDL	0.1	BDL	BDL	BDL	103	BDL	5.4	0.5	1.41	BDL	BDL
20	WIP_Jun12_H C8-1 _CO2	WIP	Coke 8-1_Jun 11	13-Jun-2007	20	E44777	0.2	BDL	BDL	BDL	BDL	BDL	0.0	0.2	BDL	BDL	BDL	129	BDL	2.5	0.6	1.47	BDL	BDL

OSPW Samples 21-49

OSPW #	Site	Pond #	Location	Date	N (UTM)	E (UTM)	Tag	pH	Cond (uS/cm)	Temp	DO	Nap Acids	NH_4	Na	K	Mg	Ca	F	Cl	SO_4	CO_3
21	SCLRecl	GP	SCL_Golden Pond	8-Aug-08	6317297	462018	E62430	8.83	1680	23.2		3.4	<0.01	225	1.1	57.6	115	BDL	38	746	0.0
22	SUN Recl	SUN_HighSO4	SUN_High SO4WL	8-Aug-08	6317201	466390	E62431	7.64	2980	23.5		15.2	<0.01	437	15.9	118	200	BDL	4.4	1590	0.0
23	SUN WL	SUN-4mCT	SUN_4m CT	8-Aug-08	6316529	467777	E62432	8.25	1953	20.4		22.3	0.22	326	13.5	58.5	83.3	BDL	43	595	16
24	SUN WL	SUN_NWL	SUN_NatWL	8-Aug-08	6315310	468982	E62433	9.11	1242	20.7		44.1	0.56	292	11.9	14.1	19.4	1.3	17	204	37
25	CNRL	CNRL_WL	CNRL	31-Jul-08			E62434	9.32	256	21.7	8.0	2.4	<0.01	22.3	0.6	8.7	23.0	BDL	4.7	22.2	0.0
26	SCL WL	SCL_SBeavWL	South Beaver	6-Aug-08			E62435	7.57	345	19.1		3.2	<0.01	30.8	0.8	10.3	41.3	BDL	6.0	5.1	0.0
27	SCL WL	SCL_NWIDWL	SCL_NWID Ditch WL	6-Aug-08			E62437	8.19	663	21.8		2.3	0.11	94.4	1.6	22.1	39.7	BDL	56	37.9	0.0
28	SUN WL	SN	SUNCTWL_Waste Area 11	7-Aug-08	6316190	467187	E62439	8.69	868	22.2		7.0	0.18	112	9.8	32.4	52.4	BDL	6.5	308	6.3
29	SCL Test	CTUPond	U-Shaped Pond	18-Aug-08	6323038	460234	E62402	8.91	342	21.2	10.1	4.6	0.17	37.1	1.0	8.0	29.1	BDL	25.0	79.4	0
30	SCL Test	1	FE1	18-Aug-08	6327088	457969	E62403	7.69	729	21.9	5.9	1.3	0.12	78.2	1.0	30.0	53.9	BDL	5.6	249	0
31	SCL Test	2	FE2	18-Aug-08	6327018	457959	E62404	8.35	688	21.1	6.8	3.2	0.70	148	1.0	15.4	14.9	0.2	33.0	51.7	20
32	SCL Test	3	FE3	18-Aug-08	6327032	457941	E62405	8.52	674	21.5	8.7	2.4	0.14	147	1.0	15.0	13.5	0.2	29.0	54.8	21
33	SCL Test	5	FE5	18-Aug-08	6327037	457909	E62407	8.96	2680	21.7	9.3	10.6	0.23	630	8.7	37.6	15.1	BDL	140	784	65
34	SCL Test	6	FE6	18-Aug-08	6327019	457931	E62408	9.03	1252	21.8	12.2	2.5	0.34	268	1.0	29.3	12.4	BDL	37.0	341	44
35	SCL Test	9	TPW POND	18-Aug-08	6326943	458066	E62411	9.20	2080	19.5	6.3	20.0	0.28	519	1.0	8.7	5.7	1.3	230	119	124
36	SCL Test	10	STOR POND	18-Aug-08	6326915	458077	E62412	8.81	3010	22.4	8.6	45.0	<0.01	780	7.6	11.0	9.7	2.2	310	275	139
37	SCL Test	11	BPIT	18-Aug-08	6326776	458206	E62413	9.06	1584	22.2	9.7	12.1	0.15	378	1.0	15.8	8.9	0.6	112	188	109
38	SCL Test	12	DEEP WL	18-Aug-08	6326603	458368	E62414	7.83	547	22.7	9.7	0.92	<0.01	72.2	1.0	23.9	30.9	BDL	12.0	78.6	13
39	SCL Test	13.1	SHALWL-Ditch	18-Aug-08	6326634	458130	E62415	8.61	748	21.2	3.1	0.55	<0.01	113	1.0	37.1	19.9	BDL	15.0	174	30
40	SCL Test	CTPd	CT POND	18-Aug-08	6330003	458807	E62417	8.72	4730	23.3	10.3	29.0	<0.01	1080	15.0	32.3	31.2	3.3	690	1260	41
41	SCL Test	CTProto	CT PROTO POND	18-Aug-08	6328848	458631	E62418	8.93	540	24.1	11.2	5.5	0.10	124	1.0	3.5	7.4	BDL	69	28.5	20
42	Seep Water	BCV-1	MSLB OP	18-Aug-08	6327923	461465	E62419	7.56	2230	11.2	0.6	68.5	2.17	570	5.5	11.7	28.1	1.3	210	84.5	36
43	Seep Water	SCP-1	SCP1	18-Aug-08	6328616	461375	E62422	7.96	2270	17.4	7.9	46.6	0.51	557	5.6	17.6	40.0	0.9	250	124	40
44	Seep Water	BCV-4	BCV-A5	18-Aug-08	6328331	461813	E62423	8.07	2280	19.2	6.1	19.8	<0.01	519	1.0	18.2	44.8	BDL	340	91.3	22
45	Seep Water	BCV-5	BCV-B16	18-Aug-08	6330255	462031	E62424	7.49	1261	16.5	5.4	5.4	<0.01	159	1.0	32.0	94.9	BDL	130	185	10
46	Seep Water	BCV-0.5	ETB POND	18-Aug-08	6327559	462034	E62425	8.94	535	23.1	11.3	12.9	<0.01	123	1.0	3.0	7.5	0.1	55	29.1	16
47	Seep Water	DykeSeep	DD B2506	18-Aug-08	6325605	463546	E62426	7.59	2850	10.1	1.8	82.3	2.81	706	9.5	14.4	18.9	4.1	250	310	30
48	Seep Water	DykeSeep	DD B2503	18-Aug-08	6325587	463549	E62427	7.20	2950	10.4	1.0	75.8	2.70	733	10.5	14.6	24.3	0.8	280	301	29
49	RefPond	ML	MLAKE	18-Aug-08	6323411	463556	E62428	8.22	287	23.3	9.1	0.4	<0.01	17.9	1.0	9.8	33.7	BDL	8.0	31.1	0

OSPW Samples 21-49 Continued…

OSPW #	Site	Pond #	Loc	Date	HCO_3	Alkalinity expressed as $CaCO_3$	Na/Cl (meq/meq)	(Ca+Mg) / HCO_3 (meq/meq)	(Ca+Mg) / SO_4 (meq/meq)	Na / (Ca+Mg) (meq/meq)	Al	B	Fe	Mn	Mo	S	Si	Sr	V	Zn
21	SCLRecl	GP	SCL_Golden Pond	8-Aug-08	163	134	9.14	3.95	0.68	0.93	BDL	0.13	BDL	BDL	BDL	266	0.5	0.8	BDL	BDL
22	SUN Recl	SUN_HighSO4	SUN_High SO4WL	8-Aug-08	239	196	153.30	5.06	0.60	0.96	BDL	0.93	BDL	BDL	BDL	539	0.6	2.2	BDL	BDL
23	SUN WL	SUN-4mCT	SUN_4m CT	8-Aug-08	512	446	11.70	1.01	0.73	1.57	BDL	1.73	0.3	BDL	BDL	210	6.4	1.0	BDL	BDL
24	SUN WL	SUN_NWL	SUN_NatWL	8-Aug-08	504	475	26.51	0.23	0.50	5.92	0.6	2.46	0.9	BDL	0.3	70.1	6.1	0.5	BDL	BDL
25	CNRL	CNRL_WL	CNRL	31-Jul-08	120	98	7.32	0.95	4.05	0.52	BDL	0.07	0.1	BDL	BDL	7.6	0.5	0.2	BDL	BDL
26	SCL WL	SCL_SBeavWL	South Beaver	6-Aug-08	231	189	7.92	0.77	27.51	0.46	BDL	0.07	0.9	0.2	BDL	1.7	2.1	0.2	BDL	BDL
27	SCL WL	SCL_NWIDWL	SCL_NWID Ditch WL	6-Aug-08	333	273	2.60	0.70	4.85	1.07	BDL	0.17	BDL	BDL	BDL	15.9	1.9	0.3	BDL	BDL
28	SUN WL	SN	SUNCTWL_Waste Area 11	7-Aug-08	169	149	26.60	1.78	0.83	0.92	BDL	0.24	BDL	BDL	BDL	109	0.7	0.4	BDL	BDL
29	SCL Test	CTUPond	U-Shaped Pond	18-Aug-08	80	65	2.29	1.62	1.28	0.76	BDL	0.23	BDL	BDL	BDL	27.3	0.7	0.30	BDL	BDL
30	SCL Test	1	FE1	18-Aug-08	173	142	21.55	1.83	1.00	0.65	BDL	0.15	BDL	BDL	BDL	85.7	1.0	0.32	BDL	BDL
31	SCL Test	2	FE2	18-Aug-08	322	296	6.92	0.34	1.88	3.17	BDL	0.35	BDL	BDL	BDL	15.9	0.8	0.12	BDL	BDL
32	SCL Test	3	FE3	18-Aug-08	308	287	7.82	0.34	1.69	3.32	BDL	0.49	BDL	BDL	BDL	16.7	0.7	0.10	BDL	BDL
33	SCL Test	5	FE5	18-Aug-08	403	439	6.95	0.44	0.24	7.04	BDL	2.00	BDL	BDL	BDL	266	0.6	0.30	BDL	BDL
34	SCL Test	6	FE6	18-Aug-08	259	286	11.18	0.53	0.43	3.81	BDL	0.73	BDL	BDL	BDL	116	0.6	0.17	BDL	BDL
35	SCL Test	9	TPW POND	18-Aug-08	553	660	3.48	0.08	0.41	22.39	0.6	1.88	0.1	BDL	BDL	41.3	4.0	0.09	BDL	BDL
36	SCL Test	10	STOR POND	18-Aug-08	896	966	3.88	0.07	0.24	24.18	0.9	2.76	0.3	BDL	BDL	91.2	6.1	0.22	BDL	BDL
37	SCL Test	11	BPIT	18-Aug-08	419	525	5.21	0.17	0.45	9.33	0.2	1.27	BDL	BDL	BDL	63.5	2.1	0.20	BDL	BDL
38	SCL Test	12	DEEP WL	18-Aug-08	258	232	9.29	0.76	2.16	0.89	BDL	0.13	BDL	BDL	BDL	29.9	1.0	0.21	BDL	BDL
39	SCL Test	13.1	SHALWL-Ditch	18-Aug-08	218	228	11.63	0.90	1.13	1.20	BDL	0.37	BDL	BDL	BDL	58.2	1.7	0.19	BDL	BDL
40	SCL Test	CTPd	CT POND	18-Aug-08	298	312	2.42	0.68	0.16	11.04	BDL	3.44	BDL	BDL	BDL	402	0.8	0.71	BDL	BDL
41	SCL Test	CTProto	CT PROTO POND	18-Aug-08	158	163	2.77	0.20	1.12	8.14	BDL	0.54	0.1	BDL	BDL	10.7	0.8	0.12	BDL	BDL
42	Seep Water	BCV-1	MSLB OP	18-Aug-08	1025	900	4.19	0.13	1.35	10.41	BDL	1.60	1.0	0.3	BDL	30.1	5.3	0.25	BDL	BDL
43	Seep Water	SCP-1	SCP1	18-Aug-08	914	815	3.44	0.21	1.34	6.99	BDL	1.51	0.2	BDL	BDL	46.0	6.1	0.33	BDL	BDL
44	Seep Water	BCV-4	BCV-A5	18-Aug-08	765	664	2.36	0.28	1.98	6.01	BDL	1.26	1.2	0.1	BDL	31.4	4.7	0.17	BDL	BDL
45	Seep Water	BCV-5	BCV-B16	18-Aug-08	351	304	1.89	1.22	1.92	0.93	BDL	0.22	BDL	BDL	BDL	67.0	3.8	0.27	BDL	BDL
46	Seep Water	BCV-0.5	ETB POND	18-Aug-08	184	177	3.45	0.18	1.03	8.57	0.4	0.44	0.2	BDL	BDL	11.4	2.2	0.10	BDL	BDL
47	Seep Water	DykeSeep	DD B2506	18-Aug-08	1020	886	4.36	0.12	0.33	14.31	BDL	2.54	0.0	BDL	BDL	110	5.7	0.60	BDL	BDL
48	Seep Water	DykeSeep	DD B2503	18-Aug-08	1040	901	4.04	0.13	0.39	13.11	BDL	2.59	BDL	0.1	BDL	109	6.0	0.62	BDL	BDL
49	RefPond	ML	MLAKE	18-Aug-08	138	113	3.45	1.11	3.86	0.31	BDL	0.06	BDL	BDL	BDL	11.4	1.6	0.25	BDL	BDL

Appendix I – Cell line responses to OSPW samples as measured by CFDA-AM and NR

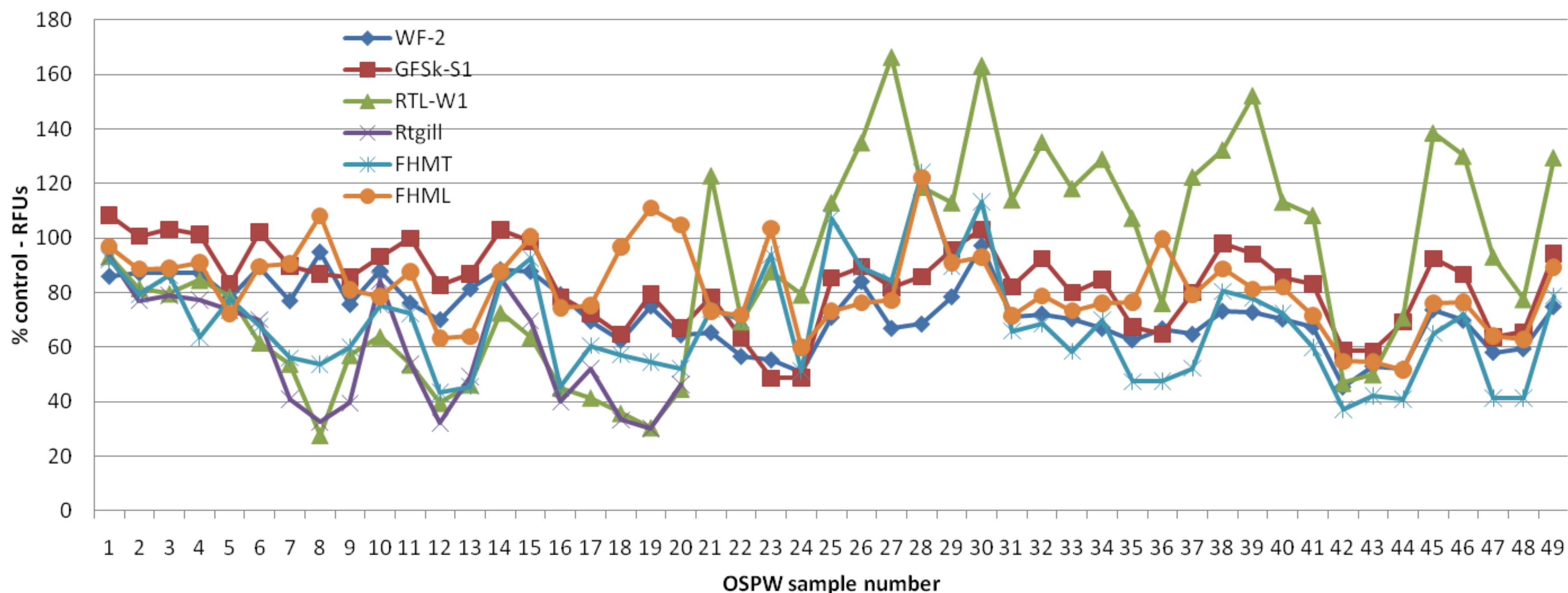


Figure 5.18 – Cell line response to 24 h exposure to OSPW samples as measured by CFDA-AM. FHML, FHMT, RTgill-W1, RTL-W1, GFSk-S1, and WF-2 cells were exposed to iso-osmotic OSPW samples for 24 h at 18°C. Cell viability was then measured by CFDA-AM. Data points represent the mean of 2 separate experiments (each experiment consisted of 6-well replicates for each OSPW sample). Cells were plated at densities ranging from $3.3X10^4$ – $8.0X10^4$ cells/well.

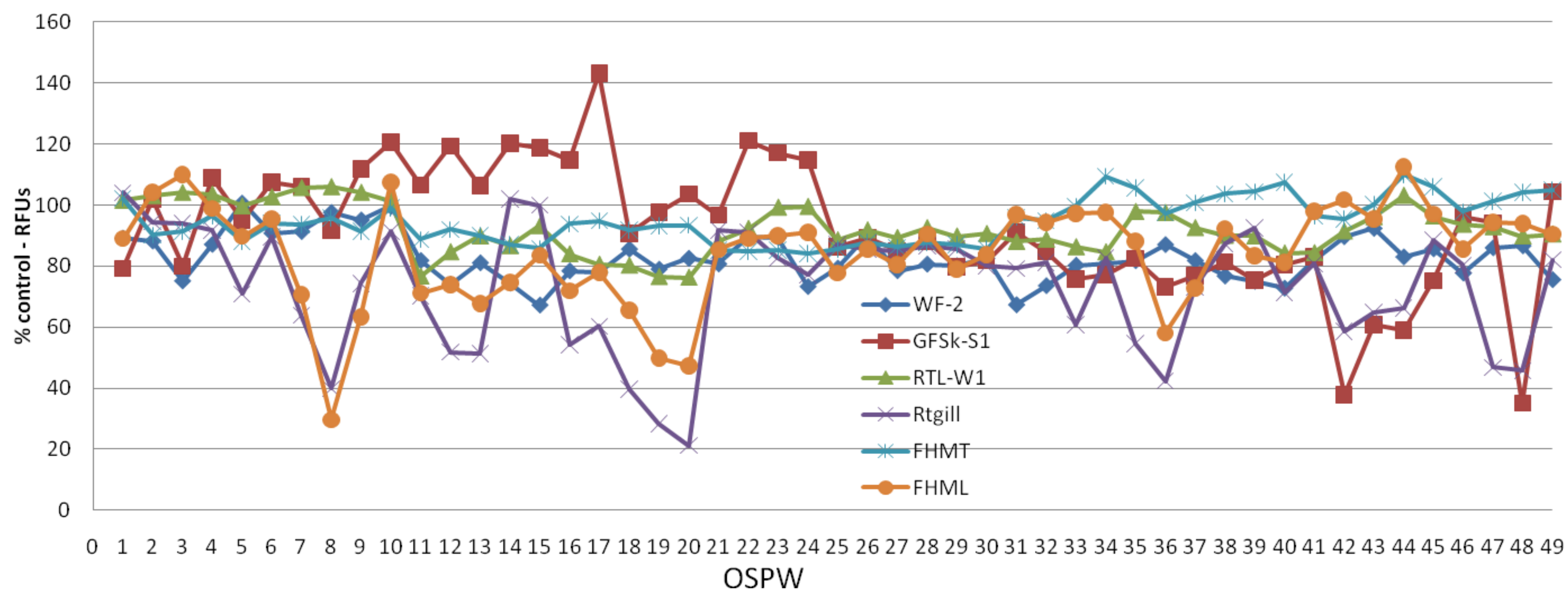


Figure 5.19 – Cell line response to 24 h exposure to OSPW samples as measured by NR. FHML, FHMT, RTgill-W1, RTL-W1, GFSk-S1, and WF-2 cells were exposed to iso-osmotic OSPW samples for 24 h at 18°C. Cell viability was then measured by NR. Data points represent the mean of 6 replicate wells. Cells were plated at densities ranging from $3.3X10^4$ – $8.0X10^4$ cells/well.