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The establishment of a fibroblastic cell line from yellow perch (Perca flavescens) and its potential applications in toxicology

Katelin W. Spiteri
Wilfrid Laurier University, katelin.spiteri@gmail.com

The establishment of a fibroblastic cell line from yellow perch (*Perca flavescens*) and its potential applications in toxicology

By

Katelin W. Spiteri

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Abstract

This thesis describes the establishment, characterization and potential toxicological applications of YPF5, a yellow perch cell line. YPF5 is a fibroblastic cell line derived from the caudal fin of yellow perch. This cell line has been maintained for over 2 years. Cultures have been passaged over 60 times, and successfully cryopreserved and thawed. YPF5 is grown at room temperature in Leibovitz-15 (L-15) media, supplemented with 10% fetal bovine serum and 3% penicillin-streptomycin. The origin of YPF5 has been confirmed as *Perca flavescens* through "DNA Barcoding" and reaffirmed through Karyotyping (2n = 48) as per literature reported karyology for percidae including the yellow perch. YPF5 tested positive for alkaline phosphatase and negative for beta-galactosidase, markers of stem cell-like characteristics and senescence, respectively, providing evidence for a continuous cell line. Immunofluorescence staining with mesodermal cell markers (vimentin and collagen type 1) also confirmed the fibroblastic origin of the cells. In agreement with related literature, the morphological response of YPF5 exposed to cortisol made cells more epithelial-like, and exposure to ascorbic acid induced extracellular collagen secretion. YPF5 responded to different contaminants (naphthenic acid, copper sulphate, ammonium chloride and OSPW samples) in a dose dependent manner. Evaluation of possible remediation methods using bentonite and UV radiation to ameliorate toxicity of oil sands chemicals showed promise *in vitro* with YPF5 cells and could be investigated further.

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List of Abbreviations

AB	Alamar Blue
AB_{50}	EC50 value as measured by AB
AP	Alkaline phosphatase
A.A	Ascorbic Acid
AOS	Athabasca Oil Sands
CF	carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
$CFDA\text{-}AM_{50}$	EC_{50} value as measured by CFDA-AM
CO1	cytochrome c oxidase subunit 1
Cr.NA	Crude Naphthenic Acid
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EPLs	End-pit Lakes
EtOH	Ethanol
FBS	Fetal bovine serum
FHML	Fathead minnow liver cell line
GFSK-S1	Gold Fish Skin Fibroblastic cell line
IHC	Immunohistochemistry
L-15	Leibovitz-15 media
MGG	May-Grünwald Giemsa
NA	Naphthenic Acid
NR	Neutral Red
OSPW	Oil sands process-affected water

RFU	Relative fluorescence unit
PBS	Phosphate buffered saline
RT	Room temperature
TE	TrypLE
TC	Tissue culture
YPF5	Yellow Perch Fin from Fish Five
ZEB2J	Zebra fish embryonic blastula cell line

1. Introduction

1.1. Fish Cell Culture

1.1.1. General background

Cell culture is a biological technique by which viable cells are successfully maintained in a controlled environment outside of the animal (Schaeffer, 1990). Cells can be cultured for either short term (hours or days) or long term (weeks, months and even years). Cell culture was first reported by Wilhelm Roux who in 1885 was able to sustain (in a saline solution) a chick neural tissue (Wolf and Quimby, 1969). In 1914, the first account of fish tissue culture occurred with trout frys and embryos (Wolf and Quimby, 1969) and in 1962, RTG-2, derived from rainbow trout gonads, was the first documented permanent fish cell line (Wolf and Quimby, 1962). Since the establishment of the RTG-2 cell line, there have been several hundreds of fish cell lines reported in the past five decades (Fryer and Lannan, 1994; Lakra et al., 2011). However, this value could be potentially higher given the assumption that there are still unpublished reports of fish cell lines.

In general, cell culture may be classified as: (1) primary cultures and (2) cell lines. A primary culture is developed from isolated or clumps of cells from freshly derived tissues of the specimen and are considered finite and not routinely subcultured (Schaeffer, 1990). A cell line is developed from cells that initially resulted from a primary culture and have been maintained and subcultured *in vitro* with either finite (if cells have a tendency to reach senescence) or indefinite growth (Schaeffer, 1990; Bols and Lee, 1991).

1.1.2. General applications of fish cell culture

Fish cell cultures have been used extensively for research in virology (Bols et al., 1992; Fryer and Lannan, 1994). However, many other potential applications of fish cells have been undertaken because they are effective biological research tools. Fish cell lines have biomedical applications such as in the study of fundamental basis of DNA repairs and the effects of mutagens in non-mammalian vertebrate cells (Grist et al., 1986; Kocan et al., 1979). In radiation biology, fish cell cultures are useful to investigate the effects of radiation on ecosystems and how radiation-induced 'by-stander' signals are transmitted in water bodies (Mothersill et al., 2006). Another common application of fish cell lines is in the field of toxicology. Numerous studies have been conducted using fish cell lines to investigate the cytotoxicity of different contaminants: metals, industrial effluent, and oil sands process affected waters (Dayeh et al., 2002; Dayeh et al., 2005; Sansom et al., 2013 Castano et al., 2003; Bols et al., 2005; Goodale et al., 2008; Wang et al., 2004).

1.1.3. Cell line characterization

Characterization of a cell line is critical for obtaining knowledge about the cells that can confirm their origin, identify special cellular and biochemical properties, benefit future studies and help determine the research disciplines that the specific cells may be useful in (Freshney, 2010). This characterization process is especially necessary for new cell lines. In general, cell line characterization could be categorized in the following four aspects (Figure 1.1). Firstly, cell morphology and function is described. Examining the cell morphology in the cultures can contribute to the initial basic categorization of the cell lines into possible cell types like fibroblastic, epithelial and neuronal for adherent

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cells, or round, oval and irregular for non-adherent cells. For example, if cells display the shape, organization and culture behaviors of fibroblasts, further experiments can be conducted to determine if fibroblastic properties are present. These further experiments could look at expression of vimentin (a possible fibroblastic marker) or collagen type 1 (the form that is predominantly expressed in fibroblasts) (Challa and Stefanovic, 2011; Chan et al., 1990).   Secondly, cell growth characteristics are also studied because each cell line may have different requirements such as concentrations of serum, passaging frequency and temperature requirements for optimal growth and success. Normally, the thermobiology of fish cell lines overlap that of the fish species they are derived from (Bols et al., 1992). This provides valuable information on the temperature ranges that could terminate cell viability as well as promote or cease cell proliferation. Thirdly, cell line authentication is another characterization that should be performed (Freshney, 2010). Cell lines may be subjected to cross-contamination. This is imperative to discern as it may invalidate any contaminated findings (Freshney, 2010). Confirming the identity of the fish cell lines is critical because it ensures that their presumed species of origin is accurate. Testing if cells are undergoing senescence is another important factor since it can help in determining if cell lines have the potential to become immortal or finite. Fourthly, quality assurance is another aspect of the characterization process. It is important to ensure that cells are free of exogenous contaminants as cultures could contain mycoplasma, viruses, bacteria, yeast or fungi. A smaller part of quality assurance is ensuring cells are frozen in liquid nitrogen to act as a

contingency plan in case something goes wrong with the presently growing cultures

(Lannan, 1994).
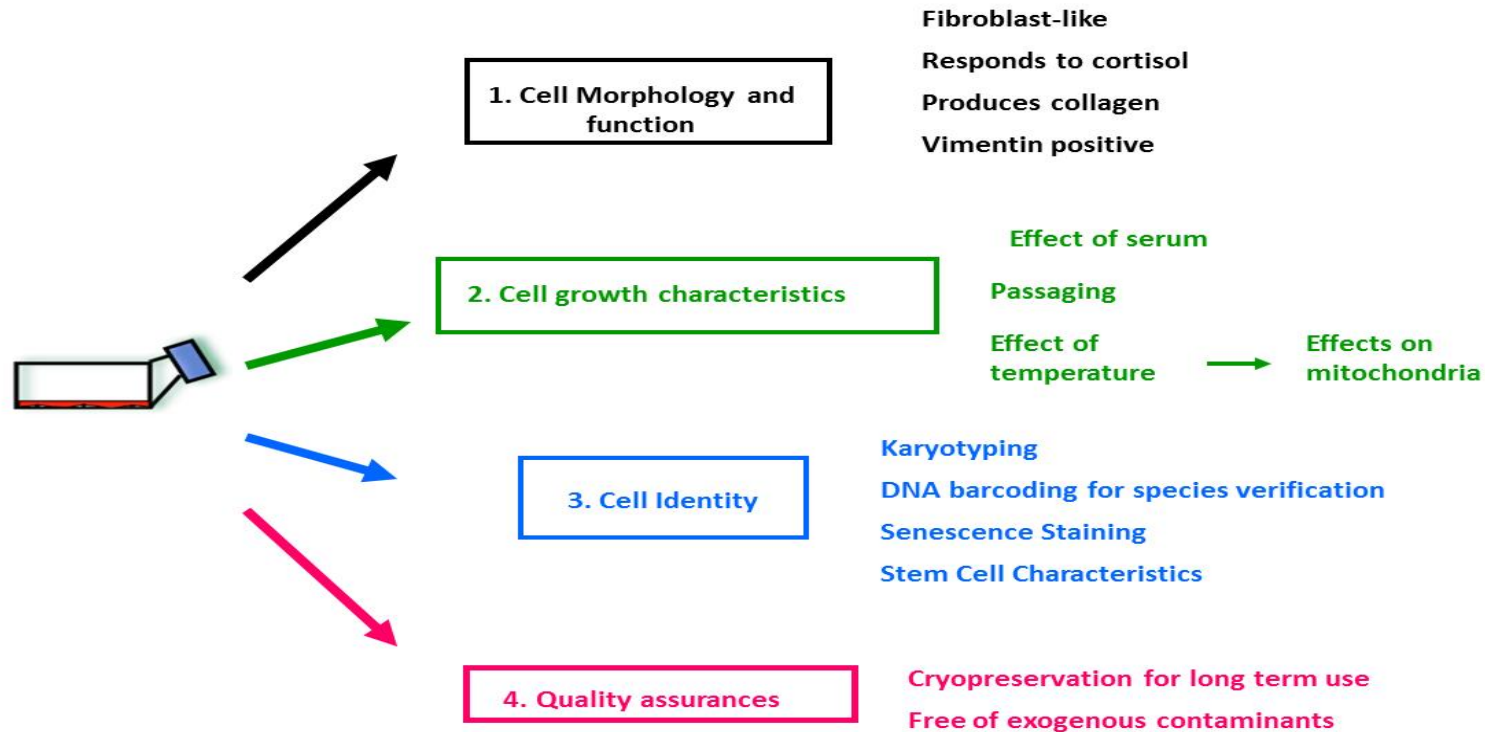
Figure 1.1: Schematic for Characterization of a Cell Line. Common approaches for characterizing new cell lines include morphological and functional evaluation, growth characteristics, authentication of cells for species of origin and quality control. Some of the experiments conducted specifically on YPF5 in this thesis are outlined in this chart.

1.1.4. Perch cell lines

Taxonomically, yellow perch (*Perca flavescens),* European perch (*Perca fluviatilis*) and Balkhash perch (*Perca schrenkii*) share the genus Perca (Brown et al., 2009). However, there are other perch-like species that do not belong to this genus. Such fish include, but are not limited to the silver perch (*Bidyanus bidyanus*) and the sea perch (*Lateolabrax japonicus*). Cell lines from both of these species have been established (Tong et al., 1998; Wharton et al., 1977; Ye et al., 2006). In China, sea perch is an important marine fish species that has led to the development of at least eight sea perch cell lines originating from various tissues including spleen, heart, liver and head kidney (Nicholson et al., 1987; Tong et al., 1998; Ye et al., 2006). Additionally, a swim bladder cell line was derived from the marine species, the silver perch (Wharton et al., 1977). The primary cultures of these cell lines were initiated by disinfecting/washing the tissue with a salt solution containing antibiotics. Then the tissues were minced into small pieces that were cultured in either of two methods: (1) explant outgrowth method and (2) enzyme dissociation method. In the explant outgrowth method, minced tissues were placed into culture flasks with minimal amount of growth media and primary adherent cells migrated out from the tissues over time (Tong et al., 1998; Wharton et al., 1977). In the enzyme dissociation method, minced tissues underwent at least one round of cell dissociation by trypsin in combination with other enzymes (Ye et al., 2006). The dissociated cells were seeded in tissue culture flasks along with media and eventually resulted in a confluent monolayer of cells in the flask.

Despite its economic value (Section 1.2.2) and its frequent use as model in toxicological studies, no yellow perch cell lines are currently available and thus their development is of high interest.

1.1.5. Fish fin cell lines

In teleost fish, the fin skeletal structure is composed of 2 parts: lepidotrichia and actinotrichia (Becerra et al., 1983; Duran et al., 2011; Montes et al., 1982). The lepidotrichia are segment rays that originate at the fin base (Becerra et al., 1983; Duran et al., 2011; Genten et al., 2009). These rays then proceed by extending along the entire fin (Becerra et al., 1983; Duran et al., 2011; Genten et al., 2009) with the main function of helping the fin maintain its shape (Duran et al., 2011). The composition of lepidotrichia includes two collagen containing hemisegments that enclose an intrasegmental area (Becerra et al., 1983; Genten et al., 2009; Lanzing, 1976; Montes et al., 1982). In the intrasegmental region, loose connective tissue, blood vessels as well as nerve bundles are present (Becerra et al., 1983; Genten et al., 2009; Lanzing, 1976; Montes et al., 1982). The second fin skeleton component is actinotrichia which emerges in the lepidotrichia intrasegment space and can be found distally in the lepidotrichia (Becerra et al., 1983; Duran et al., 2011).

Successful fish cell lines have been developed from numerous different tissues including the fin (Bols and Lee, 1991; Fryer and Lannan, 1994; Lakra et al., 2011). The fin is a tissue that has a high success rate for establishing primary cultures and cell lines, possibly due to the well-known regenerating potential of the organ (Akimenko et al.,

2003). The majority of fin cell lines are fibroblastic in morphology and have been established from a variety of fish. Table 1 contains information on some of the reported fin fish cell lines found in the peer reviewed literature. In most cases, the establishment of these primary fin cell cultures utilized the explant tissue method in which the fin is cut up into small pieces and then placed into flasks (Babu et al., 2011; Dong et al., 2011; Kapoor et al., 2013; Lakra et al., 2010; Lin et al., 2013; Swaminathan et al., 2010; Swaminathan et al., 2012; Vo et al., in press; Yan et al., 2011; Zhou et al., 2008; Zhu et al., 2013). For the establishment of fish fin primary cultures, it appeared that the different types of basal growth media are generally interchangeable since a variety of basal media have been used with success (Dong et al., 2011; Lakra et al., 2010; Rathore et al., 2007; Swaminathan et al., 2010; Swaminathan et al., 2012; Yan et al., 2011; Vo et al., in press; Zhou et al., 2008; Zhu et al., 2013). Thus fish fin cell cultures are relatively plastic in terms of adapting to *in vitro* environments.

Fish fin cell lines have been used for various purposes. They aid in the isolation of virus as well as act as a tool for studying cellular pathogenesis of fish viruses (Bols and Lee, 1991; Dong et al., 2011; Lakra et al., 2010; Swaminathan et al., 2012; Vo et al., in press; Yan et al., 2011). Although fin cell lines have been mainly used for comparative virology, they have also been used in the investigation of cytotoxic and genotoxic effects of compounds such as sodium chromate and sodium arsenite (Goodale et al., 2008; Wang et al., 2004).

Table 1: Summarized information of established fin cell lines found in literature. There are many published fish fin cell lines in the literature and this table summarizes key information of some examples.

Fish Name	Cell line Abbreviation	Organ	Number of Passages	Cell Morphology	Reference
Megalobrama amblycephala (blunt snout bream)	MAF	Fin	>95 passages in almost a year	Fibroblastic and epithelial-like	(Zhu et al., 2013)
Clarias batrachus (Indian walking catfish)	ICF	Fin	>110 passages since 2007 initiation	Fibroblast-like	(Babu et al., 2011)
Cyprinus carpio (koi carp)	KF-101	Caudal Fin	>90 passages over 3 years	Fibroblast-like before confluency and epithelial at confluence	(Lin et al., 2013)
Chitala chitala (knifefish)	CF	Caudal Fin		Fibroblastic-like	(Kapoor et al., 2013)
Cyprinus carpio koi	KCF-1	Caudal Fin	>100 passages since 2006	Fibroblastic-like	(Dong et al., 2011)
Cyrinus carpio (common carp)	CCF	Fin		Epithelial	(Lakra et al., 2010)
Puntius denisonii (red-line torpedo)	RTF	Caudal Fin	>52 passages	Fibroblastic-like	(Swaminathan et al., 2012)
Carassius auratus (goldfish)	GFTF	Tail Fin	>50 times over 15 months	Fibroblast-like	(Yan et al., 2011)
Oryzias Latipes (medaka)	OL-17 and OL-32	Fin		Fibroblast-like	(Komura et al., 1988)
Acipenser sinensis (Chinese sturgeon)	CSTF	Tail Fin	>60 passages since 2005	Epithelial	(Zhou et al., 2008)
Scophthalmus maximus (turbot)	TF	Fin	133 passages	Fibroblast	(Fan et al., 2010)
Etroplus suratensis (Green	PSF	Caudal Fin	35 passages	Epithelial-like	(Swaminathan et al., 2010)

chromide)					
Sander vitreus (walleye)	WE-cfin11f	Caudal Fin	>60 passages	Fibroblastic-like	Vo et al., in press

1.2. Yellow Perch

1.2.1. Yellow perch biology

Yellow perch, (*Perca flavescens*), belongs to the Percidae family that includes walleye, darter and sauger (Holtan, 1990; NCRAC, 2006). *P. flavescens* inhabits fresh waters across North America, including the Athabasca Oil Sands area (Fisheries and Oceans Canada, 2010; NRCAC, 2006; van den Heuvel et al., 1999a). Yellow perch is considered a cool water fish species since it prefers temperatures between 21-24 °C (NRCAC, 2006). This fish is known for its ability to tolerate unfavorable conditions which include surviving in turbid as well as nutrient rich waters and more notably surviving with low dissolved oxygen levels (NCRAC, 2006; Holtan, 1990). A typical *P. flavescens* fish will range in size from 15-30 cm in length and weigh between 4-16 oz. (NCRAC, 2006).

P. flavescens can be identified by a number of different characteristics. This fish has an elongated oval form that is olive green on the back and golden coloured throughout the body. Across the body is the most distinguishable yellow perch feature, six to eight vertical dark stripe markings covering the yellow body and ending in a white belly (Fisheries and Oceans Canada, 2010; Holtan, 1990; NCRAC, 2006). The belly fins of the yellow perch are usually a reddish orange colour while the caudal fin are green, with a slight fork, and a combination of spines and rays compose the two dorsal fins, (Holtan, 1990; NCRAC, 2006).

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P. flavescens has a relatively short life span, averaging about seven to ten years (Holtan, 1990; Brown et al., 2009). As a result, yellow perch enter sexual maturation at approximately 1-2 years for males and approximately 2-3years for females (NCRAC, 2006; Holtan, 1990). For sexually mature female yellow perch, the ideal location for spawning includes an area containing vegetation, although places with just sand and gravel are sufficient (Holtan, 1990; NCRAC, 2006). Yellow perch eggs are released from the female in a gelatinous accordion-like form. Once the eggs are expelled, the male immediately releases milt (seminal fluid) to attempt fertilization (Holtan, 1990; NCRAC, 2006). The yellow perch eggs usually attach onto any substrate in the water (vegetation, debris etc.) and are left to survive without any parental care (Holtan, 1990; NCRAC, 2006). The hatching time for these eggs will vary, depending on the temperature of the water (Holtan, 1990; NCRAC, 2006). Once hatched, yellow perch fry are born with a yolk sac that will sustain them for approximately 2-5 days, after which they will then begin to eat algae and early life stages of zooplankton, (Holtan, 1990; NCRAC, 2006). Eventually, their diet will lead to the consumption of insects or small invertebrates and as adults, larger insects and other fish (Holtan, 1990; NCRAC, 2006).

1.2.2. Relevance of yellow perch

The natural range of the yellow perch covers waterways from Nova Scotia to Alberta and most of the North Eastern states in the US, especially along the Atlantic Coast (NCRAC, 2006; Holtan, 1990; Brown et al., 2009). Non-native distributions of yellow perch now include British Columbia as well as parts of North Western United States (NCRAC, 2006; Holtan, 1990; Brown et al., 2009). Yellow perch is considered to be

a relatively sedentary fish species within its habitat distribution (Aalto and Newsome, 1990; Giguere et al., 2004; Hontela et al., 1995; Levesque et al., 2002). This is one reason why it is a model organism for studies that investigate water quality (Aalto and Newsome, 1990; Giguere et al., 2004; Hontela et al., 1995; Levesque et al., 2002).

In both the commercial and recreational fishing industries, *P. flavescens* is a valued fish species since it possesses desirable characteristics from a consumption perspective (Hinshaw, 2006; Manci, 2001 NCRAC, 2006). Although *P. flavescens* fisheries in the Great Lakes have been on the decline since around the 1950-1970s, purchasing prices of this fish were still between $9-16/lb. in 2006 (Hinshaw, 2006; Manci, 2001; NCRAC, 2006). It is apparent that there is a high commercial demand for yellow perch, despite the fact that supplies having not fully recovered (Manci, 2001; NCRAC, 2006). A possible solution for meeting market demand may be aquaculture. However, a thorough understanding of *P. flavescens* needs to be available since aquacultured fish may encounter occasions for exposure to harmful factors such as environmental contaminants and opportunistic pathogens (Manci, 2001). One of the widely accepted scientific approaches to assessing fish health is through the use of fish cell cultures. A fundamental understanding of *P. flavescens* using cell cultures derived from this species may be achieved by studying how diseased tissue extracts, intracellular and extracellular pathogens as well as toxic environmental contaminants affect the viability and biochemical functions of cultured cells. These valuable results may collectively contribute to the overall understanding on how these harmful factors may

compromise yellow perch health. Currently no yellow perch cell line is reported, despite

potential toxicology and aquacultural applications.

1.2.3. Uses of yellow perch in toxicology

1.2.3.1 In vivo

Perca flavescens has been used in whole organism toxicological studies,

particularly in the investigation of metal toxicity. Studies using *P. flavescens* have looked

at oxidative stress, bioaccumulation, the effects on organs and metabolism as well as

endocrine dysfunction resulting from exposure to metals such as copper, cadmium,

nickel, zinc, arsenic and mercury (Audet and Couture, 2003; Giguere et al, 2004; Giguere

et al., 2005; Larose et al., 2008). In addition to metal toxicity, yellow perch has been

used to investigate the effects of organic compounds, oil sands processed-affected

waters and naphthenic acid (Hontela et al., 1995; Nero et al., 2006a, Nero et al., 2006b;

Peters et al., 2007; van den Heuvel et al., 1999a). In particular, since yellow perch is

native to the Athabasca Oil Sands region (van den Heuvel et al., 1999a), it is a desirable

in vivo model for these types of toxicity assessments.

1.2.3.2. In vitro

In vitro work using *Perca flavescens* cell culture has been explored. Studies

evaluating the effects of steroidogenic compounds have been conducted using yellow

perch oocyte cells (DeManno and Goetz, 1987; Goetz and Bergman., 1978a; Goetz and

Bergman, 1978b). Dodson et al. (2008) have studied yellow perch skeletal muscle

cultures and adrenocortical cells have been used to evaluate the toxicity of cadmium as

well as uptake (Lacroix and Hontela, 2004; Raynal et al., 2005). Despite studies using

Perca flavescens cell culture, there appear to be no reported yellow perch cell lines.

Evaluating actions of environmental contaminants on yellow perch cells can be more rapidly obtained and can provide pre-information that might suggest their plausible local and systematic effects on whole fish. Since yellow perch is found in waters across North America and inhabit areas that could potentially be metal or oil sands contaminated (Pyle and Wood, 2008; van den Heuvel et al., 1999a), the use of yellow perch cell cultures to evaluate the toxicity of metal and oil sands waters were evaluated in this study.

1. 3. Fish Cell Lines in Aquatic Toxicology Methods

Cell lines have beneficial applications in toxicology as they can be used for supplementing *in vivo* methods and are economically efficient for testing the effects of contaminants using cell viability assays in terms of costs (if an existing cell culture lab is readily available and fully equipped) and especially in terms of convenience and obtaining rapid results (Castano et al., 2003; Bols et al., 2005). In the literature, cell viability assays have been used with various cell lines including, but not limited to rainbow trout cell lines from the gill (RTgill-W1) and liver (RTL-W1), goldfish skin cell line (GFSk-S1) bluegill cell line (WF-2) and fathead minnow cell lines from the liver (FHML) and testis (FHMT) (Dayeh et al., 2002; Dayeh et al., 2005; Sansom et al., 2013). These cell lines were used to test the toxicity of different environmental contaminants like paper mill effluent, mining effluent, naphthenic acids and OSPW samples (Dayeh et al., 2002; Dayeh et al., 2005; Sansom et al., 2013)

There are a variety of *in vitro* parameters to assess cytotoxicity. Impairment of metabolic activities, disruption of cell membrane integrity and damage of lysosomal

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functions often resulted from (bio)chemical insults and can be measured quantitatively by the sensitive fluorescent indicator dyes Alamar Blue, CFDA-AM and Neutral Red, respectively (Bols et al., 2005). Cell-based bio-assays using these dyes have been designed and the procedures have been optimized to measure cell viability (Dayeh et al., 2013).

1.3.1 Cell viability assays

Alamar Blue (AB), is a dye that is used in cell viability assays to investigate cell cytotoxicity by determining metabolic impairment and can be measured both fluorometrically or spectrophotometrically (Bols et al., 2005; Dayeh et al., 2013; Evans et al., 2001; O'Brien et al., 2000; Schreer et al., 2005). Alamar Blue contains resazurin, a non-fluorescent blue dye that can be reduced by cytoplasmic and mitochondrial esterases in viable cells and becomes a fluorescent pink form called resorufin (Dayeh et al., 2013; Schreer et al., 2005; O'Brien et al., 2000). By exposing cells to environmental contaminants, viability and growth can diminish and result in a decrease in the amount of resazurin being reduced when added to cells (Evans et al., 2001). Therefore, with the AB assay, cells that are viable will possess increased amounts of resorufin while compromised cells will not, thus providing strong fluorescence output in viable cells compared to low or no fluorescence in dying cells.

Measuring cell membrane integrity may involve using an esterase substrate as well as a fluorescent marker and this is a method that has been used since the 1960s (Schreer et al., 2005). 5-carboxyfluorescein diacetate acetoxymethyl ester or CFDA-AM is a non-fluorescent dye commonly used in cytotoxicity assays. CFDA-AM can enter into

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the cells by diffusion and if cells are viable, the dye is converted by non-specific

esterases into 5-carboxyfluorescein (CF), the fluorescent form (Bols et al., 2005; Dayeh

et al., 2013; Schreer et al., 2005). This fluorescent dye then slowly diffuses out of the

cells (Bols et al., 2005; Dayeh et al., 2013; Scheer et al., 2005). A decrease in esterase

activity and therefore a decrease in the amount of CF fluorescent dye could be due to

toxicants causing the cells to lyse. This would allow the esterases to be lost from the cell

and released into the medium during the assay and be low or absent from the final step

of plate reading (Bols et al., 2005).

The Neutral Red assay was initially developed with the intention of being a

chemo sensitivity assay but it demonstrated a biomedical and environmental purpose

and now is used in cytotoxicity (Repetto et al., 2008). Neutral Red dye accumulates in

the lysosomes of viable cells by the process of diffusion (Bols et al., 2005; Dayeh et al.,

2013; Repetto et al., 2008). It is a weakly cationic dye that in the lysosomal matrix will

bind to anionic and or phosphate groups (Repetto et al., 2008). This endpoint may be

measured spectrophotometrically or fluorometrically (Bols et al., 2005; Dayeh et al.,

2013; Repetto et al., 2008). Thus, live cells retain the dye whereas damaged cells lose it

and give low signals.

1.4. Evaluating Oil Sands samples

The Athabasca Oil Sands (AOS) are located in Northern Alberta with three major

deposits encompassing approximately 140,000km^2 of land and containing an estimate of

174 billion barrels of bitumen (Allen, 2008a). Bitumen is oil existing in a semi-solid state

that is found in the oil sands and is separated from additional components through the

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Hot Water Extraction Process (Allen, 2008a; Herman et al., 1994; Holowenko et al.,

2002; Rogers et al., 2002b). This process occurs with the addition of large quantities of

hot water (approximately 50-93 ° C) and caustic soda (NaOH) to produce a slurry and the

bitumen can be separated while the processed water waste proceed into settling or

tailing ponds (Holowenko et al., 2002; Rogers et al., 2002b). These tailings waters are of

concern as they contain contaminants, including Naphthenic Acids (NA) which are

naturally occurring mixture of carboxylic acids in bitumen that possess surfactant

properties (Allen, 2008a; Herman et al., 1994; Holowenko et al., 2002; Peters et al.,

2007; Rogers et al, 2002b; Young et al., 2007). As a result of the Hot Water Extraction

Process, especially with the usage of the NaOH causing an increase in alkalinity, NA are

concentrated (Allen, 2008a; Herman et al., 1994; Holowenko et al., 2002; Nero et al,

2006b; Rogers et al., 2002a; Rogers et al., 2002b; Young et al., 2007). Tailings ponds

were used for on-site storage of the extraction process byproducts (i.e. tailings and

process waters) (Allen, 2008a; Rogers et al., 2002a; Rogers et al., 2002b). Upon

completion of bitumen extraction, these tailings ponds must be reclaimed (Allen,

2008a). The use of cell lines could help monitor the toxicity of these tailings ponds by

using a high throughput screening method that could be completed in a fast and cost

effective manner (Castano et al., 2003; Bols et al., 2005). Sansom et al. (2013) has

recently showed the possibility of using fish cell lines for preliminary screening methods

of OSPW samples and that by utilizing fish cell lines, it could help indicate if *in vivo*

remediation such as End-pit Lakes (EPLs) were ready for whole fish testing (Sansom et

al., 2013).

1.5 Remediation Strategies

Using a cell line could be a convenient method to evaluate remediation strategies *in vitro*. Remediation strategies could involve the use of ameliorating compounds such as adsorbents (activated carbon, clays etc.). Clay adsorption to metals has been investigated as a way of remediating contamination (García-Sánchez et al., 1999; Quintelas et al., 2011). In particular, a type of montmorillonite clay called bentonite has been used for the purpose of ameliorating oil and grease (WHO, 2005). Utilizing a clay as a remediation strategy is a feasible remediation solution as its cost is low and its availability is high (Quintelas et al., 2011).

Ozonation is another remediation strategy that has been investigated with OSPWs. This process has shown the potential to decrease the concentration of Naphthenic Acids and subsequently reduce the toxicity (Gamal El-Din et al., 2011; He et al., 2012; Martin et al., 2010; Scott et al., 2008). Since ozonation would be a relatively costly process (Scott et al., 2008), studies have been conducted to examine partial or light ozonation in conjunction with another biodegradability process such as microbial degradation (Martin et al., 2010). McMartin et al. (2004) looked at using UV $_{254}$ radiation to decrease Naphthenic Acid amounts but results show that this process did not fully reduce NA concentrations. Further investigations using UV radiation as a possible remediation solution could still be considered, especially in *in vitro*.

1.6 Objectives

There were three objectives for this thesis. The main objective was to develop and characterize a yellow perch cell line. After the yellow perch cell line was successfully

stable, the next objectives was to use it to test the cytotoxicity of environmental contaminants such as crude naphthenic acid, ammonium chloride, copper sulphate and OSPW samples as well as investigating possible remediation strategies.

1.7 Integrative Focus

As per requirement by the Biology Department at Wilfrid Laurier University, the integrative focus of the thesis was considered. Research at the cellular level could be integrated to the whole organismal level and applied to ecosystems. The development of a yellow perch cell line is novel and the cells may be utilized not only in toxicology but also in other aspects of biology, such as virology and parasitology, as well as for a variety of economic (e.g. *in vitro* production of fish collagen, a popular ingredient in nutraceutical cosmetics), health (testing host-parasite interactions and evaluating possible chemotheraupetic agents) and environmental applications (screening physicochemical parameters that could be integrated to whole organism).

In this thesis, integrating cell biology findings to toxicology has been studied since the potential toxicological applications of a yellow perch cell line were considered. The use of this cell line focused specifically on OSPWs applications as a yellow perch cell line would be a very economical means to test the environmental progression of tailing ponds and end-pit lakes as it would provide rapid results.   However, a yellow perch cell line could have additional toxicology applications. As mentioned, yellow perch are common in metal contaminated waters (Audet and Couture, 2003; Giguere et al., 2004; Giguere et al., 2005; Larose et al., 2008; Pyle and Wood, 2008). Studies have used other

cell lines (including fish cell lines from rainbow trout) for the purpose of testing mining

effluent (Dayeh et al., 2005) and a rapid indication test using yellow perch cell lines

could be adapted and be beneficial for testing water bodies in the mining

environment. Additional environmental contaminants have been evaluated using fish

cell lines (Dayeh et al., 2002; Sansom et al., 2013; Schirmer et al., 2004) and conclusions

have been drawn that cell line methods could be used as a convenient indication or

screening method and as a way to integrate the uses of cellular and organismal findings.

As previously indicated, cell lines could help facilitate aquaculture efforts as it

can be used to investigate the physiology, nutritional and cell biology requirements of

the whole organism. Since cell lines are convenient and cost effective (Castano et al.,

2003; Bols et al., 2005), the utilization of a yellow perch cell line in developing a strong

aquaculture for the yellow perch consumption market could have advantageous

economic benefits. In addition, cell lines could be key tools in the investigation of fish

viruses: how they work and propagate inside the fish (Bols et al., 1992; Fryer and

Lannan, 1994). By utilizing a yellow perch cell line for virology studies, it could help

further the current knowledge of viruses that threaten the susceptibility of yellow perch

which can help protect the health of both wild and aquaculture stocks of this fish

species. With respect to human health, a recent study by Brennan et al. (2012)

described using a rainbow trout gill cell line as a cell-based biochips for determining if

drinking water was contaminated. Currently, this has military applications but

eventually, it has the potential to be used in the general population. In the future, a

yellow perch cell line could be used as bio-indicators to test for the health of streams,

rivers and lakes used for human consumption and recreation.

2. Materials and Methods

2.1 Developing a Yellow Perch (*Perca flavescens*) Cell Line

2.1.1. Primary cultures of yellow perch (*Perca flavescens*)

Primary cultures of yellow perch were derived from eight fish (13-17cm long) caught in August and September of 2011 from Pigeon Lake in the Kawarthas near Peterborough, Ontario. The fish were kept on ice and donated for cell culture 2 – 3 days post mortem. Two trials were performed: Fish 1 to 5 were processed on August 8 and 9th, 2011 and Fish 6-8 were processed on September 6, 2011. All fish were rinsed in tap water and sprayed with 70% Ethanol prior to dissection. See Table 1 for details of various tissues and organs processed from the various fish. Primary cultures were established by explant outgrowth (Figure 2.1). In brief, each organ was cut into small pieces and separately washed with 1X Dulbecco's Phosphate Buffered Saline solution 3-4 times. Pieces of organ tissues were placed into 12.5 cm^3 flasks with approximately 1mL of Leibovitz's L-15 media (various sources were used) with 10-20% fetal bovine serum (FBS) (Sigma) and 2% Penicillin/Streptomycin (Invitrogen). Cultures were refreshed with the same growth media each day for the first 6 days and then approximately every 6 days afterward to avoid contamination. See Appendix A for detailed instructions on processing and making solutions.
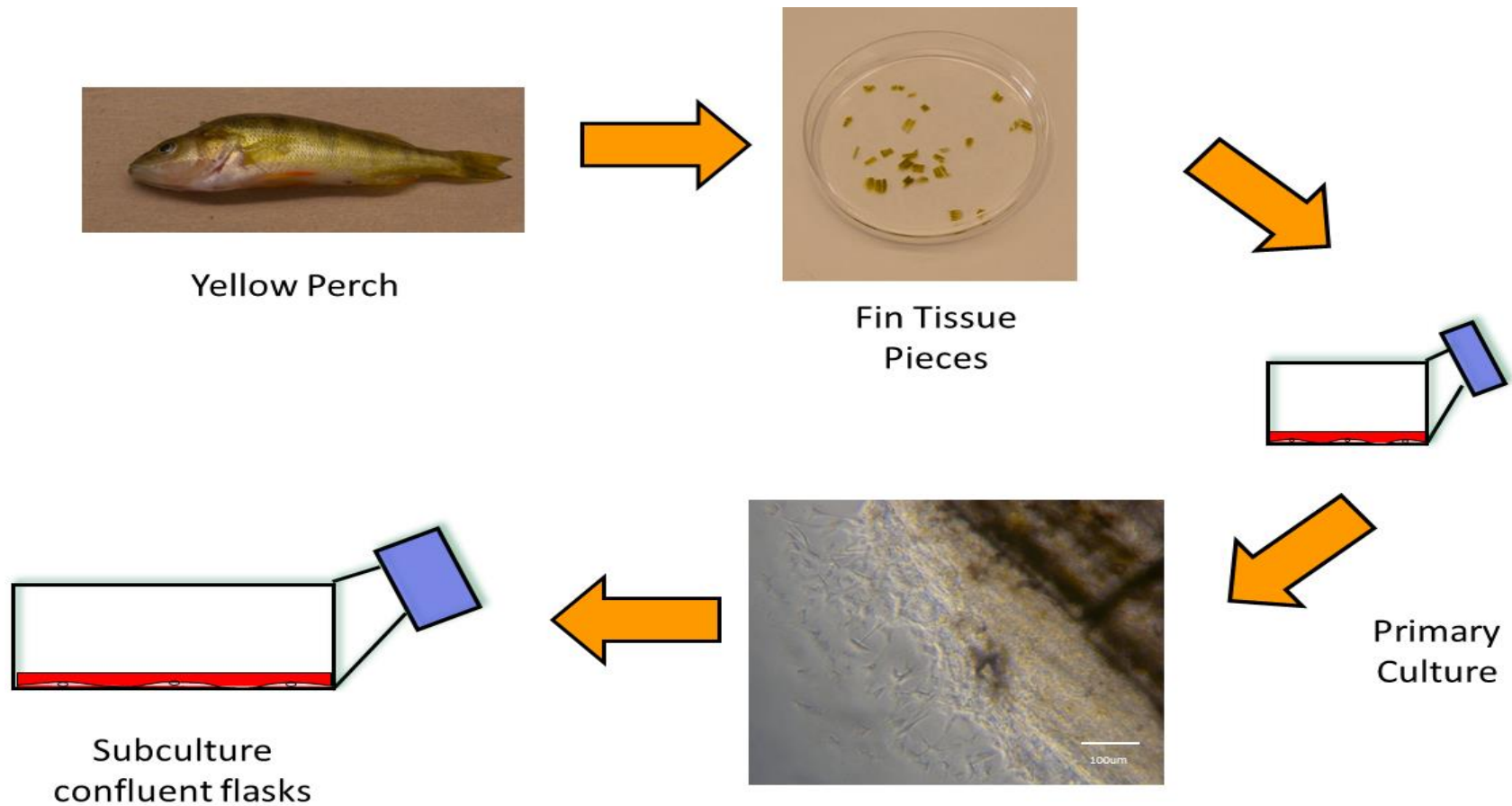
Figure 2.1: The explant outgrowth method for developing a yellow perch cell line. Fish were sprayed with ethanol, the desired tissues were dissected and rinsed 3-4 times with 1X Dulbecco's Phosphate Buffered Saline solution. Organ pieces were placed in 12.5 cm^3 flasks containing approximately 1mL of Leibovitz's L-15 media, 10-20% FBS and 2% Penicillin/Streptomycin. Media was changed daily for the first 6 days and then approximately once a week. If cell growth lead to a confluent flask, subculturing/passaging occurred

2.2. Maintenance of Yellow Perch (*Perca flavescens*) Cell Line

The primary fin culture from fish 1 (YPF1) and fish 5 (YPF5) produced continuous growth that led to two continuous cell lines YPF1 and YPF5. This thesis characterized the YPF5 cell line. The majority of yellow perch (YPF5) cells were maintained at Room Temperature (RT) using L-15 media supplemented with 10% Fetal Bovine Serum (FBS) and 3% penicillin/streptomycin (P/S). The cell line was passaged approximately every 7-10 days in 75 cm^2 flasks (BD 353135) and 10 mL of media for over two years. All cell culture maintenance and experiments were conducted following proper aseptic techniques, including using autoclaved/sterilized equipment. Cultures were passaged with TE, a dissociation solution used to detach cells from flask surfaces, (TE, Invitrogen 12604) once they reached confluency.

2.2.1. Cryopreservation

In addition to being maintained or used for experiments, confluent and healthy flasks were cryopreserved for future use. Cells were processed as if they were to be passaged but instead of being re-suspended with 10 mL of media, cells were re-suspended in only 1 mL of media. The cells were added to a cryovial containing 100 μL of DMSO, dimethyl sulfoxide, (final concentration of 10% DMSO) and mixed well. The cryovial was then immediately placed into a liquid nitrogen tank or -80°C freezer. When required, cells were thawed and regular cell culture maintenance was followed.

2.3. Characterizing a Yellow Perch (*Perca flavescens*) Cell Line

2.3.1 Authentication of YPF5

2.3.1.1 DNA Barcoding

DNA Barcoding is a process used for the identification of species in a rapid, yet accurate manner (Hebert and Gregory, 2005; Ivanova et al., 2007). YPF5 cells were dissociated with TE, centrifuged, washed with PBS, and then pelleted again in a microcentrifuge tube at 500 g for 3 min. Approximately 100 µL of absolute ethanol was added into the bullet tube. The samples were transported on ice to the University of Guelph's Fish Barcode of Life (FISH-BOL) (Guelph, ON, Canada). At FISH-BOL the DNA barcoding occurred with cytochrome c oxidase subunit 1 using a PCR primer cocktail that was designed for teleost fish , as previously described for fish cell lines by Kawano et al. (2010), Sansom et al. (2013) and Vo et al. (in press). The procedure conducted followed previous work by Cooper et al. (2007) and Ivanova et al. (2007). DNA barcoding was analyzed on the cells at passage 4 and 22 on separate occasions.

2.3.1.2 Karyotyping

Karyotyping was performed in the Lee Lab by an undergraduate student, Mike Mikhaeil to determine chromosome numbers for YPF5 to see if it agreed with the reported chromosome numbers for *Perca flavescens*. Cells at passage 17 were processed as follows: Cells were passaged and allowed to grow to 80% confluency. The day before karyotyping, media in the flask was changed. Before karyotyping occurred, preparation steps were first completed. A fixative 3:1 of methanol: acetic acid was made and kept ice cold. As well, microscope slides were immersed in absolute methanol (cold)

for 5-10 minutes and wiped with lint-free wipes. These slides were placed in a refrigerator for several hours or in a freezer for 30 minutes. Demecolcine was added to the flask to create a concentration of 0.4 µg/mL and left in flask at RT for 2.5 hours. Then, the culture was washed with 'Hank's Balanced Salt Solution' (HBSS) and TE was added. Once cells were dissociated, they were very gently transferred into a 15mL conical test tube and centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the pellet re-suspended by flicking the base of test tube. 10mL of cold 0.075M KCl was very gently added to the test tube and sat for 30-45 minutes in the fridge (usually 35 minutes). The test tube was then centrifuged at 1000rpm for 5 minutes and supernatant removed. A few drops of fixative were gently added to the test tube (let drops run down the side while shaking). This caused the pellet to re-suspend and should be repeated twice. Approximately 1-2mL more of the fixative were added and samples were left for 2 hours in the refrigerator (0-4°C). After incubation, centrifuging at 1000rpm for 5 minutes occurred and the supernatant was removed and 0.2-0.5mL (usually 0.4mL) of ice cold fixative added. The slide was held at a 40° angle over a few sheets of paper towels and 3 separate droplets of cell solution were dropped onto the slide. This could be done using a Pasteur pipette from 0.6-0.9m or by using a micropipettor to suck 100 µL and slowly release droplets of 10-20 µL size and let them fall. This step was repeated onto other slides changing heights starting at ~50cm then increasing in 10-20 cm increments per slide to approximately a meter. The slide was quickly passed over a Bunsen burner flame to evaporate the fixative (avoid letting the slide become too hot). The slide was allowed to dry completely and then either make a

1:20 dilution Giemsa stain in tap water (0.1mL giemsa in 2mL water) and add a few

drops to cover the cells or use a DAPI stain at 0.3 μg/mL. Slides were incubated for 20-

25minutes at RT, rinse with deionized water and let dry. Examination occurred using a

phase contrast microscope at 40X magnification and a determination was made as to

whether or not countable spreads were obtained.

2.3.2 Effects of Fetal Bovine Serum (FBS) on cell growth

Varying concentrations of Fetal Bovine Serum (FBS) were used to determine the

optimal FBS percentage for YPF5 growth. YPF5 at passage 12 were dissociated and re-

suspended with 10% FBS L-15 media. Cells were plated equally in 12 well TC plates (BD

353043) with 70,000 cells/well and incubated at RT for 24 hours. After 24 hours, media

was changed in all wells and FBS in L-15 at the following concentrations were added into

triplicate wells: 0%, 5%, 10% and 20% FBS, except for 6 wells for Day 0 which were

counted using a Z2 Coulter particle counter (Beckman Coulter, Brea, CA). Cells were

then counted on Days 2, 5, 9, and 11 in triplicate wells (media was changed in remaining

wells each time counting occurred).

2.3.3 Effects of Temperature on cell growth

As with different fish species, different fish cell lines have optimal temperatures

for growth. YPF5 cells were grown at varying temperatures to determine which

temperature was optimal for their growth. YPF5 at passage 7 were dissociated and re-

suspended with 10% FBS L-15 media. They were then plated equally, meaning each well

had the same cell concentration, in 12 well TC plates (BD353043) with 70,000 cells/well

and incubated at RT for 24 hours. After 24 hours, plates were incubated at 4, 14, 20, 26,

29 and 37 ° C. Media was changed and cell counts were done (using the Coulter Counter as per section 2.3.2) on Day 0, 2, 4, 7, and 12.

2.3.4 May-Grünwald Giemsa (MGG) staining

MGG was performed to allow better visualization of the morphology of YPF5 cells. A confluent YPF5 flask at passage 53 was used in this staining. Media was removed and cells were rinsed with 10 mL of PBS. A 1:1 mixture of Methanol: Acetone was used to fix cells for 20 minutes at RT. This fixative was prepared in a glass container. After 20 minutes, the fixative was removed. 10 mL of May-Grünwald stain (Harleco) was added to be incubated for 3 minutes and subsequently removed. Next, 10 mL of Giemsa (Harleco) was added to the flask (1:50 Giemsa: MilliQ Water) for 10 minutes. The flask was rinsed with MilliQ water and left to air dry. Pictures were taken with Nikon TS100 microscope.

2.3.5 Alkaline phosphatase staining

Alkaline phosphatase is an enzyme that is located in the cell membrane (Deniz and Yüce 2012). Elevated levels of alkaline phosphatase can be found in various types of stem cells like embryonic (Wakamatsu et al., 1994; Sun et al., 1995), testicular (Hong et al., 2004) and primordial (Resnick et al., 1992; Deniz and Yüce, 2012). Therefore, this enzyme and subsequently this stain can be used as a possible indicator of stem cells in test cell cultures.

YPF5 cells were dissociated at passage 33 and plated equally into 25-cm^2 Falcon flasks. The flasks were incubated at 26°C for 48 hours. Goldfish skin fibroblast (GFSk-S1) (Lee et al., 1997), Zebra fish embryo (ZEB2J) (Xing et al., 2008) and American Eel brain (Eel Brain) cells were used as controls in the senescence staining experiment. Eel Brain

cells were developed in our laboratory (Wagg, undergrad thesis, 2005). GFSk-S1, ZEB2J and Eel Brain cells were used at passage 61, 101, and 78 respectively. After the desired incubation, media was removed from the flasks and cells were fixed with a Citrate-Acetone-Formaldehyde fixative. For this staining, the Leukocyte Alkaline Phosphatase kit protocol was followed (Sigma-Aldrich 85L3R-1KT) using fast red violet and without hematoxylin counterstaining. Pictures were taken using both a phase-contrast Olympus (CK40) and Nikon Eclipse (TE300) microscope. The amounts of positive Alkaline Phosphatase cells were quantified. Each picture was divided into a 3 X 3 grid and three random squares were chosen to be counted. For each square, the number of red coloured cells were counted and added together. This quantity was then divided by the total number of cells counted in all three squares and a percentage could be calculated. For a more accurate total, the percentages from each picture were all combined and then averaged.

2.3.6 Senescence-associated β-galactosidase activity

Senescence-associated β-galactosidase (SA-β-gal) expression is induced when cultured cells enter replicative senescence (Kruz et al., 2000). A substrate called X-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside) will produce a blue colouration if it is cleaved by β-galactosidase (Debacq-Chainiaux et al., 2009) Therefore if a blue colour is produced in cells being treated with X-gal, it suggests that senescence-associated β-galactosidase is expressed and thus implies that the cells are undergoing senescence. YPF5 cells at passage 33 were plated in 25 cm^3 flasks (BD 353014) and incubated at 26∘C for 48 hours. Goldfish skin fibroblast (GFSk-S1), Zebra fish embryo (ZEB2J) and Eel Brain

cells were again used as controls, with similar plating techniques as in AP staining. The Senescence Cells Histochemical Staining Kit protocol was followed (Sigma-Aldrich S0030-1KT). After staining was concluded, flasks were covered and incubated overnight in the fume hood. Pictures were taken using both a phase-contrast Olympus CK40 and Nikon Eclipse TE300 microscope. A similar quantification method to the Alkaline Phosphatase was followed (2.3.5 for details); however, the number of blue senescent cells were quantified instead.

2.3.7 Effects of cortisol on cell morphology

Exposure to cortisol has been shown to cause fibroblastic cells to become morphologically epithelial-like (Lee et al., 1986). YPF5 at passage 38 were plated at 600,000 cells/well in six-well TC plates (Thermo Scientific 130184) and incubated for 24 hours at 26°C. After 24 hours media was removed, and 10, 100 and 1000 ng/mL of cortisol in L-15 without serum were added to the cells. A cortisol stock of 10 mg/mL was dissolved in absolute ethanol and kept at 4°C until used to prepare the serial dilutions of 10,100 and 1000 ng/mL. A control culture was exposed to serum-free L-15 with 0.1% absolute ethanol. Pictures were taken using a Nikon TE300 inverted phase contrast microscope on Days 3, 6 and 9. Each time pictures were taken, media was removed from all wells and fresh dilutions were added.

2.3.8 Effects of RU486 and cortisol on cell morphology

RU486 is an antagonist that has been shown to block the effect of cortisol on fibroblastic cells (Lee and Bols, 1989b). The effect of RU486 on YPF5 cell morphology was investigated in tandem with the effect of Cortisol (2.3.7.). The experiment was set

up to determine if RU486 blocked the morphological effect of cortisol. To determine this, it was necessary to investigate if RU486 alone had any effect and then look at the combination of RU486 and Cortisol together. As indicated in section 2.3.7, YPF5 cells were plated equally (600,000 cells/well) in six-well TC plates (Thermo Scientific 130184) and incubated for 24 hours at 26°C. After 24 hours media, was removed and either 10, 100 and 1000 ng/mL of RU486 in L-15 without serum (prepared by serial dilutions) was added to wells or 10, 100 and 1000 ng/mL of Cortisol, in L-15 without serum plus 100ng /mL of RU486 (in each cortisol concentration) was added to wells. A control was included in both situations. On Days 3, 6 and 9, pictures were taken using a Nikon TE300 inverted microscope and all wells received fresh dilutions.

2.3.9 Effects of cortisol on cell growth
The effect of cortisol on cell growth was determined via a VIACOUNT program in a Guava Flow cytometer. Cell counts using this flow cytometer were initially calibrated with hemocytometer counts. YPF5 cells at passage 41 were plated at 70,000 cells/well in a 12 well TC plate (Thermo Scientific 130188) for 24 hours at 26°C. After 24 hours, media was removed and 10, 100 and 1000ng/mL of Cortisol (prepared by serial dilutions), with L-15 and 10% FBS, were added to wells. YPF5 cells were counted (Total Cells in Original Samples) on Days 3 and 7.

2.3.10 Effects of Ascorbic Acid on cell growth
The majority of vertebrates and invertebrates possess the ability to synthesize Vitamin C (Ascorbic Acid) through the kidneys or liver (Drouin et al., 2011). Ascorbic Acid is of importance as it is a necessary component in collagen synthesis (Drouin et al.,

2011). There has been controversy regarding whether fish have the ability to synthesize

this water soluble compound (Drouin et al., 2011). However, it is now believed that

teleost fish cannot produce Ascorbic Acid (Drouin et al., 2011). According to Drouin et

al., 2011, teleost fish are not the only vertebrate species with the inability to synthesis

vitamin C and it has been theorized that this loss is a result of mutations in the GLO gene

(L-gulono-γ-lactone oxidase) which eventually impacts the vitamin's biosynthesis. If it

cannot be synthesized, then a diet rich in vitamin C must be necessary (Drouin et al.,

2011) which is the case for teleost fish (Dabrowski and Ciereszko, 2001). YPF5 at

passage 9 were plated equally in 12 well TC plates (BD353043) with 50,000 cells/well

and incubated at RT for 24 hrs. After 24 hours, a 1000 µg/mL of Ascorbic Acid (Sigma A-

4544) was made using MilliQ Water that was autoclaved. This concentration was filter-

sterilized in a flow hood using a 0.2-µm filter. The test tube was wrapped in aluminum

foil as Ascorbic Acid (A.A) is light sensitive. From this stock solution, serial dilutions of 0,

5, 25, 50, and 100 µg/mL of A.A were made using L-15 with 10%FBS. They were added to

the respectively labeled wells. Wells were counted on Days 0, 2, 6, 8, 12 using a Coulter

Counter (Refer to 2.3.2). Fresh A.A dilutions were made from 1000 µg/mL stock solution

every day that counting occurred.

2.3.11 Immunostaining for collagen
Type I collagen is the major constituent found in the extracellular matrix of

dermal connective tissues that are made of mostly fibroblasts (Chan et al., 1990).

Exposing fibroblasts to Ascorbic Acid (A.A) has been demonstrated to increase collagen

synthesis and induce collagen secretion in the fibroblasts (Chan et al., 1990).

Immunohistochemistry (IHC) was conducted on YPF5 to investigate if these yellow perch fin fibroblastic-like cells contained intracellular collagen and if the addition of A.A induces the secretion. YPF5 cells at passage 34 were dissociated and then re-suspended in L-15 with 5% FBS, 1% P/S. Cells were then equally plated into four slide flasks at 8×10^5 cells/flask (ThermoScientific 170920 Denmark). Slide flasks were incubated at RT for 24 hrs. After 24 hours, 5% FBS media was changed in three slide flasks and 50 μg/mL A.A (Sigma A-454) in 5% FBS was added to the last slide flask. The 5% FBS growth medium in the first three slide flasks and the A.A-containing medium in the last slide flask were changed every 3 days. All slide flasks were kept in the dark at all times during the course of the experiment because A.A is light-sensitive. On Day 13, an immunostaining protocol was performed. Primary antibody was a rabbit polyclonal anti-salmon collagen type 1 IgG (CedarLane CL50171AP-S1) used at 1:250 diluted in blocking buffer. The secondary antibody used was goat anti-rabbit IgG conjugated with AlexaFluor 488 (Invitrogen A-11008) used at 1:1000. Two different negative controls were conducted. The first was with the use of blocking buffer and the omission of the 1° antibody. The second control was similar but used rabbit serum instead of the 1° antibody. Both negative controls used 2° antibodies. DAPI fluoroshield (Sigma F6057) was used as a method of counterstaining the nucleus of YPF5. Stained cultures were examined under the Zeiss LSM 510 laser scanning microscope and confocal images were analyzed using a ZEN lite 2011 software.

2.3.12 Immunostaining for anti-vimentin

Vimentin is an intermediate filament protein and a marker for fibroblastic cells (Challa and Stefanovic, 2011). YPF5 cells at passage 20 and goldfish fibroblasts GFSk-S1 (Lee et al. 1997) at passage 55, acting as a positive control, were plated at 150,000 cells/well and 250,000 cells/well respectively in a four-chamber glass chamber slide (Lab-Tek 177437). The slide was incubated overnight at room temperature and then the immunohistochemistry protocol was performed. The primary antibody was mouse monoclonal anti-vimentin (Sigma V5255) at 1:250, and the secondary antibody was AlexaFluor 488 Goat Anti-Mouse IgG 1:1000 (Invitrogen A-11001). The negative control used was blocking buffer and the omission of the 1° antibody. Cells were counterstained with DAPI present in the mounting medium Fluoroshield (Sigma F6057). Stained cultures were examined under the Zeiss LSM 510 laser scanning microscope and confocal images were analyzed using a ZEN lite 2011 software.

2.3.13 Rhodamine 123 staining

Rhodamine 123 is a fluorescent dye that is used to stain viable mitochondria (Collier et al., 1993; Johnson et al., 1980; Rube and van der Bliek, 2004). In the past, mitochondria were thought to be organelles that possessed only a "sausage-like" shape (Rube and van der Bliek, 2004) but recent studies using these vital dyes have shown that mitochondria have dynamic and plastic morphologies (Rube and van der Bliek, 2004; Johnson et al., 1980). The metabolic state of a cell may affect the morphology of these organelles (Johnson et al., 1980). YPF5 at passage 37 were equally plated in separate twelve-well TC plates (Thermo Scientific 130185) at 75,000 cells/well and incubated at 26°C for 24 hrs. On Day 0, cells were moved and incubated at 10°C, 18°C and 26°C. A

Rhodamine 123 stain was performed on Day 0, Day 3 and Day 6. 0.5mL of 1µg/mL

solution of Rhodamine 123 (R8004 Sigma) was added to 50mL of L-15 no serum to make

the Rhodamine 123 Solution. After media was removed from wells, 1 mL of this solution

was added to each well of YPF5 cells and incubated at room temperature for 30 minutes

to 1 hr. After incubation, pictures were taken at each temperature using 20X

magnification on a Nikon Eclipse TE300 inverted phase contrast and fluorescence

microscope.

2.4. Using a Yellow Perch (*Perca flavescens*) Cell Line to investigate toxicity

2.4.1. Chemical preparation

The chemicals tested using the YPF5 cell line included $CuSO_4$, NH_4Cl and Crude

naphthenic acid (CrNA) extract.

A stock solution of (1000 µg/mL) $CuSO_4$ was prepared by weighing 0.01g of

$CuSO_4$ (Sigma) and adding 10 mL of L15ex, and then filter sterilized using a 0.2µm filter

(VWR) and a syringe. L15ex or L-15 exposure is a salt solution that does not contain FBS,

amino acids and vitamins that could potentially interact with the exposure yet it has

enough sugar to provide nutrients so cells can survive for a short period of time (See

Appendix B for protocol).Concentrations used for $CuSO_4$ were 0.1, 0.5, 1, 5, 10, 25, 50,

100 and 250 µg/mL and were prepared through serial dilutions.

CrNA extract, with an initial concentration of 2770 µg/mL, was used to make a

stock solution of 1000 µg/mL which was then filter sterilized using a 0.2µm filter (Pall

Corporation) and a syringe. From the 1000 µg/mL stock solution, additional

concentrations were made using L15ex through serial dilutions. The concentrations for

CrNA were 5, 10, 25, 50, 100, 250, 500, 1000μg/mL.

A stock solution of (1000 μg/mL) NH_4Cl was prepared by weighing 0.01g of NH_4Cl

(AL-DON AA2351) and adding it to 10mL of L15ex. This 10 mL of stock solution was filter

sterilized using a 0.2μm filter and a syringe. The concentrations used for experiments

were 5, 10, 25, 50, 100, 250, 500, 1000μg/mL prepared by serial dilutions.

2.4.2. OSPW sample preparation
Oil Sands Processed Water Samples (OSPWs) were shipped to the Lee Lab from

Syncrude Canada in 100 mL vials. These samples were kept in a cardboard box as to

avoid light and stored at 4 ° C. Nine samples were chosen to be tested using YPF5:

Sample 1, 2, 3, 4, 5, 12, 14, 15 and 16. These samples were tested at 80% concentration

and utilizing 5X concentrated L15ex. 5X L15ex preparation instructions can be seen in

Appendix B. 12 mL of each OSPW sample was taken and added to 3 mL of 5X L15ex and

then filter sterilized using a 20 mL syringe (BD 309661) and 0.2μm filter.

2.4.3 Remediation Strategy Preparations
Two remediation strategies were investigated: the use of bentonite, a clay and

the use of UV radiation. Samples were first prepared regularly as indicated in section

2.4.1 and 2.4.2 in 15 mL test tubes. Bentonite powder used in the experiments was

obtained from health food store supplement capsules and had a purity of greater than

90%. The amount of bentonite added to the chemicals and OSPWs samples was kept

consistent. For every 1 mL of solution 1 mg of bentonite was added. The bentonite was

allowed to stay in the solutions for a specific amount of time overnight, in these

experiments bentonite pretreatment intervals of 18 and 48 hrs were used. It was important to shake each test tube at least once before leaving for the night as the bentonite will eventually settle to the bottom. Once bentonite was added, it was normal for the solutions to turn cloudy. After the specified time had passed, the test tubes were shaken again and then poured through a 40 µm nylon cell strainer (BD 352340) and into a labeled 50 mL test tube. The bentonite tended to clog the 0.2µm filters if a syringe filter was used; thus, the cell strainer was used. The bentonite was collected in the cell strainer and the "now remediated" solutions were transferred into the 50 mL test tube. The solutions could now be syringe filtered using 0.2µm filters. A new syringe, filter and cell strainer needed to be used for each concentration of OSPW sample.

Utilizing UV radiation required fewer steps than using bentonite. Samples were first prepared regularly as indicated in section 2.4.1 and 2.4.2 in 15 mL test tubes. These samples were then put into test tube holders (making sure they were evenly spaced) and placed into the laminar flowhood that has a UV lamp incorporated inside. The flowhood (power off) was properly covered and the UV light switched on. These samples were kept exposed to UV light (254nm) for 18 hrs overnight. The next day the samples were ready to be used for exposures.

2.4.4. Exposure of YPF5 cell line to chemical and OSPW samples

Alamar Blue and CFDA-AM Assay
YPF5 cells were plated approximately between 40,000-60,000 cells/well in 96 well plates using L-15 with 10%FBS and 3% P/S (BD 353072 or ThermoScientific 130188) for 24 hours at 26°C. Each set up of the 96 well plates for the Alamar Blue and CFDA-AM

assay included a control of 4-6 wells of cells that were not exposed to toxicants, and 6

wells with no plated cells that acted as the blanks. After 24 hours, plates were checked

to make sure cells formed a confluent layer in the wells. If confluent, plates were

inverted onto a paper towel to remove the L15 media. Cells were then exposed to 100µl

of concentrations of chemicals (Refer to section 2.4.1 for exact concentrations used) or

OSPW samples. Controls were exposed with 100µl of L15-ex and exposed plates were

left for 24 hours at room temperature. For the extended $CuSO_4$ cell viability assays (48-

96hrs), YPF5 was exposed to $CuSO_4$ and L15ex for 48-96 hrs.

Neutral Red Assay

YPF5 cells were again plated with a well concentration of approximately 40,000-

60,000 cells/well in 96 well plates using L-15 with 10%FBS and 3% P/S (BD 353072 or

ThermoScientific 130188) for 24 hours at 26°C. However, this Neutral Red assay

included more control wells (between 8-10) which were not exposed to the toxicant as

well as 10 wells which contained no cells and were called blanks. After 24 hours, all

plates were checked for confluency and if determined confluent, the plates were

inverted onto a paper towel and the L15 media removed. Controls and cells were

exposed with either 100µl of L15-ex or varying concentrations of chemicals and then left

at room temperature for 48- 96 hours.

2.4.5 Preparation of Fluorometric Indicator Dyes

Cell viability was measured using the following three fluorometric dyes: Alamar

Blue (Invitrogen DAL 1100), 5-carboxyfluorescein diacetate acetoxymethyl ester or

CFDA-AM (Sigma) and Neutral Red (Sigma N2889). Preparation protocols were adapted

from (Dayeh et al., 2013)

2.4.6 Cell Viability Assays

Cell viability was measured using different assays that investigate specific cellular

parameters such as cellular metabolic activity by investigating the activity of

intracellular and mitochondrial diaphorases (Alamar Blue Assay), integrity of cellular

membranes by measuring activity of membrane esterases (CFDA-AM Assay) and

lysosomal functions by measuring acid phosphatase activity and an ability to retain dye

within the lysosome (Neutral Red Assay) (Bols et al., 2005; Dayeh et al., 2013). After 24

hours of exposure for AB and CFDA-AM or 48-96 hours of exposure for Neutral Red,

plates were inverted onto paper towels to remove the chemicals. The fluorometric AB

and CFDA-AM dyes as well as NR working solution prepared in section 2.4.5, were added

to all wells including the blank and were incubated at room temperature for 1 hour.

With the exception of the NR assay which required additional steps, immediately after

incubation time, the SpectraMAX Gemini XS microplate reader was used to quantify the

fluorescence of each well. Each fluorometric dye required a different excitation and

emission wavelength. The excitation and emission wavelengths were as follows: AB 530

and 590nm, CFDA-AM, 485 and 530nm, and 530 and 645nm for NR. For the NR assay,

cells were incubated with the NR working solution for an hour. Then the solution was

removed and wells were rinsed with the NR fixative solution. After rinsing, the NR

extraction solution was added and then fluorescence was measured using the

SpectraMAX and expressed in Relative Fluorescent Units (RFUs). Cell viability assay

protocols were adapted from Dayeh et al (2013) and can be found in Appendix C.

2.4.7 Data Analysis

Statistical analysis was conducted using GraphPad InStat (California). An

unpaired t-test was used when comparing the effect of remediation strategies versus no

remediation strategies. A one way ANOVA with a Dunnett post test was conducted

when comparing the results from all concentrations to the control (P value <0.05 was

considered significant). Liner regression analysis was conducted to determine if there

was a relationship between the levels of Naphthenic Acid in the OSPW samples and a

decrease in YPF5 cell viability. The Relative Fluorescent Units (RFUs) of 4-6 well results

were imported into Microsoft Excel where the means and standard deviations were

calculated. The RFUs of 4-6 wells were averaged and expressed as a percentage of the

L15ex control. These percent control results were used to calculate the Effective

Concentrations values (EC_{50}) of cell viability with GraphPad Prism 5.02 for each

environmental contaminant (except the OSPW samples).

3. Results

3.1. Development of a yellow perch (Perca flavescens) cell line

3.1.1. Primary Cultures developed from Yellow Perch (*Perca flavescens*)

Primary cultures were first processed using five different yellow perch fish, then 3 other fish were processed after a month. Various tissues were taken from each fish and in total nine different tissues were used (Table 2). Not all attempted tissues attached and/or had cell outgrowth. Many tissues appeared necrotic, especially the liver, spleen, brain and heart tissues because these fish were kept on ice for 2-3 days before processing. In general, contamination was also a hindering factor; among the tissues, the gill from fish 4 was the first tissue to produce growth but was quickly overrun by bacterial contamination.

Table 2: Yellow perch tissues used for the establishment of primary cultures. Eight fish were used for primary cultures. The green boxes indicate that cell outgrowth occurred.

Tissue	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Fish 7	Fish 8
Gill	YPG1#1	YPG2#1		YPG4#1		YPG6#1		YPG8#1
	YPG1#2	YPG2#2		YPG4#2		YPG6#2		YPG8#2
	YPG1#3			YPG4#3		YPG6#3		YPG8#3
Brain	YPB1#1	YPB2#1	YPB3#1	YPB4#1				
	YPB1#2	YPB2#2	YPB3#2	YPB4#2				
	YPB1#3		YPB3#3	YPB4#3				
	YPB1#4							
Liver	YPL1#1		YPL3#1	YPL4#1		YPL6#1		
	YPL1#2		YPL3#2	YPL4#2		YPL6#2		
	YPL1#3		YPL3#3			YPL6#3		
			YPL3#4			YPL6#4		
			YPL3#5			YPL6#5		
			YPL3#6			YPL6#6		
Gut	YPGut1#1							
	YPGut1#2							
	YPGut1#3							
Fin	YPF1#1			YPF4#1	YPF5#1			YPF8#1
	YPF1#2			YPF4#2	YPF5#2			YPF8#2
	YPF1#3			YPF4#3	YPF5#3			YPF8#3
								YPF8#4
Spleen	YPS1#1					YPS6#1		
	YPS1#2					YPS6#2		
Heart			YPH3#1				YPH7#1	
			YPH3#2				YPH7#2	
Bulbus Arteriosus			YPBA3				YPBA7	
Gonad						YPGon6#1		YPGon8#1
						YPGon6#2		YPGon8#2
						YPGon6#3		

As seen in Table 2, sixteen primary cultures demonstrated cell attachment, outgrowth and, in some cases, proliferation but this did not guarantee a successful cell line. Table 3 is a summary of the status of some successful primary cultures. The majority of these primary cultures were passaged at least once. Adherent cells were

viable but not sufficient enough to produce a confluent cell monolayer. These

unsuccessful primary cultures were eventually discarded because the tissues ultimately

died, became detached and stopped producing cell outgrowth or the viable cells

stopped growing. The most successful growth was found to be from the fin tissues of

the yellow perch. Primary cultures from the fin of three different yellow perch (YPF1,

YPF5 and YPF8) produced confluent flasks that were passaged more than once. Two fin

cell lines in particular (YPF1 and YPF5) were successfully maintained at over 20 passages.

Table 3: Detailed yellow perch primary culture information. Primary cultures that showed
proliferation were further passaged as indicated below. Three successful fin cell lines were
developed, and currently two are still being maintained.

Cell Line	Initiation Date	Organ Derived From	Number of Passages	Successful Cell Line
YPF1#2	August 2011	Fin	>20	Yes
YPG4#1	August 2011	Gill	0	No
YPG4#2	August 2011	Gill	0	No
YPG4#3	August 2011	Gill	0	No
YPF5#2	August 2011	Fin	>60	Yes
YPF8#1	September 2011	Fin	0	No
YPF8#2	September 2011	Fin	>6	Yes
YPF8#4	September 2011	Fin	0	No
YPH7#1	September 2011	Heart	1	No
YPBA3	August 2011	Bulbus Arteriosus	1	No
YPBA7	September 2011	Bulbus Arteriosus	1	No
YPGon6#1	September 2011	Gonads	1	No
YPGon6#2	September 2011	Gonads	1	No
YPGon6#3	September 2011	Gonads	1	No
YPGon8#1	September 2011	Gonads	0	No
YPGon8#2	September 2011	Gonads	0	No
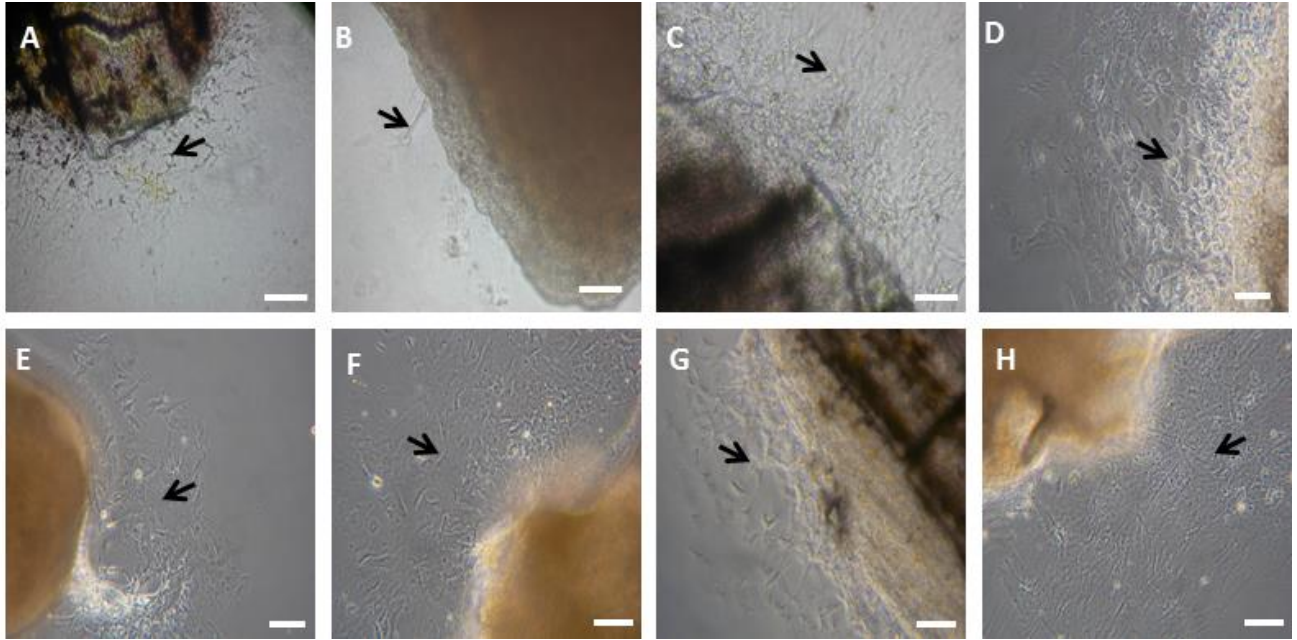
Figure 3.1: Phase contrast micrographs of yellow perch tissue explants. Panels depict various tissue explants after several days of culture in L-15 media under phase contrast microscopy. Yellow perch tissue explants of A) YPF1#2, B)YPBA3, C)YPF5#2, D)YPGon6#1, E)YPBA7, F)YPH7#1, G)YPF8#1, H)YPGon8#1, can be seen at 10x magnification. Arrows indicate cell outgrowth areas from the explants. Bar = 100 μm

Cryopreservation

YPF5 was routinely cryopreserved over two years at passages ranging from six to forty- eight for an approximate total of 60 vials. Cell viability was determined when cells were thawed. Counting was done using a hemocytometer and viability was noted with Trypan Blue exclusion test (dead cells are stained blue). The following is an example of how cell viability was calculated as two counts were always averaged.

Count 1: Cell Viability = [live cell number / total (live + dead cell number)] * 100%
= [34 / (12 + 34)] * 100%
= 73.9% viable

Count 2: Cell Viability = [live cell number / total (live + dead cell number)] * 100%
= [17 / (16 + 17)] * 100%
= 51.5% viable

Average Cell Viability: (73.9 +51.5)/2 = **62.7% cell viability**

(Due to the recent lab move of Dr. Lee's cryostocks, the containers that held my samples were accidentally left out from the cryotanks when shipments were made and the YPF5 cells were inadvertently thawed. This was discovered on October 30, 2013 and may have resulted in the cryovials becoming compromised.)

3.2. Characterization of YPF5

3.2.1. Authentication of YPF5

3.2.1.1. Karyotyping of YPF5

Karyotyping was performed on YPF5 by Mike Mikhaeil, a student from the Lee Lab, to determine if this cell line had corresponding chromosome numbers to Yellow Perch (*Perca flavescens*). From a confluent culture at passage 17, chromosome spreads were prepared and a total of 187 spreads were counted and the modal chromosome

number was determined to be 48 (32.6% of the 187 spreads) (Figure 3.2). Agreeing with

the reported karyotype for *Perca flavescens* with modal chromosome of 48 (Danzmann,

1979), YPF5 cell line appeared to remain diploid after at least 17 population divisions.
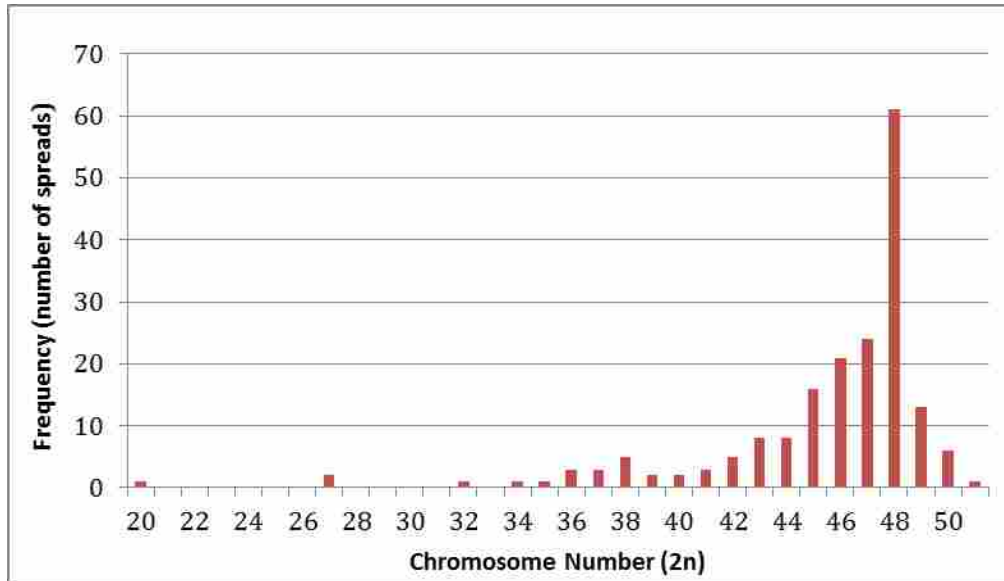
Figure 3.2: YPF5 Karyotype Frequency Distribution. Chromosomal distribution of YPF5 at
passage 17. A total of 187 spreads were counted and modal chromosomal distribution was 48
(n=61).
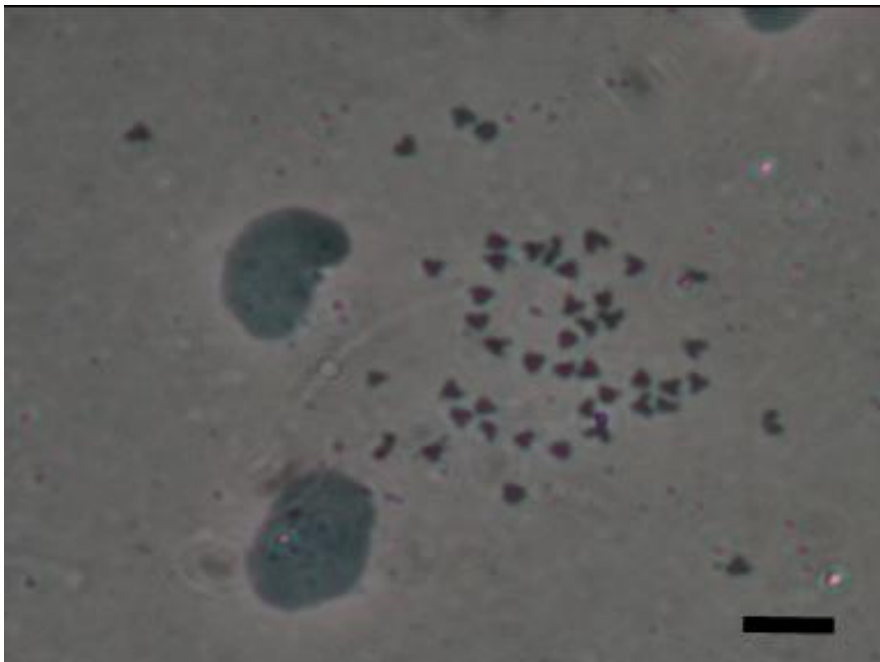
Figure 3.3: Representative Phase micrograph of the YPF5 Chromosomal Karyotype. Giemsa stained chromosomal spread from YPF5 at passage 17. Scale bar = 10μm.

3.2.1.2 DNA Barcoding of YPF5

DNA barcoding was performed at the University of Guelph (FISH-BOL). PCR amplification of a 655 bp fragment of mitochondrial cytochrome c oxidase 1 yielded as follows:

CCTTTATCTAGTATTTGGTGCTTGAGCCGGAATAGTGGGCACTGCCCTAAGCCTGCTTAT
CCGAGCAGAACTAAGCCAGCCCGGCGCTCTCCTAGGAGACGACCAGATTTATAACGTAAT
TGTTACAGCACATGCCTTCGTAATAATTTTCTTTATAGTAATACCAATTATGATTGGGGG
CTTTGGAAACTGACTAATTCCACTTATGATCGGTGCCCCTGACATAGCTTTCCCTCGAAT
AAATAATATGAGCTTTTGGCTCCTGCCTCCTTCTTTCCTTCTCCTCCTTGCTTCCTCAGG
AGTTGAAGCCGGAGCTGGTACCGGATGAACTGTTTATCCCCCTCTTGCTGGGAACTTAGC
ACATGCTGGAGCATCTGTTGATTTAACCATTTTCTCTTTACACTTAGCAGGGGTTTCCTC
AATTCTAGGTGCTATTAATTTTATTACAACCATCATTAATATAAAACCCCCTGCCATCTC
CCAATATCAAACTCCCTTGTTCGTATGGGCTGTATTAATTACCGCCGTTCTTCTCCTTCT
TTCACTACCTGTTCTTGCCGCTGGCATTACAATGCTTCTTACAGACCGAAATTTGAACAC
CACTTTCTTCGATCCTGCAGGAGGGGGTGATCCCATCCTTTACCAACACTTATTC

By comparing to NCBI BLAST and FISH-BOL databases, the match was 100% with *Perca flavescens*, confirming that YPF5 was derived from *Perca flavescens*.

3.2.2 Cell morphology

3.2.2.1. YPF5 cell line is a fibroblastic cell line

The morphology of YPF5 was typically fibroblastic, with cells assuming an elongated fusiform shape with time in culture (Figure 3.4). After passaging, cells attached rapidly onto the tissue culture surfaces and spread within a few hours, elongating into a swirl of fibroblasts, then overlapping onto one another, once a monolayer was formed. The cells contained well-defined oval nuclei as observed by fluorescence staining with DAPI (Figure 3.4.F). This typical fibroblastic morphology can be seen at both 10X and 20X magnification and at both low and high passage numbers (Figure 3.4).
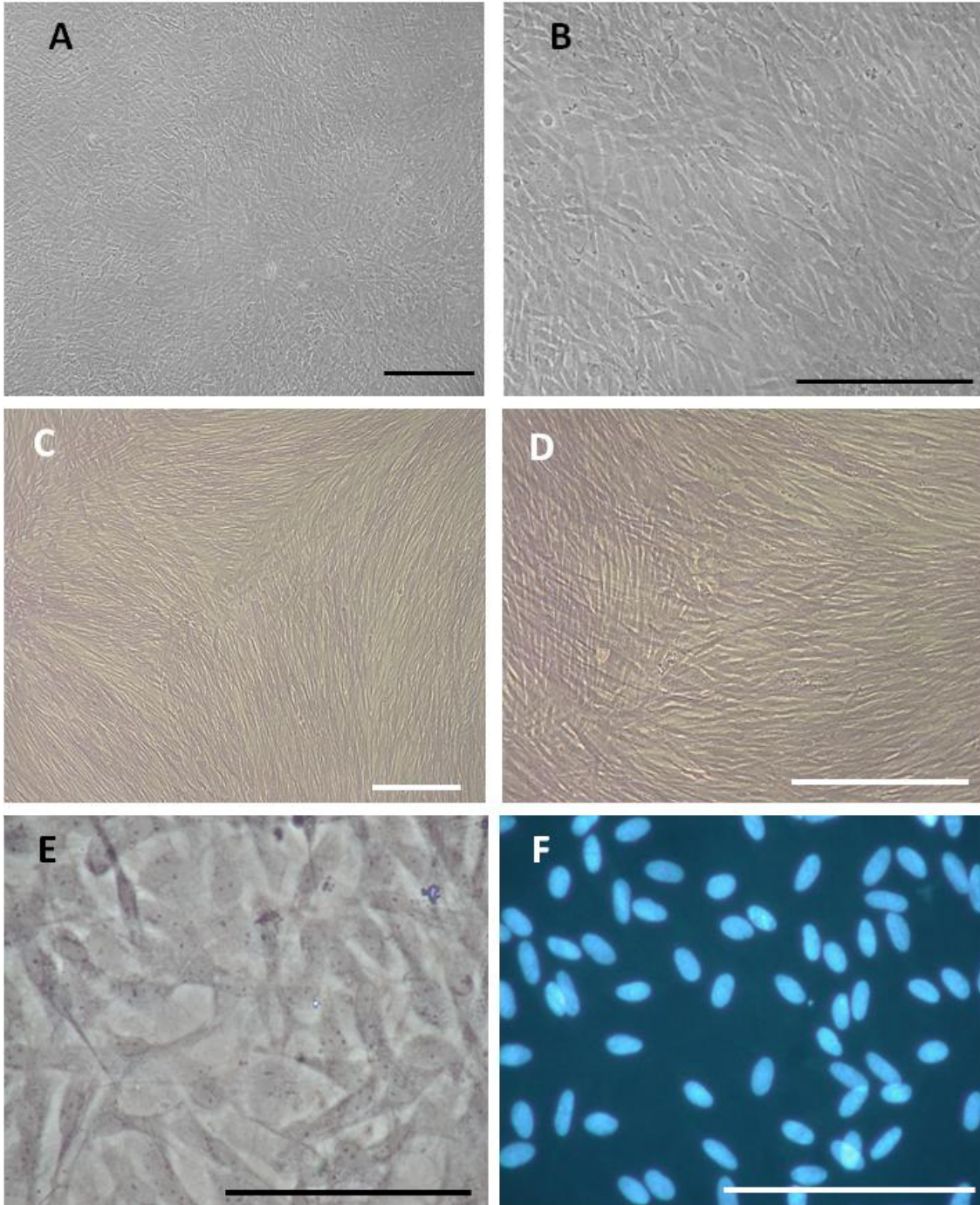
Figure 3.4: Typical morphology of YPF5, a fibroblastic cell line. Panels depict YPF5 at confluency where cells take on a fibroblastic shape. **Panels A, B**, Phase contrast micrographs at 10X **(A)** and 20X magnification **(B)** at passage 44. **Panels C and D** depict YPF5 stained with May-Grünwald Giemsa stain **(C)** 10X magnification **(D)** 20X magnification at passage 53. Pictures were taken with a phase-contrast Nikon TS100 microscope. Scale bar = 200µm. **(E)** Phase contrast micrographs of YPF5 at passage 12 fixed and stained with DAPI at 20X magnification **(F)** Fluorescent view of (E) illustrating the well-defined oval nuclei stained in blue with DAPI. Pictures were taken with a Nikon TE300 microscope. Scale bar = 100µm

3.2.2.2. Effects of Cortisol

The addition of various cortisol concentrations appeared to have a morphological effect on YPF5 cell shape. Cortisol concentrations were made using L-15 media with no serum as FBS can contain cortisol. These concentrations were added to the cells and changed every three days. YPF5 cells exposed to of L-15 media with no addition of cortisol (or the controls) appeared to maintain their typical fibroblastic shape. However, a more epithelial-like morphology was seen in YPF5 cells that were exposed to cortisol concentrations (Figure 3.5). This was especially apparent as time of exposure continued past Day 3.
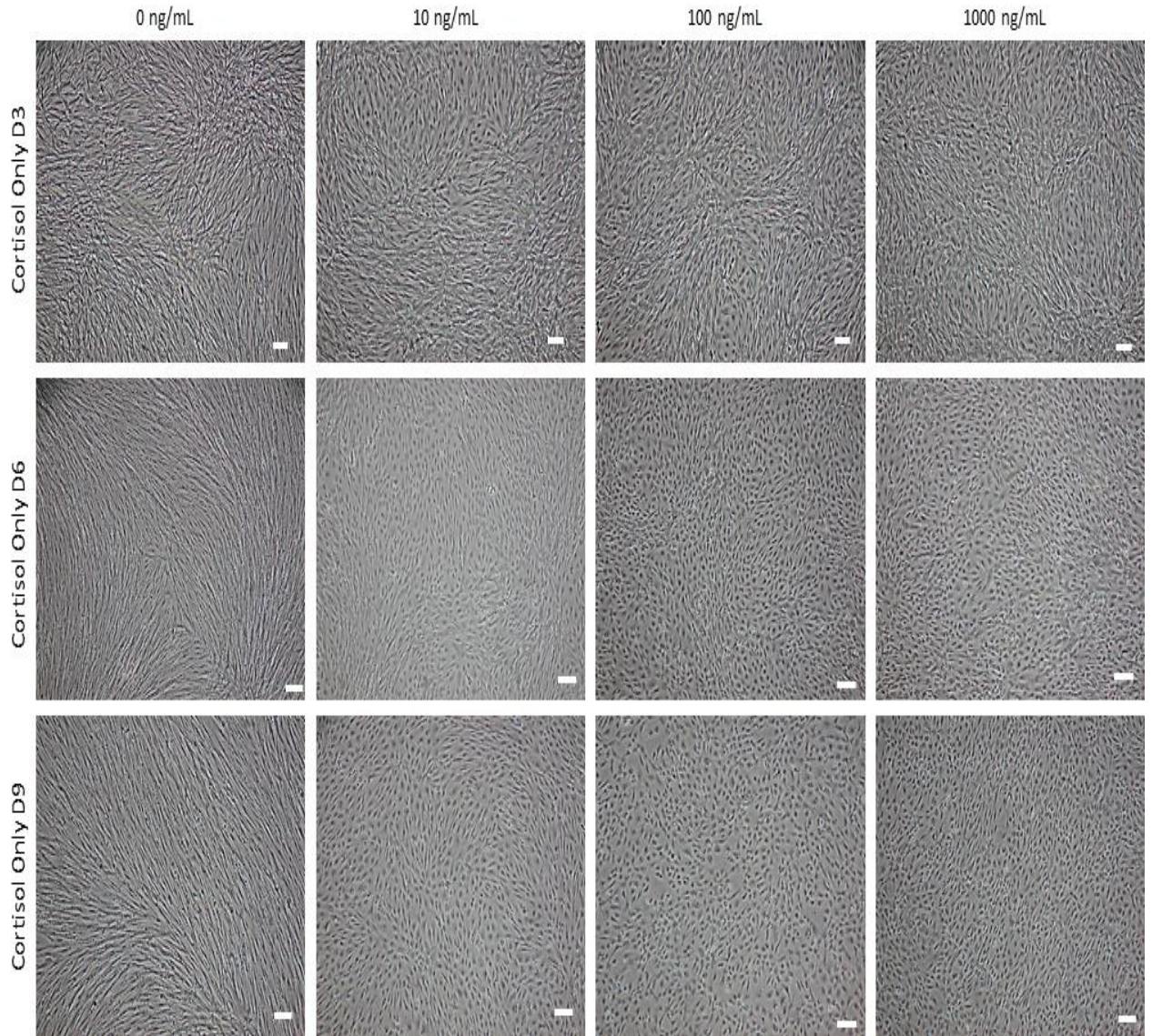
Figure 3.5: Morphology of YPF5 cells after exposure to cortisol for over 9 days. YPF5 cells were plated with L-15 10% FBS media at passage 38 with a concentration of 600,000 cells/well in 6 well TC plates. After 24 hours, these cells were exposed to different concentrations of cortisol made with L-15 no serum. Rows: days 3, 6, 9. Columns: control and exposed cells to concentrations of cortisol (10, 100, 1000 ng/mL). Pictures were taken at 4X magnification with a phase-contrast Nikon TE300 microscope. Scale bar = 100µm

3.2.2.3. Effects of RU486

The addition of various RU 486 concentrations did not have a morphological effect on YPF5 cell shape (Figure 3.6). YPF5 cells exposed to RU 486 concentrations from 0ng/mL - 1000ng/mL still maintained the typical fibroblastic morphology from Day 0 through Day 9. RU 486 concentrations were made using L-15 media with no serum.
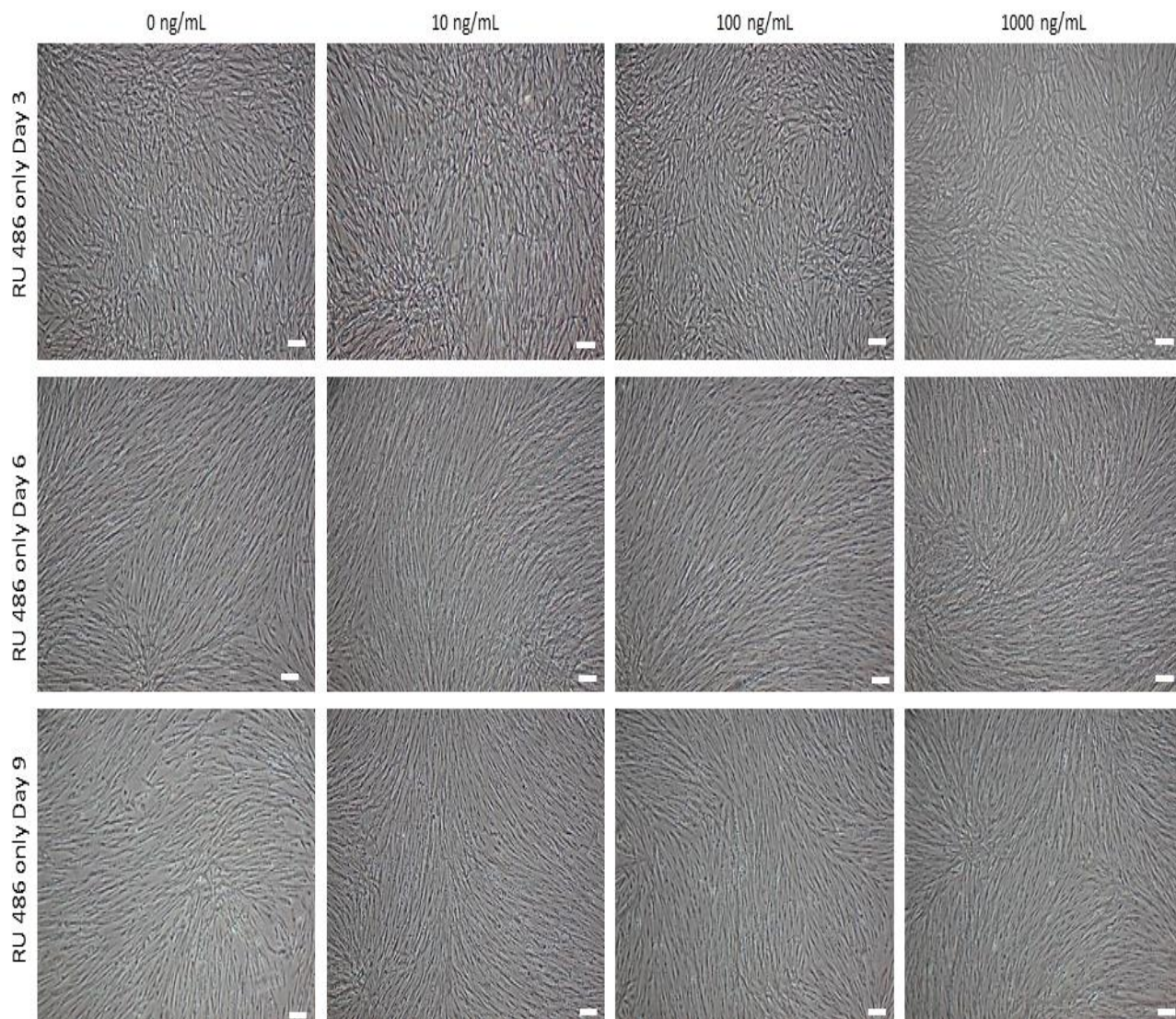
Figure 3.6: Morphology of YPF5 cells after exposure to RU 486 for over 9 days. YPF5 at passage 38 were plated at 600,000 cells/well with L-15 10% FBS in 6 well TC plates and after 24 hrs cells were exposed to different concentrations of RU 486 (made with L-15 no serum). Rows: days 3, 6, 9. Columns: control and exposed cells to concentrations of RU 486(10, 100, 1000 ng /mL). Pictures were taken at 4 X magnification with a phase-contrast Nikon TE300 microscope. Scale bar = 100μm

3.2.2.4. Effects of Cortisol and 100ng/mL RU486 together

The addition of cortisol concentrations caused a more epithelial-like morphology while

the addition of RU 486 concentrations did not. Then an investigation to see if a

combination of the two would prevent the morphological changes seen with cortisol

concentrations alone was undertaken. It appeared that the addition of 100ng/mL of RU

486 to the lower cortisol concentrations could prevent the epithelial-like morphology

change. However, at the higher cortisol concentrations some epithelial-like cell

morphology could be seen with the addition of 100ng/mL of RU 486. (Figure 3.7)
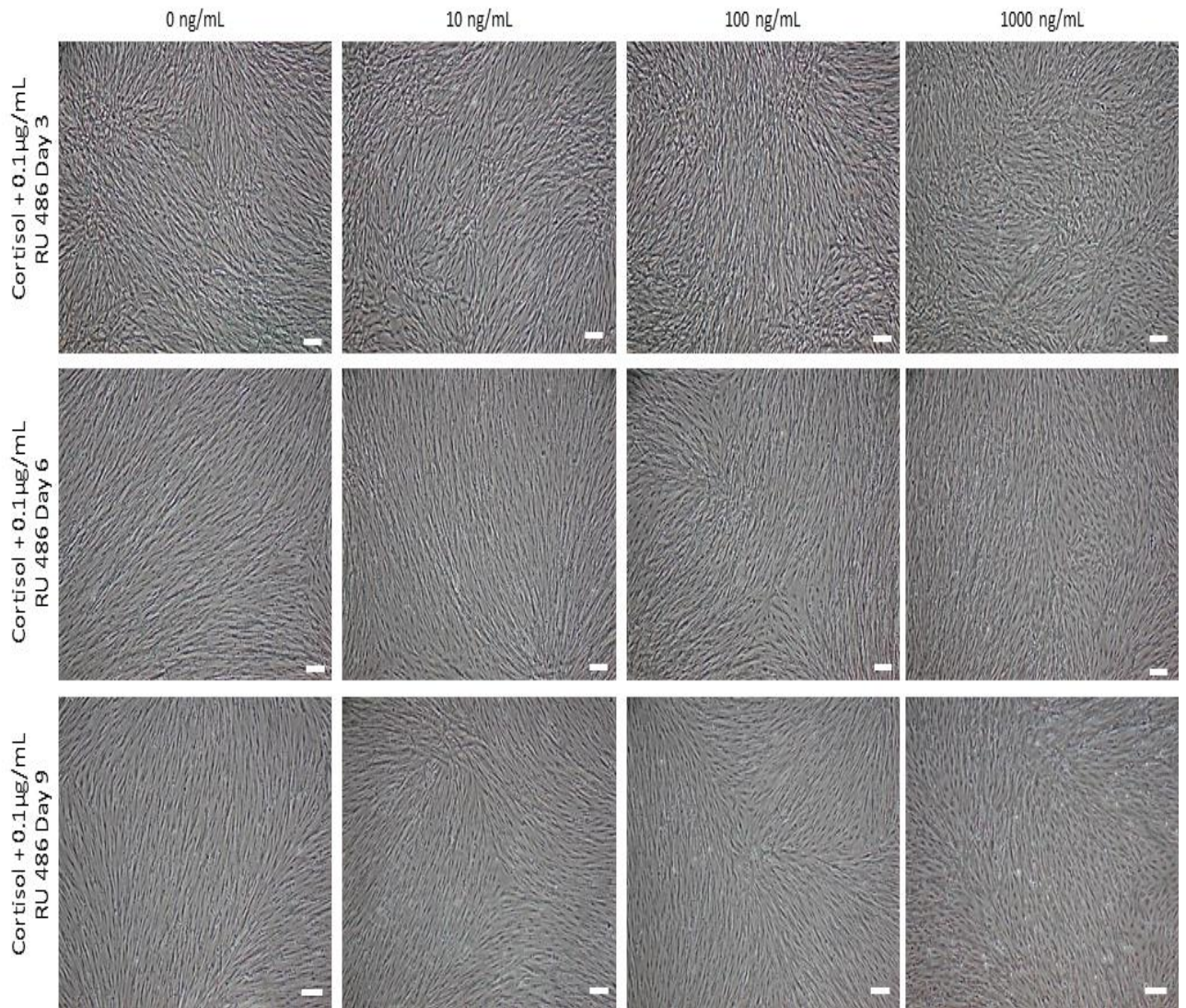
Figure 3.7: Morphology of YPF5 cells exposed to cortisol and 100ng/mL RU 486 for over 9 days.
YPF5 cells at passage 38 were plated at 600,000 cells/well in 6 well TC plates using L-15 10% FBS.
Cells were exposed to the control and three concentrations of cortisol with RU 486 at 100 ng/ml,
all made in L-15 no serum. Rows: days 3, 6, 9. Columns: control and concentrations of cortisol
(10, 100, 1000 ng/mL), with consistent concentration of 100ng/mL of RU 486. Pictures were
taken at 4X magnification with a phase-contrast Nikon TE300 microscope. Scale bar = 100μm

3.2.3 Effects of FBS concentrations on YPF5 growth

To investigate the effects different FBS concentrations have on YPF5 proliferation, 0%, 5%, 10% and 20% FBS concentrations were tested. Cells were counted on Day 0, 2, 5, 9 and 11 and expressed as a percent control of cells per mL. Day 0 through 9 showed a similar growth pattern with 5, 10 and 20% FBS. After 9 days, it was apparent that cells grew best with 20% FBS, but were still successful using 5 and 10% FBS. However, YPF5 required FBS to proliferate since the cells with 0% FBS did not increase throughout the 11 days and appeared non-viable. As shown in Figure 3.8 for graphical representation and Figure 3.9 for morphology observations. YPF5 cells were maintained with 10% FBS as they proliferate quite rapidly and to minimize lab material costs.
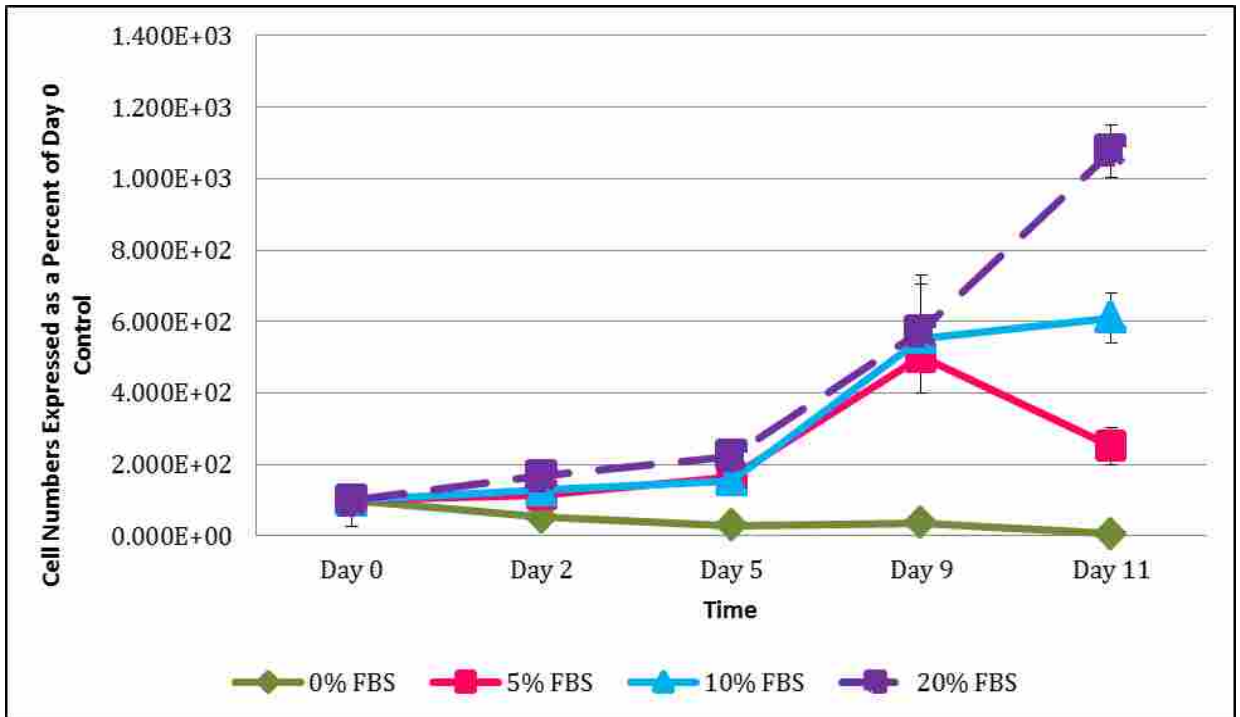
Figure 3.8: Fetal Bovine Serum (FBS) preference of YPF5 cells. YPF5 cells were plated at passage 12 and at 70,000 cells/well in 12 well plates. Cells were initially plated using L-15 with 10% FBS and incubated at RT for 24hrs. Media was then changed and various FBS concentrations of 0, 5, 10 and 20% were added on day 0. Cells were counted from 3 replicate wells in each of the indicated time points using the coulter counter (Appendix E). Media was changed in remaining plates on the days that counting occurred. All RFUs were expressed as a percentage of the Day 0 control. The mean counts ± standard errors are represented for each data point.
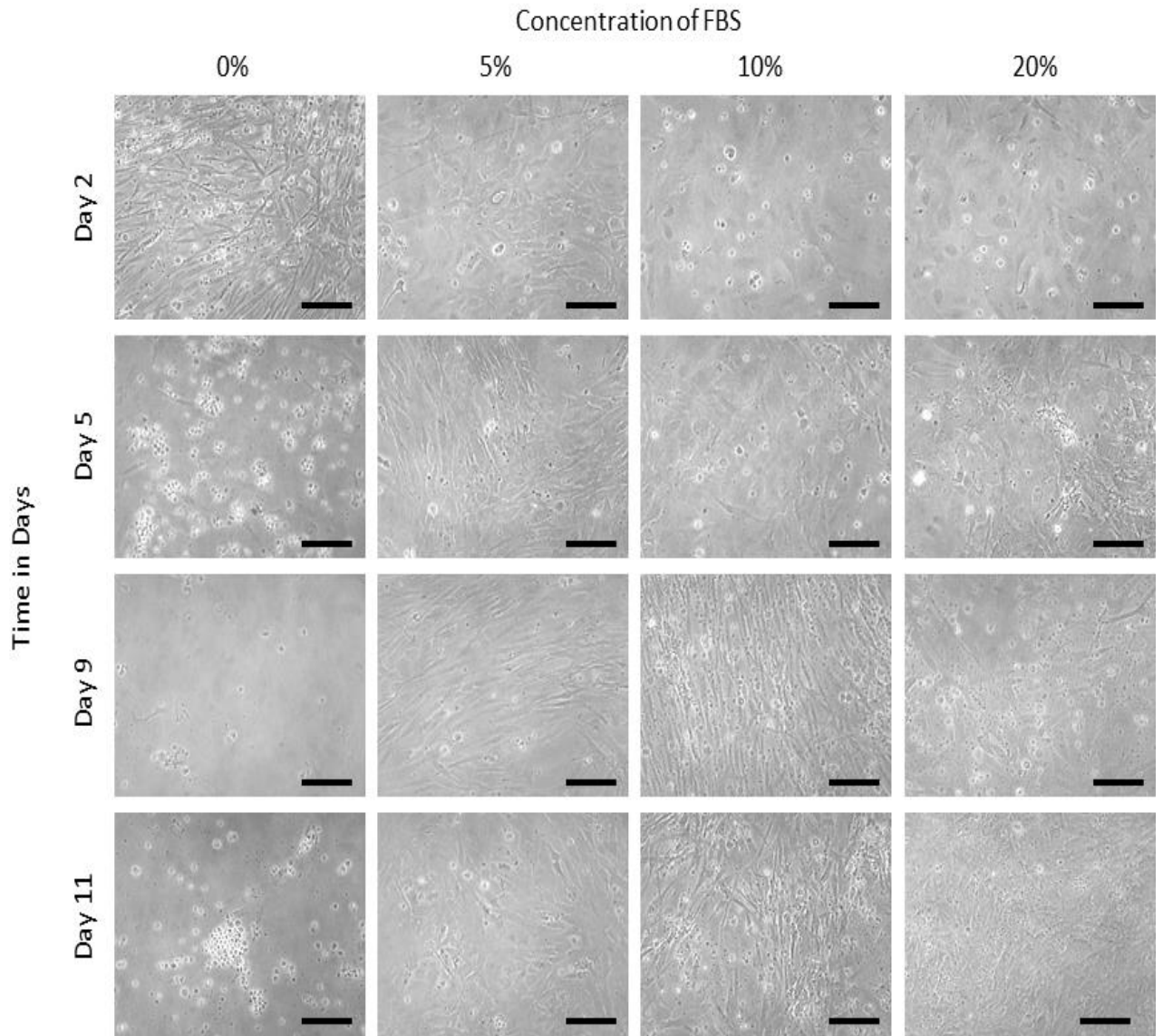
Figure 3.9: Morphology of YPF5 at various Fetal Bovine Serum (FBS) concentrations. Phase contrast micrographs were taken of YPF5 on Day 2, 5, 9 and 11 at 20X magnification using a Nikon TS100 microscope before cells were counted using the coulter counter (Appendix D). Rows: days 2, 5, 9 and 11. Columns: concentrations of FBS (0, 5, 10, 20% FBS). Scale bar = 100μm

3.2.4. Effects of temperature on YPF5 growth

Different temperatures had an effect on YPF5 proliferation. YPF5 cells were kept at 4, 14, 20, 26, 29 and 37 ° C for a 12 day period. Cells were counted on Day 0, 2, 4, 7 and 12 and expressed as a percent control of cells per mL. On Day 2, similar cell counts were recorded for all temperatures but starting at Day 4 through 12, differences in cell counts were observed. Temperatures of 20, 26 and 29 ° C enhanced proliferation while temperatures of 4, 14 and 37 ° C did not support cell growth. Figure 3.10 illustrates a graphical representation and Figure 3.11 a visual observations. 26 ° C was the temperature for optimal growth of YPF5; however, the cells were maintained at room temperature (approximately 22 °) or 18 ° C as they already grow rapidly overtime.
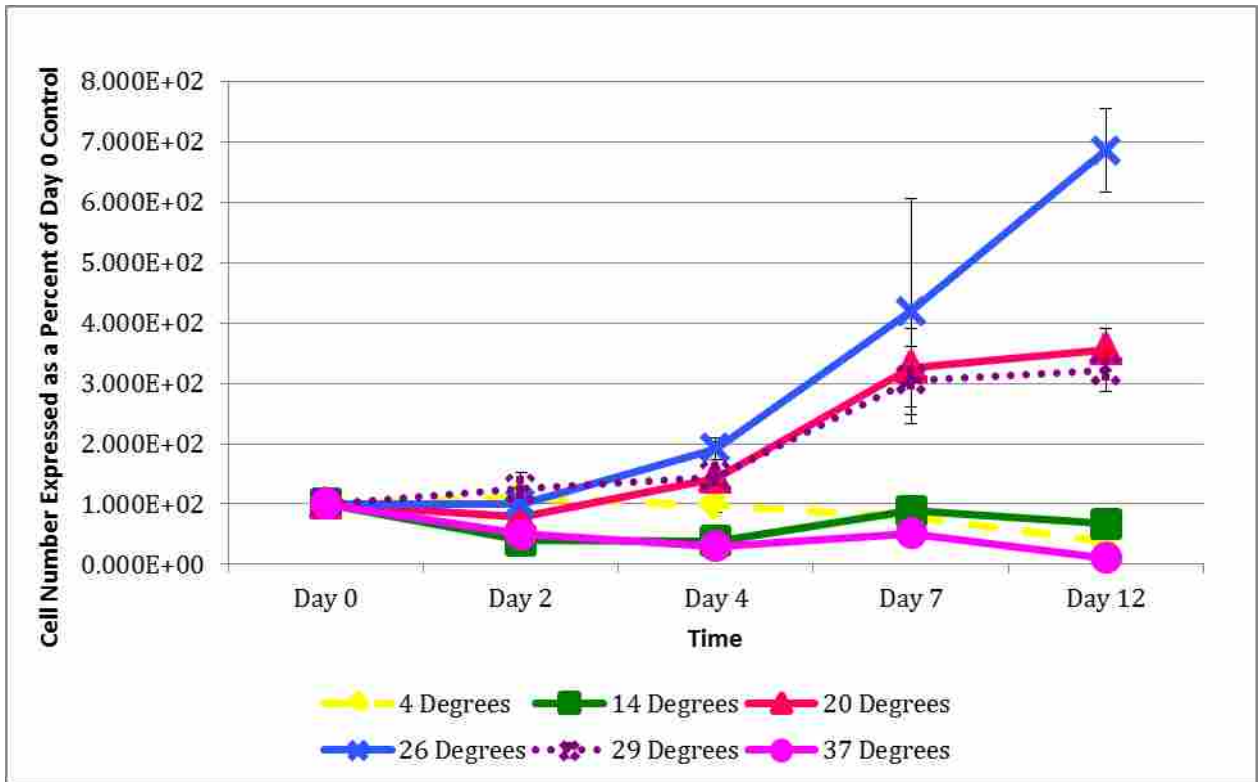
Figure 3.10: Temperature preference of YPF5 cells. YPF5 cells, passage 7, were plated at 70,000 cells/well in 12 well TC plates to incubate for 24 hours at RT. After incubation, cells were kept at six different temperatures (4, 14, 20, 26, 29 and 37 ° C) and grown with L-15 10% FBS for a period of 12 days. Plates were counted using the coulter counter (Appendix E) on days 0, 2, 4, 7 and 12 and media was changed in remaining plates on the days that counting occurred. All RFUs were expressed as a percentage of the Day 0 control. Each data point is a mean of 4 replicates ± standard deviation.
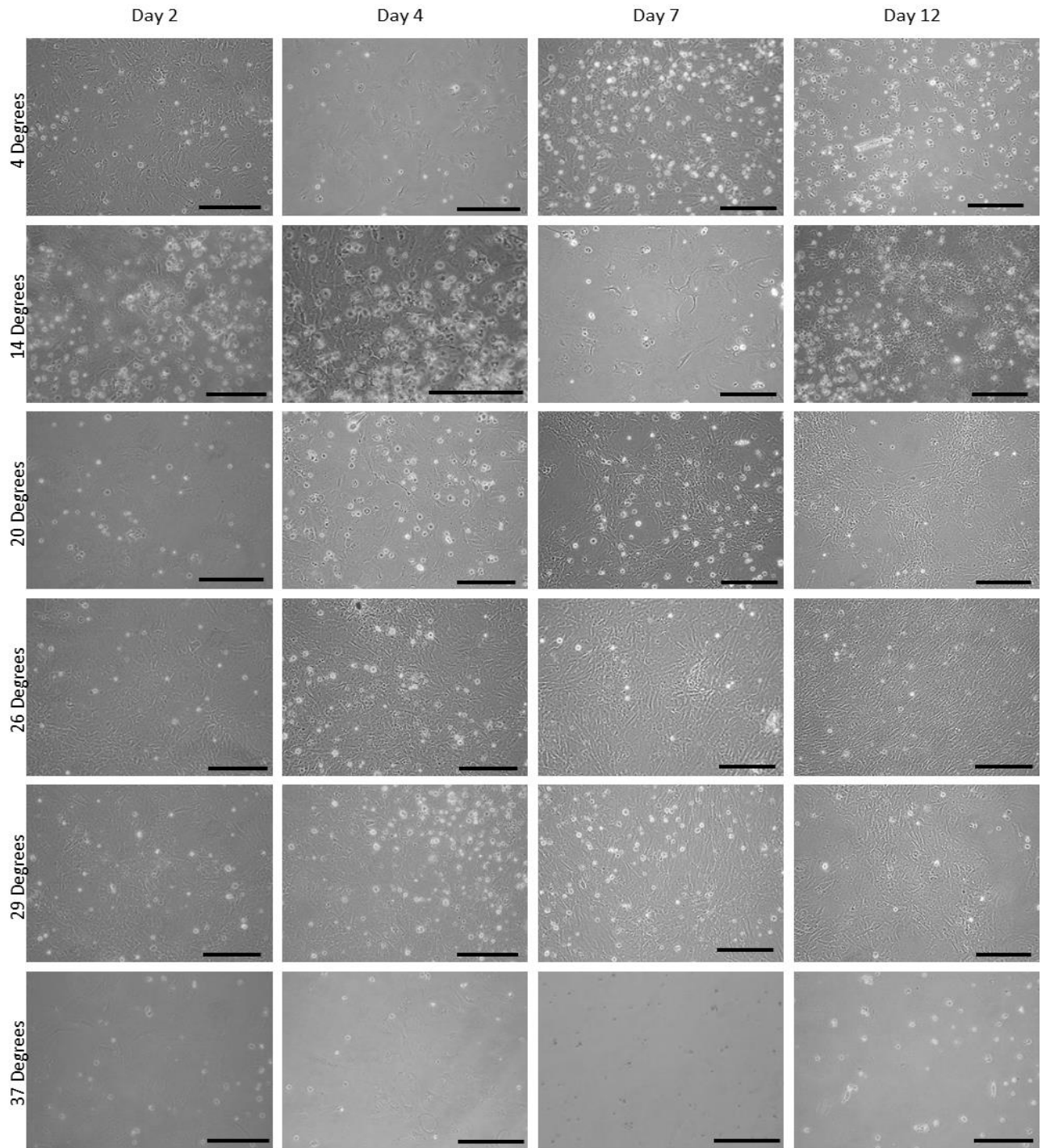
Figure 3.11: Morphology of YPF5 at various temperatures. Phase contrast micrographs were taken of YPF5 on Day 2, 4, 7 and 12 using a Nikon TS100 microscope before cells were counted using the coulter counter (Appendix D). Rows: temperatures (4, 14, 20, 26, 29 and 37 ° C) Columns: days 2, 4, 7 and 12. Scale bar = 200µm

3.2. 5 Effects of cortisol on YPF5 growth

The effects of Cortisol on YPF5 proliferation were investigated using concentrations of 0,

10, 100 and 1000ng/mL of Cortisol over 7 days. The cell counts were expressed as Total

Cells in Original Samples and as a percentage of initial cell count. It appeared that on

Day 3 there was a significant difference ($p < 0.05$*) in YPF5 growth with the presence of

cortisol versus none in the control (cells grew better without the presence of cortisol).

On Day 7, there were no significant differences between the addition of cortisol versus

the control.
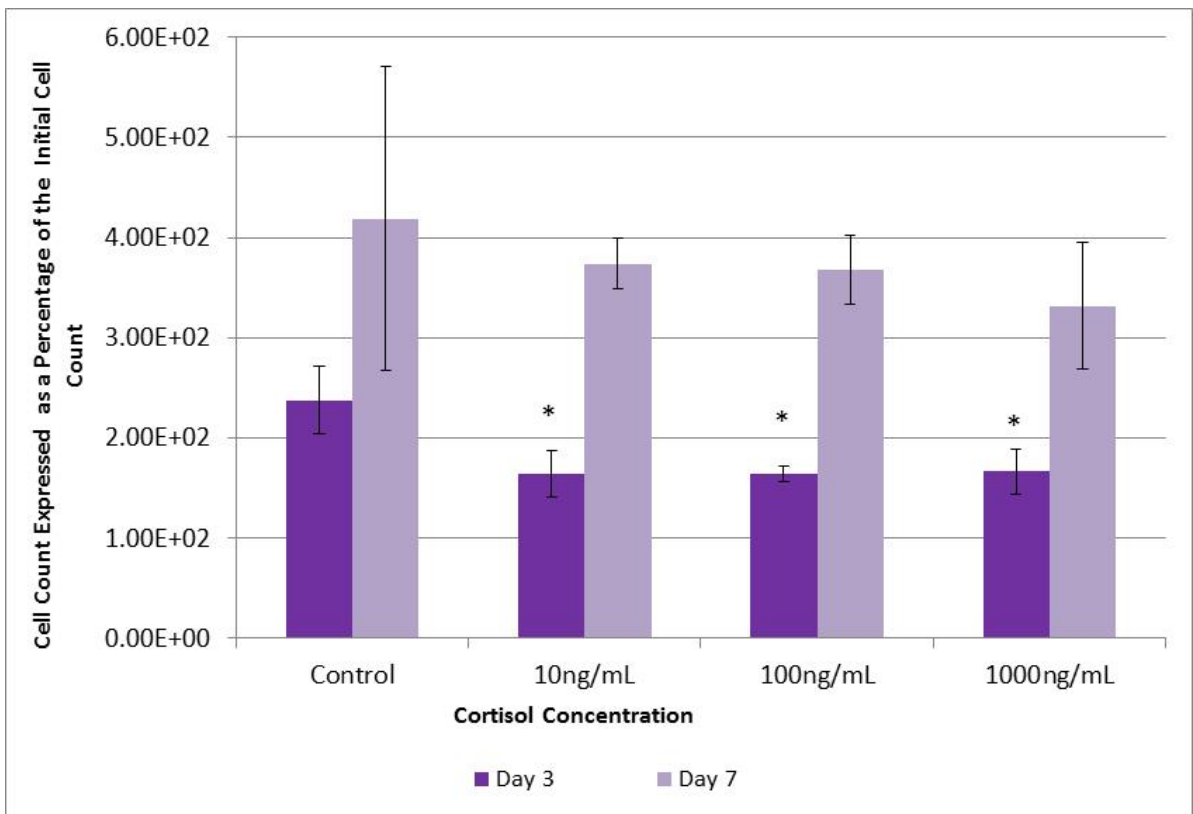
Figure 3.12: Effect of cortisol on YPF5 proliferation. YPF5 cells at passage 41 were plated in a 12 well TC plate with L-15 10% FBS at 70,000 cells/ well at 26 ° C. After 24 hours, cells were exposed to 10, 100, 1000ng/mL of cortisol. The mean counts of three replicates ± standard errors are represented. Results are RFUs expressed as a percentage of the initial cell count. Cell counting used a VIACOUNT program in a Guava Flow cytometer and cells were counted twice over a 7 day period. Media was changed in remaining plates on the days that counting occurred. Asterisks indicate significant differences of cortisol concentrations compared to control ($p < 0.05$ *)

3.2.6. Effects of Ascorbic Acid on YPF5 growth

The effects of Ascorbic Acid (A.A) on YPF5 proliferation were investigated using concentrations of 0, 5, 25, 50 and 100 µg/mL of A.A (made with L-15 and 10% FBS) for 12 days. On Days 0-8 the effect of different A.A concentrations on YPF5 was varied at each time interval and cells continued to grow with and without Ascorbic Acid. On Day 12 all A.A concentrations were found to be statistically significant from the control ($p < 0.05$ *, $p < 0.01$ **) meaning cells grew best on this day without A.A. Figure 3.13 shows a graphical representation and Figure 3.14 visual observations. A.A doesn't appear to be detrimental to YPF5 growth (especially within a one-week time frame). However, in comparison to no A.A, YPF5 cells did not proliferate as well with long term exposure.
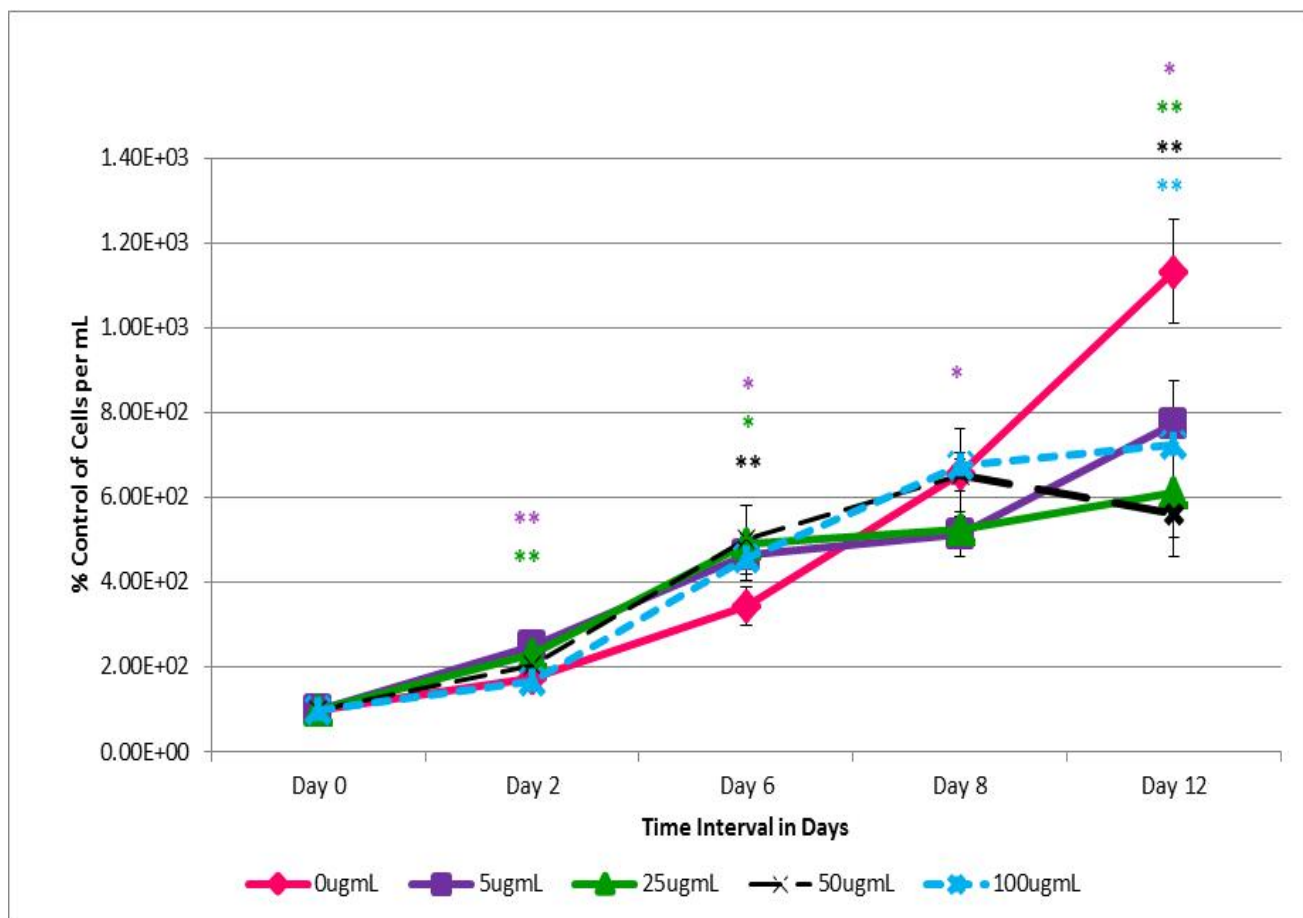
Figure 3.13: Effect of Ascorbic Acid on YPF5 proliferation over 12 days. YPF5 cells were plated at 50,000 cells/well in a 12 well TC plate at passage 9 and grown with L-15 10% FBS at RT. After 24 hrs Ascorbic Acid concentrations of 0, 5, 25, 50, 100 µg/mL were added to cells in triplicate wells. Cells were counted using a coulter counter (Appendix E) on Day 0, 2, 6, 8 and 12. All RFUs were expressed as a percentage of the Day 0 control. Each data point is a mean of 3 replicates ± standard errors are represented by each data point. Asterisks indicate significant differences between the control (0µg/mL Ascorbic Acid) and all the other concentrations for each time interval in days ($p<0.05$ *, $p<0.01$ **). Asterisk colours are Purple = 5 µg/mL; Green = 25 µg/mL; Black = 50 µg/mL; Blue = 100 µg/mL.
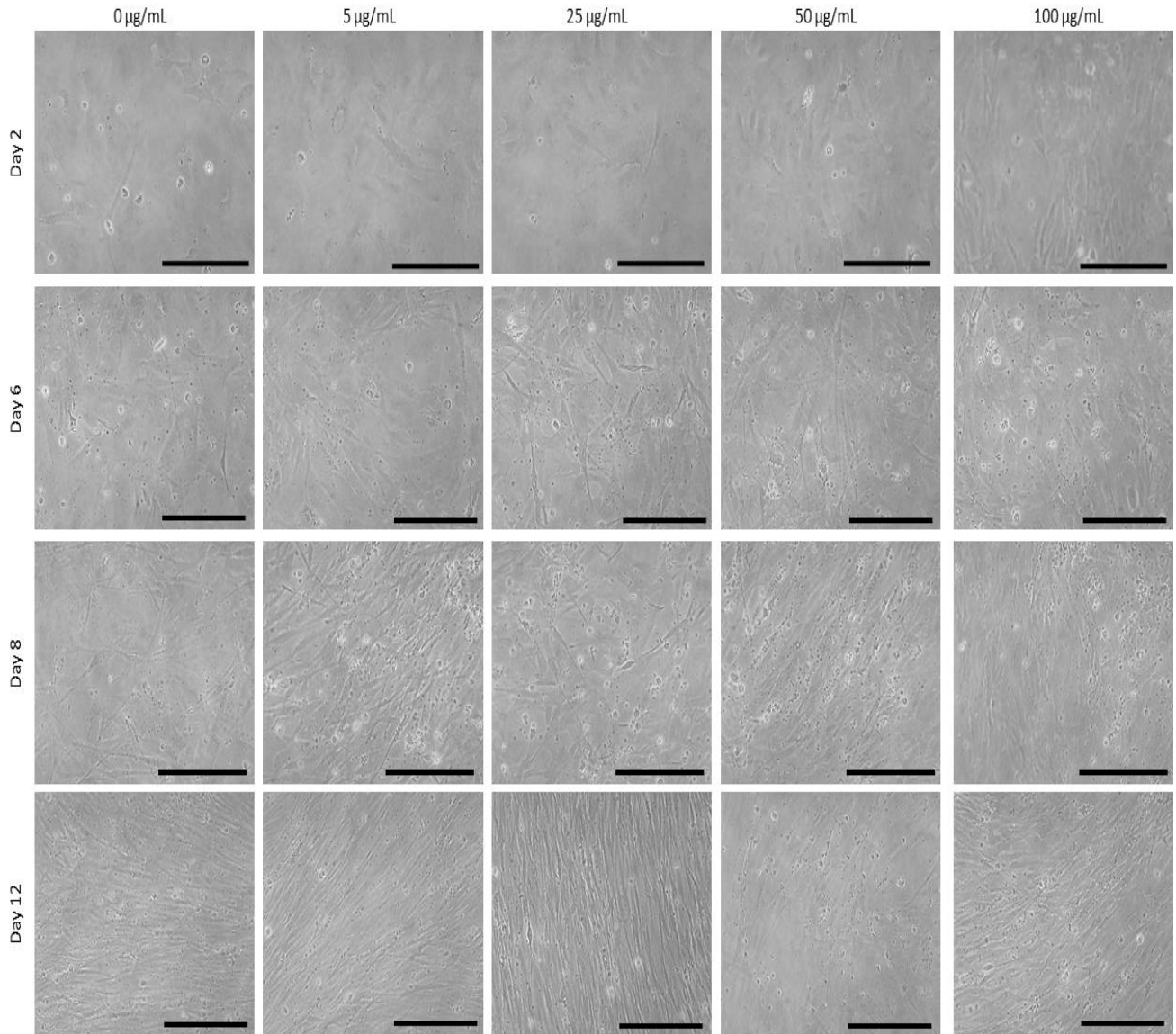
Figure 3.14: Morphology of YPF5 at various concentrations of Ascorbic Acid. Phase contrast micrographs were taken of YPF5 at passage 9 on Day 2, 6, 8 and 12. Cells were exposed to different concentrations of Ascorbic Acid over 12 days and pictures were taken at 20X magnification with a phase-contrast Nikon TS100 microscope before cells were counted using the coulter counter (Appendix D). Rows: days 2, 6, 8 and 12. Columns: concentrations of Ascorbic Acid (0, 5, 25, 50, 100 µg/mL). Scale bar = 200 µm

3.2. 7 Alkaline Phosphatase Stain for stem cell characteristics

For the alkaline phosphatase stain, two additional cell lines were used for comparison, GFSk-S1 and ZEB2J. As seen in Figure 3.15 panel B, YPF5 produced a reddish-pink colour throughout the field of view which indicated a positive stain for alkaline phosphatase. Goldfish fibroblasts GFSk-S1 derived from adult goldfish stained negative for Alkaline Phosphatase. Zebra fish embryonic ZEB2J cells that exhibited stem-like properties (Xing, et al., 2008) was also positive for alkaline phosphatase. Previously reported by Xing et al (2008), ZEB2J stained (approximately) 50% of cells as positive for AP. For this experiment it was calculated that approximately 45% of ZEB2J cells and approximately 52% of YPF5 cells were AP positive. It can be noted that AP positive YPF5 cells appeared to stain more distinctively in comparison to ZEB2J.
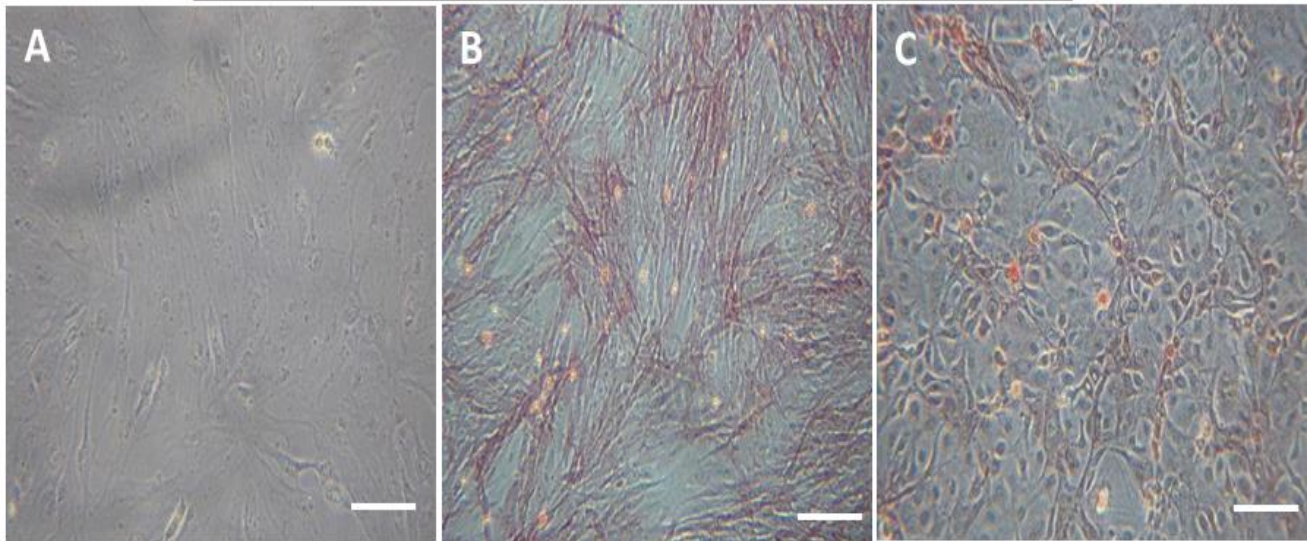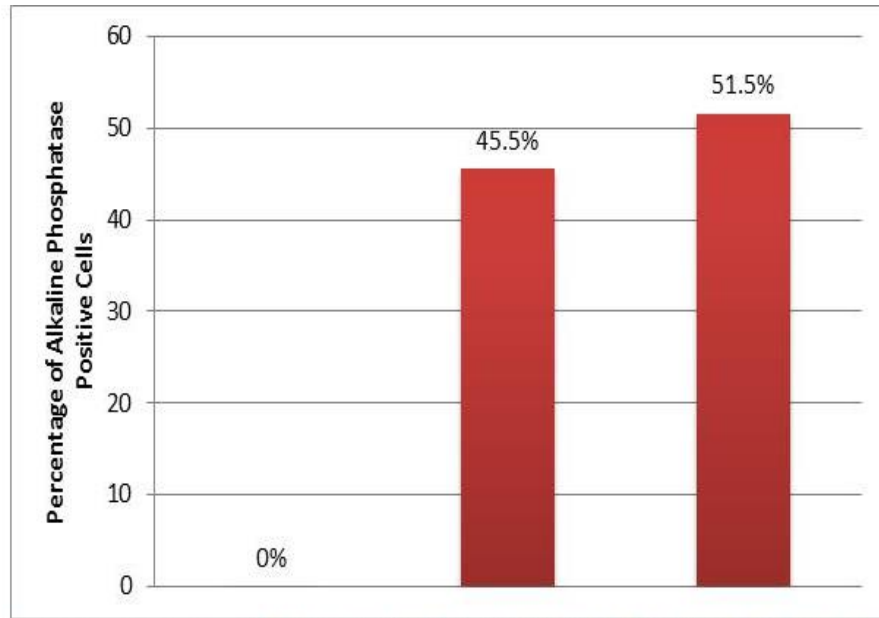
Figure 3.15: Percentage of Alkaline Phosphatase positive cells and the corresponding phase contrast micrographs. YPF5 cells were plated in 25cm^3 flasks at passage 33. These flasks were kept at 26°C for 48 hours and then fixed with a Citrate-Acetone-Formaldehyde fixative. The protocol from the Sigma Alkaline Phosphatase kit was followed. Pictures were taken with a phase-contrast Olympus CK40 microscope. Scale bar = 100µm.AP positive cells are expressed as a percentage of the total cells: **(A)** GFSK (0%) **(B)** YPF5 (51.5%) **(C)** ZEB2J (45.5%) alkaline phosphatase stain.

3.2. 8 Senescence Stain

Out of the cell lines tested, eel brain cells were positive for senescence (Figure 3.16A). After staining, these eel brain cells appeared to have approximately 88% of cells coloured blue which is similar to the previously reported 70% for this cell line (Wagg dissertation, 2005). In contrast, YPF5 cells had the lowest enzyme activity since less than 1% of the overall monolayer was stained (Figure 3.16B).
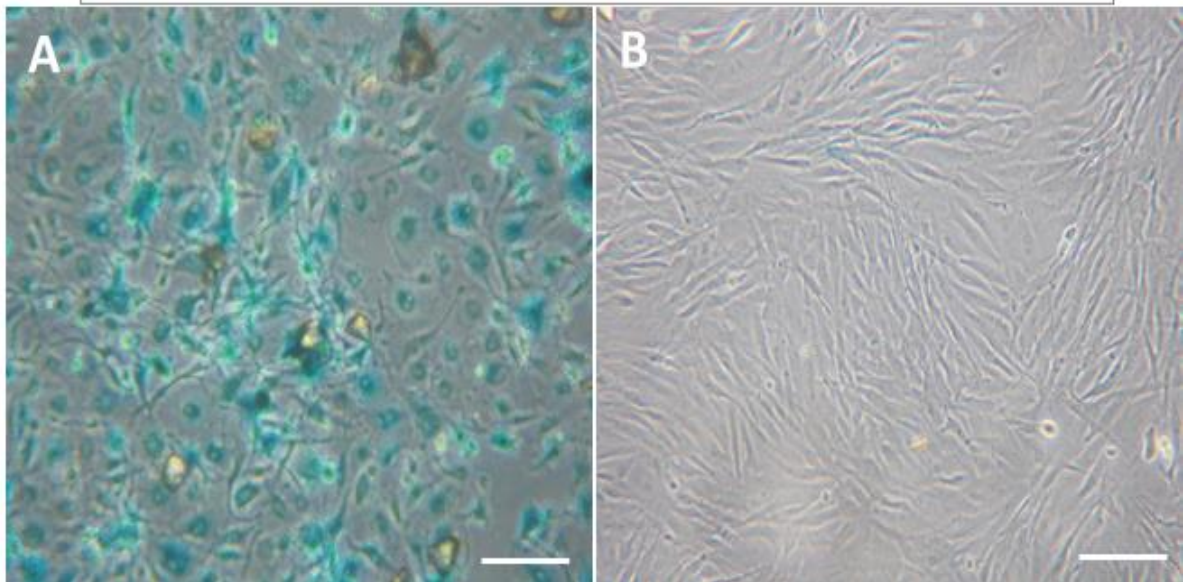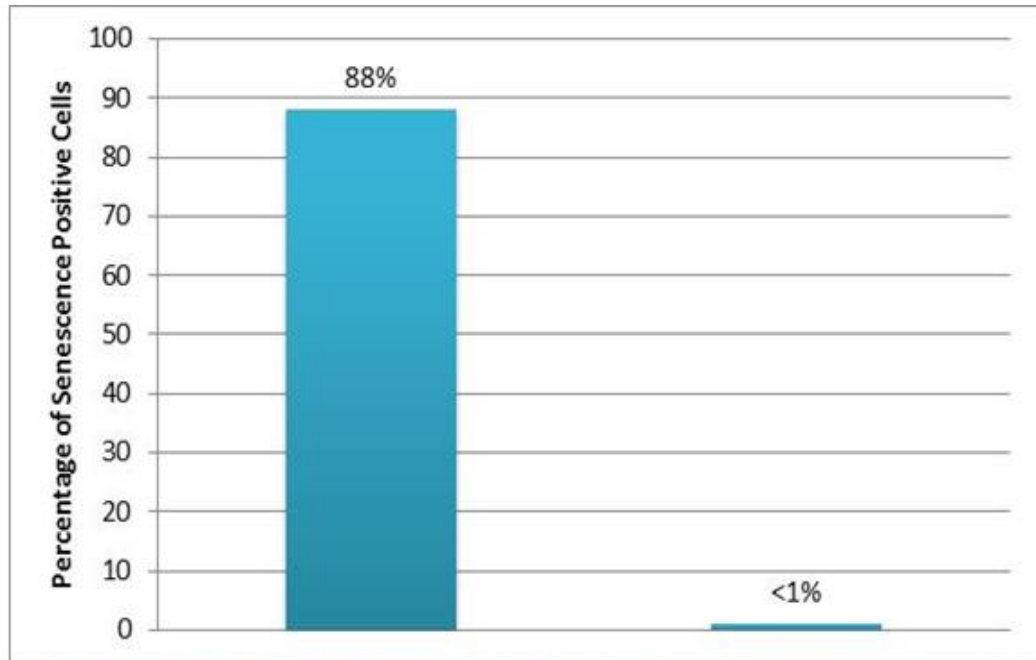
Figure 3.16: Percentage of β-Galactosidase positive Eel Brain and YPF5 cells and the corresponding phase contrast micrographs. YPF5 Cells were plated at passage 33 in 25cm^3 flasks at 26°C for 48 hours. The Sigma senescence staining kit was used and its protocol was followed. Flasks were kept overnight and pictures using an Olympus CK40 microscope were taken. Eel brain **(A)** and YPF5 **(B)** senescence stain. Scale bar=100μm. In the graph, the amount of senescent positive cells is expressed as a percentage of the total count for both cell lines. Eel brain had approximately 88% of the cell monolayer positive for senescence while YPF5 had less than 1%.

3.2. 9 Immunocytochemistry for collagen type I

After immunostaining with anti-collagen type 1, it was shown that YPF5 (not exposed to Ascorbic Acid) contained intracellular procollagen with punctated or grouping patterns, as observed in Figure 3.17B. After exposure to 50µg/mL of Ascorbic Acid for 13 days, immunostaining of YPF5 showed the presence of fibrous excreted collagen (Figure 3.17C). Thus, YPF5 cells appeared to have conserved one of the core cellular functions of fibroblasts: to synthesize, release and form collagen type 1 matrix in the extracellular space.
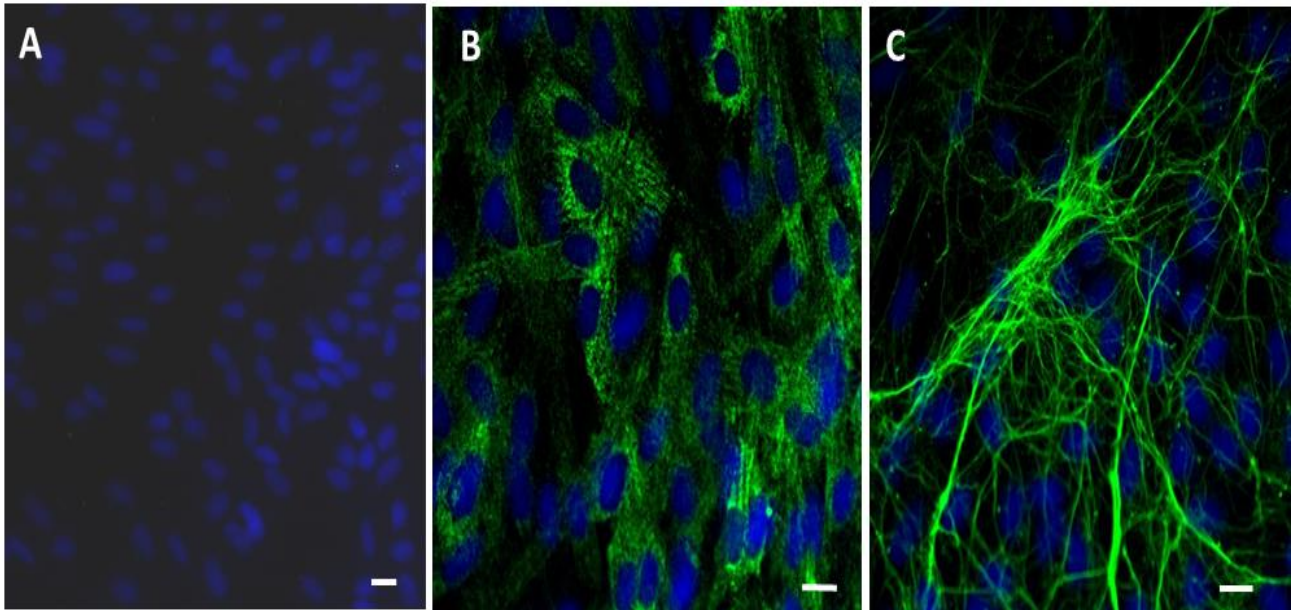
Figure 3.17: Confocal images of immunocytochemistry of YPF5 for anti-collagen. YPF5 cells at passage 34 were plated in 4 slide flasks at a concentration of 800,000 cells/flask using L-15 with 5% FBS and kept at RT. After 24 hrs, media was changed to the following: 3 flasks with L-15 media, 5%FBS and 1 flask with L-15 media, and 5%FBS and 50µg/mL of Ascorbic Acid. The media in all four flasks were changed every 3 days. On Day 13, immunostaining occurred and the following was the set up: **(A)** YPF5 control was a flask not exposed to Ascorbic Acid and with Secondary Antibody only. **(B)** YPF5 not exposed to Ascorbic Acid, but with Primary and Secondary Antibodies. The presence of punctated procollagen is apparent. **(C)** YPF5 exposed to 50µg/mL of Ascorbic Acid for 13 d, with Primary and Secondary Antibodies. Fibrous excreted collagen is present. Pictures were taken with a confocal microscope. Scale bar= 10µm. Primary antibody: rabbit polyclonal anti-salmon collagen type 1 IgG (CedarLane CL50171AP-S1) 1:250 diluted in blocking buffer. The secondary antibody: goat anti-rabbit IgG conjugated with

AlexaFluor 488 (Invitrogen A-11008) 1:1000. Blue = DAPI, Green = rabbit polyclonal anti-salmon collagen type 1 IgG.

3.2. 10 Immunocytochemistry for vimentin

Both GFSk-S1 and YPF5 were positive for immunostaining for vimentin as shown in Figure 3.18. These cell lines showed typical filamentous structures that were attached to centrosomes near the cell nuclei. These results further confirmed the mesenchymal origin of YPF5 and GFSk-S1 cells.
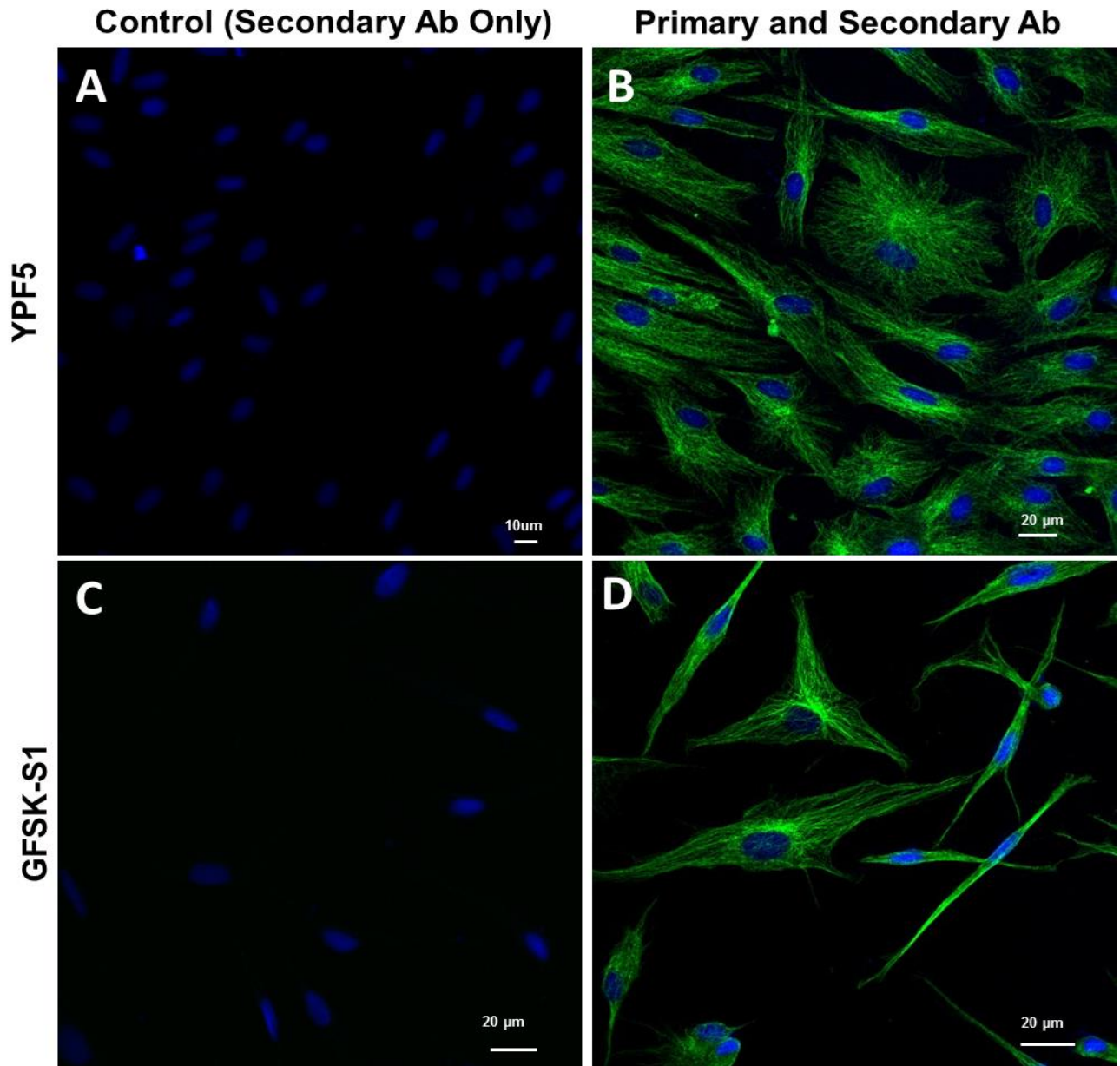
Figure 3.18: Confocal images of immunocytochemistry of YPF5 for anti-vimentin. YPF5 cells at passage 20 and GFSk-S1 at passaged 55 were plated in a four chamber glass chamber slide at 150,000 cells/well and 250,000 cells/well (respectively) using L-15 with 10% FBS at RT. After 24 hrs immunostaining occurred. Each cell line had a well for control and another well as non-control. The primary antibody: mouse monoclonal anti-vimentin (Sigma V5255) at 1:250 and the secondary antibody: AlexaFluor 488 Goat Anti-Mouse IgG 1:1000 (Invitrogen A-11001). **(A)** and **(C)** are the controls of YPF5 and GFSK-S1 respectively, meaning secondary antibody only was used during immunostaining. **(B)** and **(D)** are the respective cell lines but with both primary and secondary antibodies used. Blue = DAPI, Green = mouse monoclonal anti-vimentin.

3.2.11 Mitochondrial morphology

YPF5 cells kept at different temperatures (10, 18 and 26 ° C), changed in both cell and mitochondrial morphology. These cells were kept for 6 days before a fluorescent mitochondrial rhodamine stain was conducted. Figure 3.19 show the mitochondria indicated in green. YPF5 cells at lower temperatures possessed more punctated and wide mitochondria and epithelial-like morphology, while cells kept at higher temperatures had more fibrous mitochondria and cell shape.

Phase Contrast

Fluorescent

10 Degrees D6
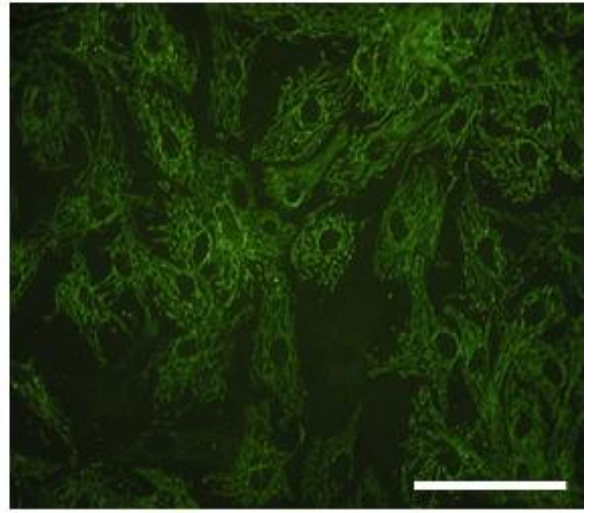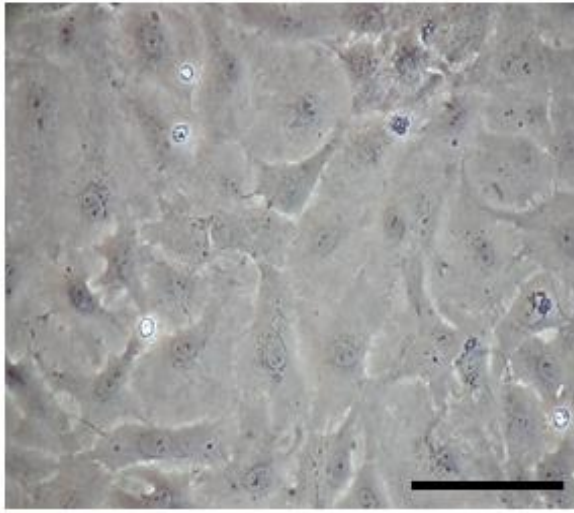
18 Degrees D6
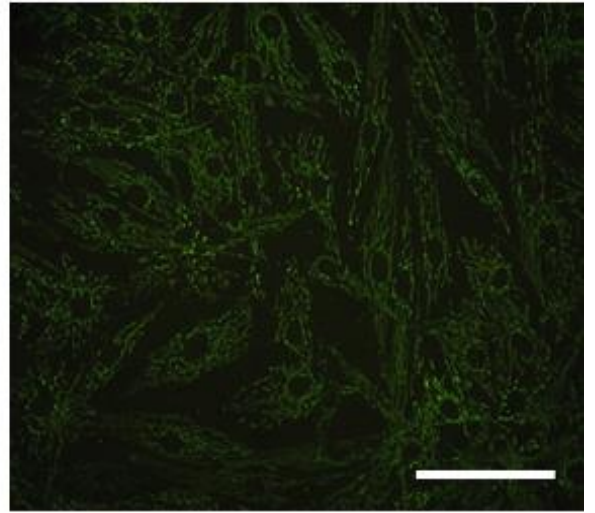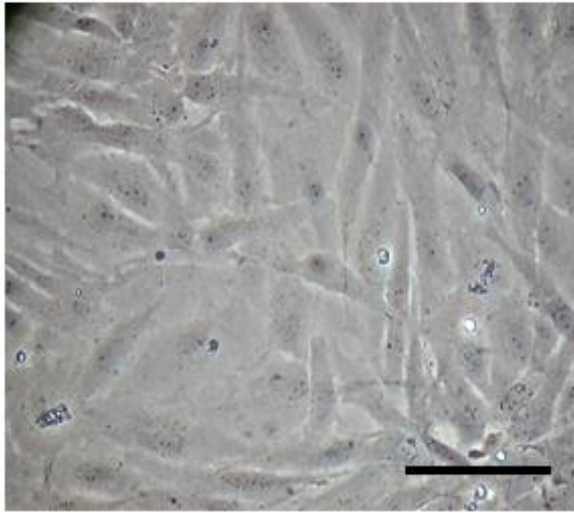
26 Degrees D6
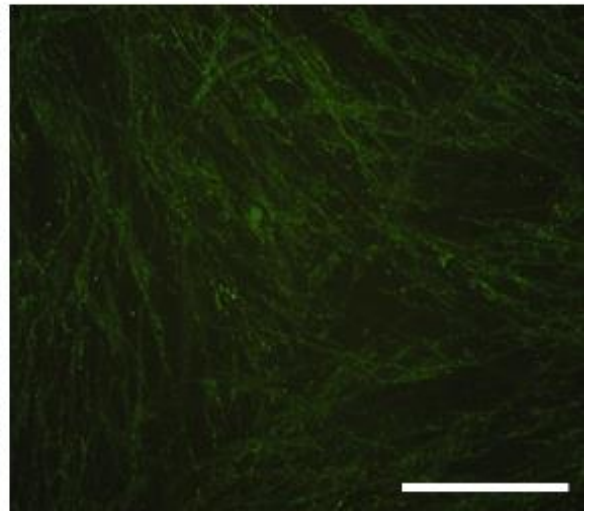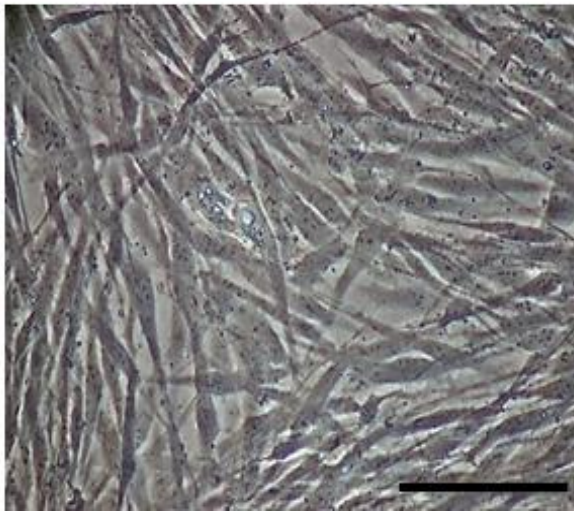
Figure 3.19 Morphology of YPF5 kept at various temperatures and stained with Rhodamine 123 mitochondrial stain. YPF5 cells at passaged 37 were plated at 75,000 cells/well in 12 well TC plates at 26 °C. After 24hrs plates were moved to 10, 18 and 26 °C temperatures. On Day 0, 3 and 6 a 30-60 min Rhodamine 123 stain was performed on cells from each of the aforementioned temperatures. Pictures shown in Figure 3.19 were taken on Day 6 at 20X magnification with a Nikon TE300 microscope. **(Left)** Phase contrast and **(Right)** fluorescent micrographs of YPF5 stained with rhodamine 123 mitochondrial stain. YPF5 cells at lower temperatures appeared epithelial-like while cells at the optimal temperature were more fibroblastic-like in morphology. Rows: temperatures, 10, 18 and 26 °C. Scale bar = 100µm

3.3. Toxicity Investigations utilizing YPF5

3.3.1. Exposure of YPF5 to chemicals

The YPF5 cell line was used to evaluate the cytotoxicity of copper sulphate ($CuSO_4$),

ammonium chloride (NH_4Cl), crude naphthenic acid (CrNA) and oil sands processed

waters (OSPW) using the Alamar Blue (AB) and CFDA-AM Assay (NH_4Cl is the only

chemical which was evaluated using the Neutral Red Assay). For each contaminant both

Alamar Blue and CFDA-AM EC50 values were calculated and the average values were

recorded in Table 4.
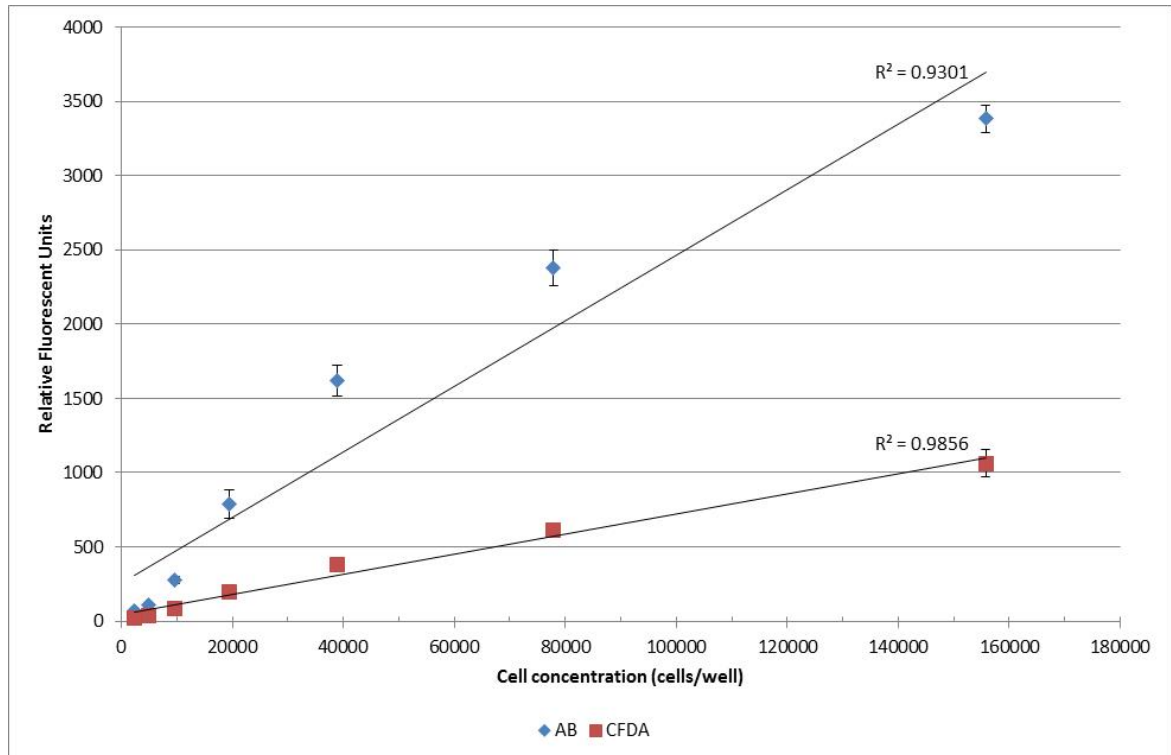
Figure 3.20: Standard Curves for Alamar Blue and CFDA-AM Assays with YPF5. YPF5 cells were plated using L-15 media with 10% FBS in a 96 well plate (100 µl/ well) and allowed to attach for 24hrs at 26 °C. The cell concentrations were serially diluted 1:2 and ranged from 2,433.59-155,750 cells/well. After 24 hours of attachment the media was removed and AB and CFDA fluorescent dyes were added. After a 60 min incubation with the dyes, Relative Fluorescent Units (RFUs) were read using a fluorometric plate reader. RFUs are shown the Y axis and cell concentration (cells/well) on the X axis.

Table 4: Effects of model chemical contaminants on YPF5 as measured by two viability assays.
YPF5 cells were exposed to the indicated contaminants below for 24h (unless indicated otherwise) and EC_{50}'s were calculated for both Alamar Blue and CFDA-AM assays. Mean EC_{50} values ± SD are presented. n = the number of trials (each trial had 4-6 replicate wells).

Environmental Contaminant	Average Alamar Blue EC_{50} (n) µg/mL	Average CFDA-AM EC_{50} (n) µg/mL	*in vivo* 96hLC_{50} for Yellow Perch (unless stated otherwise)
Crude Naphthenic Acid	34.90 ± 8.57 (n = 5)	40.27 ± 6.82 (n = 5)	6.8mg/L <96Hr 100% mortality (Nero et al., 2006b) 96-h LC_{50} 5.6mg/L Commercial Sold CAS no. 1338-24-5 (fathead minnow) (American Petroleum Institute, 2012)
Ammonium Chloride	793.77 ± 296.37 (n = 3)	718.70 ± 126.61 (n = 3)	0.66-1.02mg/L unionized ammonia (Espey, 2003 dissertation)
Copper Sulphate	2.18 ± 0.52 (n = 5) 24Hrs 1.113 (n=1) 48Hrs 0.9018 (n=1) 72Hrs 0.5081 (n=1) 96Hrs	2.25 ± 1.30 (n = 5) 24Hrs 0.7922 (n=1) 48Hrs 0.8888(n=1) 72Hrs 0.4706(n=1) 96Hrs	0.132µg/mL 48-h LC_{50} (soft water) (0.53µmol/L Taylor et al., 2003) 1.03µg/mL 96-h LC_{50} (hard water) (4.16µmol/L Taylor et al., 2003)

3.3.1.1. Ammonium Chloride

Ammonium chloride did not appear to be particularly toxic to YPF5 cells. Average

calculated EC_{50} values for NH_4Cl with the Alamar Blue and CFDA-AM assays were 793.77

± 296.37 µg/mL and 718.70 ± 126.61 µg/mL (n=3 trials with 4-6 well replicates)

respectively. Appendix D Figure 6.3 is a representative NH_4Cl graph. The asterisks

indicates the concentrations which provided average Relative Fluorescent Units that were significantly different than the control ($p<0.05^*$, $p<0.01^{**}$).

For the Neutral Red assay, YPF5 was exposed to 0, 25, 50, 100, 250 and 500 µg/mL of NH_4Cl. The duration of exposure varied and it was found that, if kept for approximately 4 days, cells formed vacuoles at a concentration of 250µg/mL or higher of NH_4Cl. Figure 3.21 (A-C) show YPF5 after 4 days of exposure before the conduction of NR assay and (D-F) after. The numerical results of the NR assay indicate that NR is increasingly sequestered in lysosomes from control until approximately 250µg/mL.
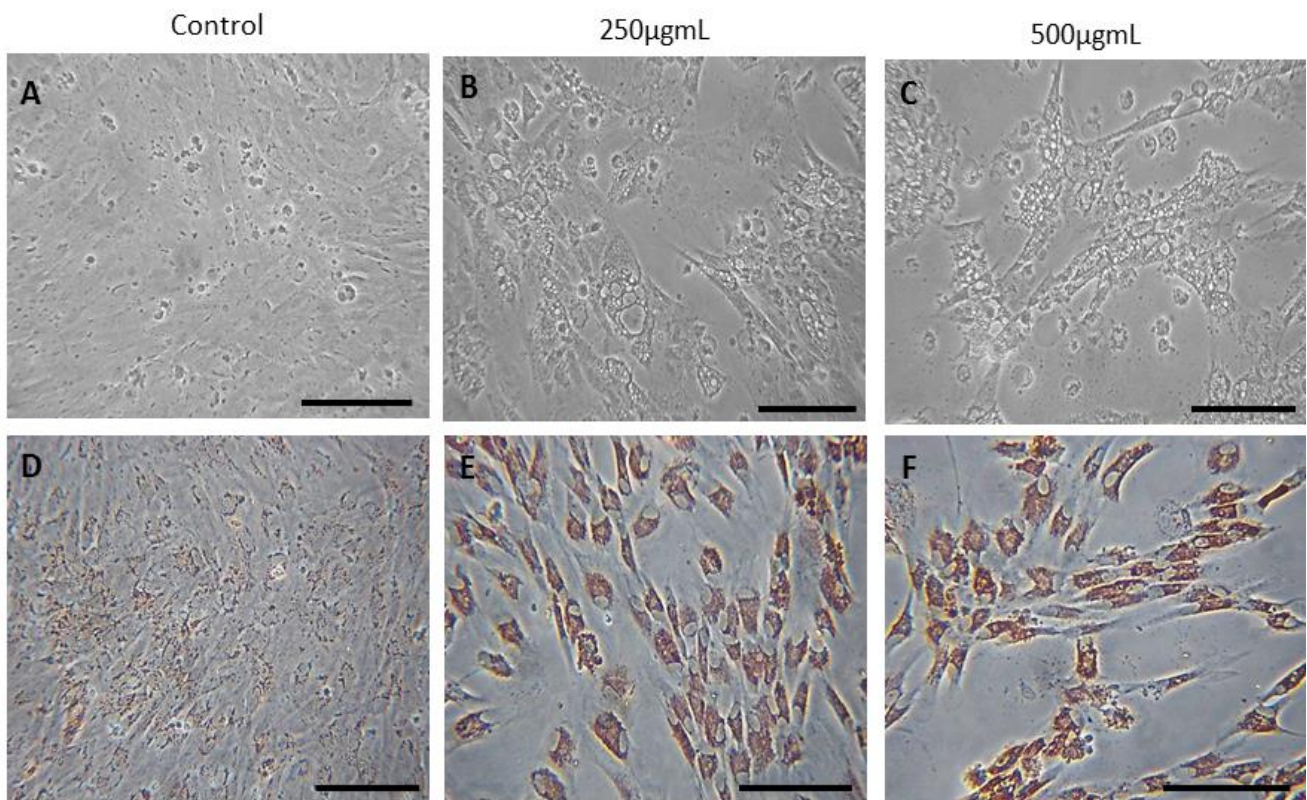
Figure 3.21: Phase contrast micrographs of YPF5 exposed to NH_4Cl before and after undergoing Neutral Red assay. YPF5 at passage 25 were exposed to 0, 250 and 500 µg/mL of NH_4Cl. Pictures of before **(A-C)** and after **(D-F)** the Neutral Red assay were taken with a phase contrast Olympus CK40 microscope. Scale bar = 100µm.
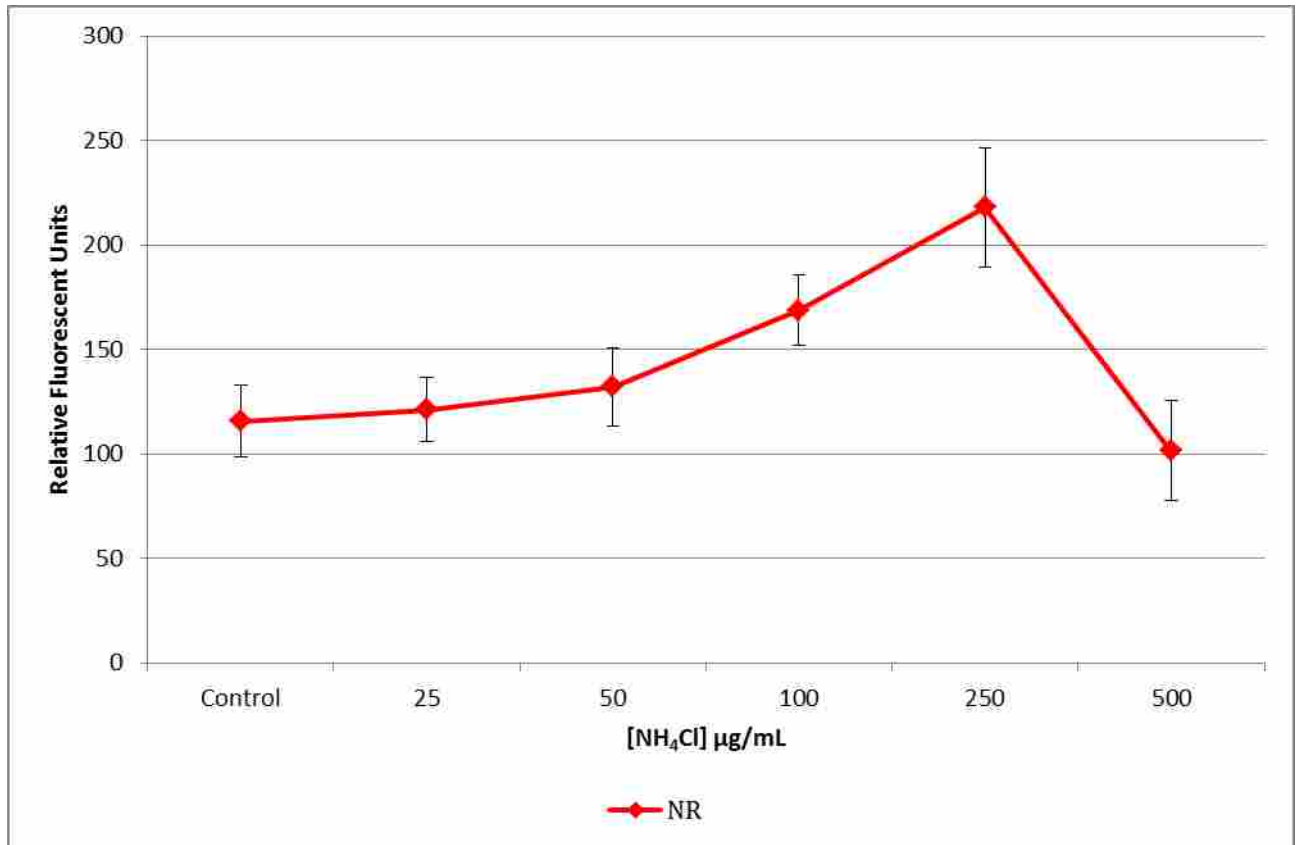
Figure 3.22: Effects of Ammonium Chloride using the Neutral Red Assay. YPF5 cells were exposed to NH_4Cl at 5 different concentrations for 4 days. NR cell viability assays were conducted with a cell concentration of 40,000-60,000 cells/well in 96 well plate. Following 24h attachment cells were exposed with increasing concentrations of NH_4Cl. After 4 days of exposure, Relative Fluorescent Units (RFUs) were read using a fluorometric plate reader. RFUs are shown on the Y axis of this graph and concentrations of NH_4Cl in in µg/mL on the X axis. Error bars are standard deviations. n= 4-6 well replicates

3.3.1.2. Copper Sulphate

Exposures to $CuSO_4$ resulted in decreasing viability of cells as measured by AB and CFDA, in a dose-dependent manner above the 0.5 μg/ml nominal dose. The average 24h EC_{50} values for both viability assays were similar with AB being 2.18 ± 0.52 μg/mL and CFDA-AM 2.25 ± 1.30 μg/mL n=5 (Table 4). Longer exposure times resulted in lower EC_{50} values. Thus, toxicity of copper sulfate was time and dose dependent. Appendix D Figure 6.4 (panels A-H) illustrates a representative $CuSO_4$ graph of 24-96hr AB and CFDA-AM results. The asterisks indicate the concentrations which provided average Relative Fluorescent Units that were significantly different from the control (p<0.05*, p<0.01**). Limited $CuSO_4$ 48-96 h (panels C-H) assays were conducted and the extended assay results had lower EC50 values (Table 4) in comparison to the 24h assay.

3.3.1.3 Crude Naphthenic Acid

YPF5 responded in a consistent dose-dependent manner to CrNA. This environmental contaminant was tested on YPF5 singularly as well as in conjunction with bentonite (a possible remediating component). Before exposure to YPF5, bentonite was added to the CrNA for two different lengths of time: either 18 hours or 48 hours. The average EC50 values of CrNA without bentonite were 34.90 ± 8.57 μg/mL (AB) and 40.27 ± 6.82 μg/mL (CFDA-AM) (Table 4). The EC50 values of CrNA with the addition of bentonite were broken down based on the pretreatment length (Figure 3.23). Pretreatment of bentonite for 18 hours yielded an EC50 of 71.36±4.31 μg/mL (AB) and 74.5±20.44 μg/mL (CFDA-AM). For the 48 hour pretreatment, the EC50 was 87.6±32.05 μg/mL (AB) and 93.72±25.33 μg/mL (CFDA-AM). These EC50 values were then compared separately to the EC50 CrNA only values of 34.90 ± 8.57 μg/mL (AB) and 40.27 ± 6.82

µg/mL (CFDA-AM) and were found to be statistically significant (Figure 3.23). Appendix

D Figure 6.6 are representative graphs demonstrating that YPF5 cell viability decreased

as the CrNA concentration increased. The asterisks indicates concentrations which

provided average Relative Fluorescent Units that were significantly different from the

control ($p < 0.05*$, $p < 0.01**$, $p < 0.001***$, $p < 0.0001****$). Panels B and D indicate results

after CrNA was exposed to bentonite for 18hrs and Panels F and H show results after

48hrs of bentonite pretreatment. Appendix D Figure 6.5 are representative bar graphs

comparing the use of bentonite (panels A-B 18 hrs, C-D 48 hrs) versus no bentonite.

Asterisk indicate the CrNA with bentonite concentrations which provided average

Relative Fluorescent Units that were significantly different from the CrNA only

concentrations ($p < 0.05*$, $p < 0.01**$, $p < 0.001***$, $p < 0.0001****$).
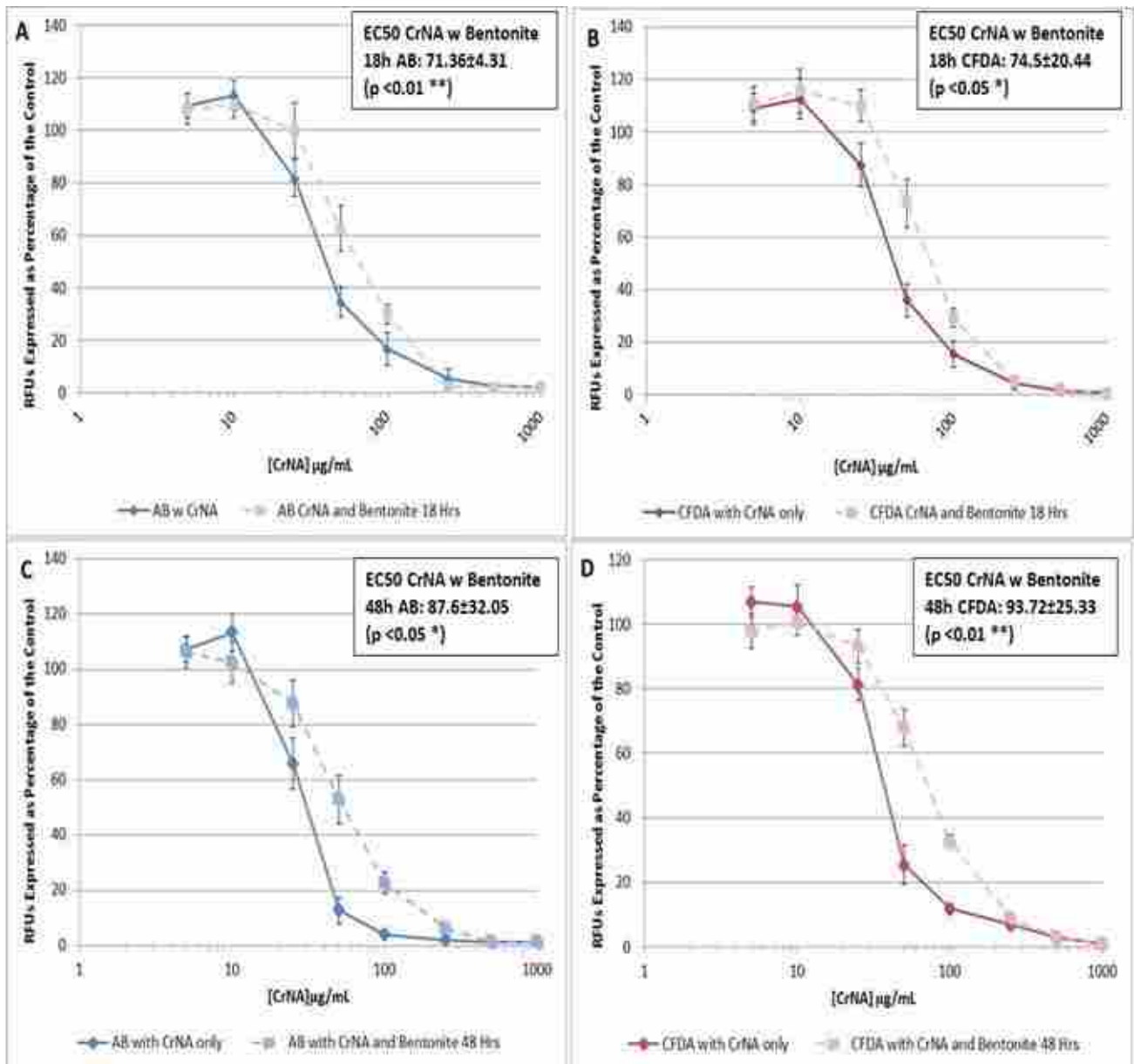
Figure 3.23: Effects of CrNA with and without Bentonite pre-treatment for testing effectiveness of amelioration strategy on viability of YPF5 cells. Representative dose response curves for YPF5 exposed to CrNA (with or without prior bentonite incubation) at the indicated concentrations for 24h. Panels **A, B** depict responses to CrNA with or without 18h pre-treatment to Bentonite. Panels **C, D** depict responses to CrNA with or without 48h pre-treatment to Bentonite. Alamar Blue viability assays are depicted in panels **A,C** and CFDA-AM results are depicted in panels **B,D**. Assays were conducted in 96 well plates. Following a 24h attachment, cells were exposed with increasing concentrations of CrNA with or without bentonite pre-treatment. After an additional 24hrs, indicator dyes were added and Relative Fluorescent Units (RFUs) were read using a fluorometric plate reader. RFUs expressed as a percentage of the control (no chemical addition) are shown on the Y axis of this graph and concentrations of CrNA in μg/mL on the X axis. 18 hours of bentonite pretreatment had n = 2 trials with 4-6 well replicates in each trial. 48 hours of bentonite pretreatment had n = 3 trials with 4-6 well

replicates in each trial. The EC50s were calculated using GraphPad Prism software and then averaged and expressed in μg/mL. **Asterisks indicate significant differences between EC50 CrNA only (AB EC50 =34.90 ± 8.57 or CFDA EC50 = 40.27 ± 6.82) and either EC50 CrNA with bentonite pre-treatment for 18hr or 48hr ($p < 0.05$ *, $p < 0.01$ **)**

3.3.2.1 Exposure of YPF5 to OSPWs

The toxicity of OSPW samples in relation to naphthenic acid content could also be identified using YPF5. In a previous study, Sansom et al., (2013) evaluated the toxicity of 49 OSPW samples. In this study, a subset of 9 samples (picked from among 49 samples that contained most volume) were evaluated #1, 2, 3, 4, 5, 12, 14, 15 and 16. Viability data demonstrated that samples, #5, 12 and 16 were the more toxic OSPW samples to YPF5 (Figure 3.26). These samples were evaluated in blind without prior knowledge of toxicity as reported by Sansom et al 2013., and in agreement with their data, these 3 samples had the higher NA content, correlating the toxicity with NA (Table 5)

For the OSPW samples, remediation methods (bentonite and UV radiation) were also tested. Although sample 5 with the lowest NA content among the 3 toxic samples, could be remediated, samples 12 and 16 did not appear to be remediated with neither bentonite or UV exposure. There were no significant differences in cell viability (AB or CFDA-AM) when considering the results of these remediation strategies compared to the results of OSPW samples only (Figure 3.26).
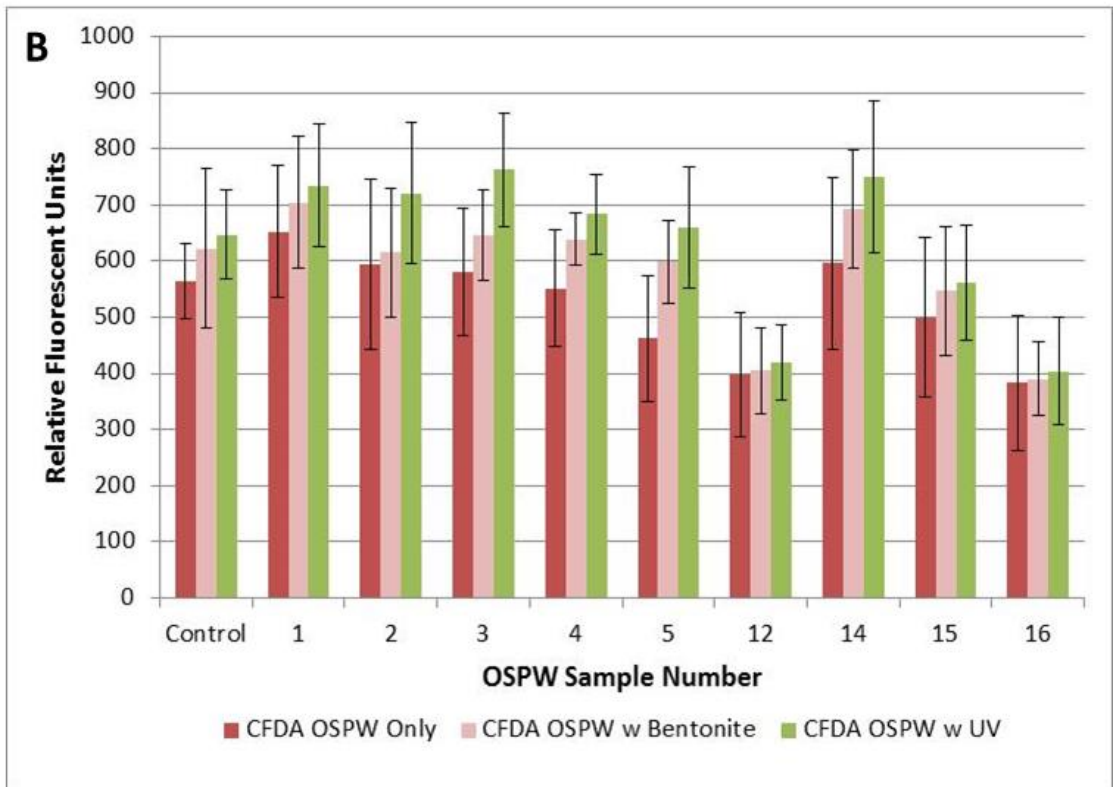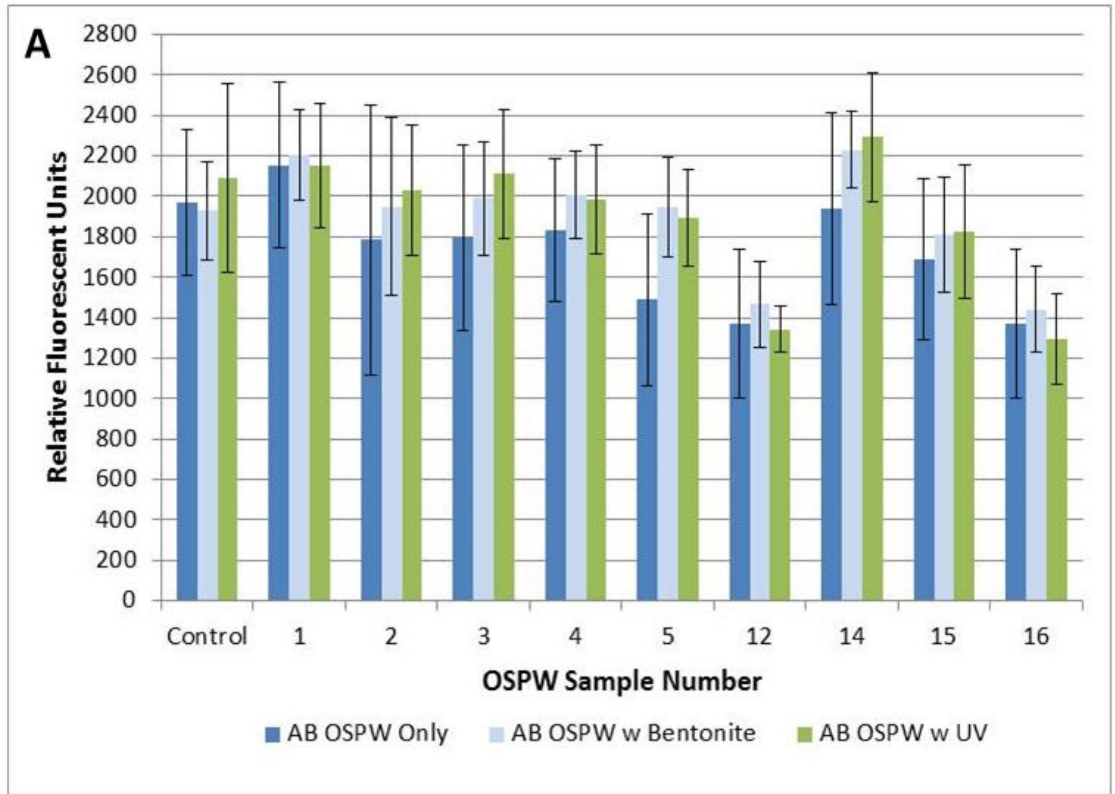
Figure 3.26: Effects of 9 OSPW samples on the viability of YPF5 with or without remediation treatments. Relative Fluorescent Units (RFUs) results of three separate trials with OSPW sample exposure for 24 hours are presented (Alamar blue in blue bars and CFDA-AM in pink bars). Viability was measured by **(A)** Alamar Blue assay and **(B)** CFDA-AM assay on YPF5 cells. Error bars indicate standard deviations. In addition to the OSPW samples, remediating strategies (UV exposure – green bars and addition of bentonite – light blue bars for Alamar Blue and light pink bars for CFDA-AM) were performed for 24 hours before exposure on the tested samples. Assays were conducted in 96 well plates. Following a 24h attachment, cells were exposed to OSPW samples (with and without remediation strategies). After an additional 24hrs, indicator dyes were added and RFUs were read using a fluorometric plate reader. RFUs are shown on the Y axis of this graph and OSPW sample numbers on the X axis. Each trial had 4-6 well replicates.
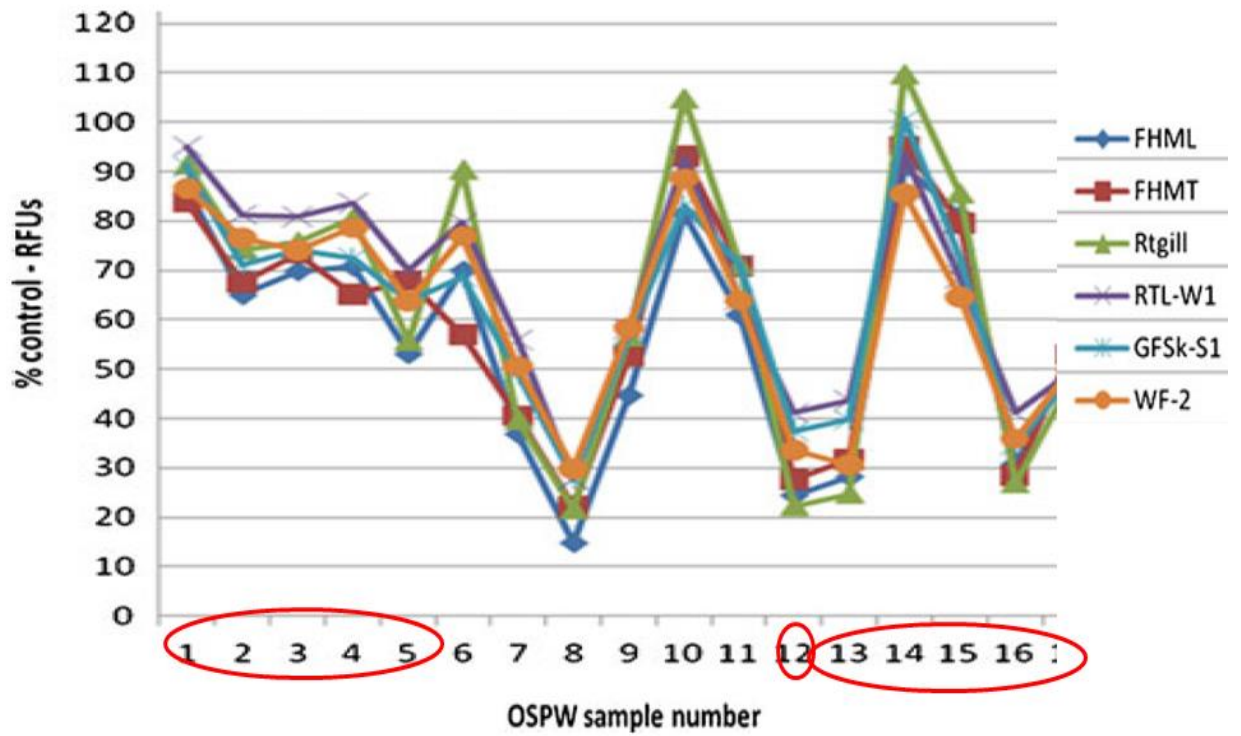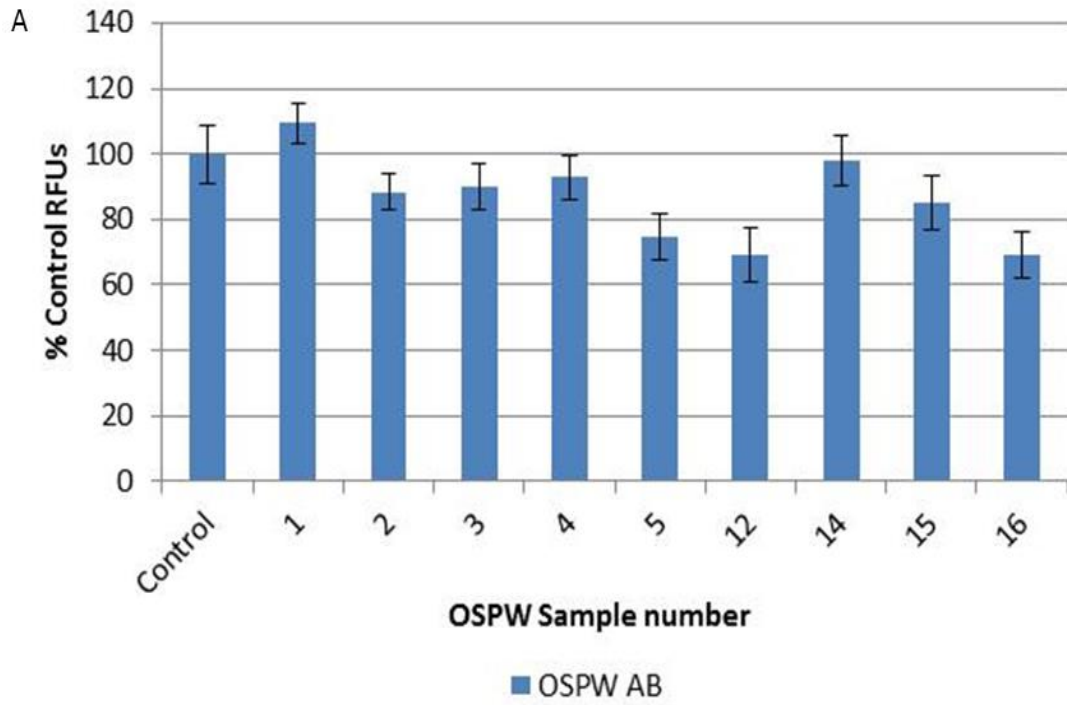
Figure 3.27: An Alamar Blue Assay comparison of OSPW samples between YPF5 and cell lines from Sansom et al., 2013. Panel A) shows the Relative Fluorescent Units (RFUs) expressed as a percentage of the control for three OSPWs Alamar Blue Assay trials using YPF5 cells. Assays were conducted in 96 well plates. Following a 24h attachment, cells were exposed to OSPW samples. After an additional 24hrs, indicator dyes were added and RFUs were read using a fluorometric plate reader. RFUs expressed as a percentage of the control (no chemical addition) are shown on the Y axis of this graph and OSPW sample numbers on the X axis. Each trial had 4-6 well replicates. **Panel B)** shows the RFUs expressed as a percentage of the control for results from Sansom et al., 2013 using six different fish cell lines exposed to OSPW samples. The numbers circled in red correspond to the samples used on YPF5.

Table 5: Naphthenic Acid content of tested OSPW sub-Samples. Naphthenic Acid content in the tested OSPW samples (data from Sansom et al., 2013 and provided by Dr. Mike MacKinnon). Samples with NA content above 10 mg/L are highlighted.

Sample #	Source	NA (mg/L)
1	FE1	1.35
2	FE2	3.01
3	FE3	2.85
4	FE4	3.56
5	FE5	11.20
12	MLSP-OP	44.22
14	MLAKE	0.30
15	BCV-B16	1.94
16	DD B2506	65.63

4. Discussion

Perca flavescens is a valuable freshwater fish widely distributed in North America (Fisheries and Oceans Canada, 2010; NRCAC, 2006; Holtan, 1990; Brown et al., 2009; Hinshaw, 2006; Manci, 2001). They are not only sought after for their high value in commercial and recreational fisheries (Manci, 2001) but also as model aquatic vertebrates in toxicology (Audet and Couture, 2003; Giguere et al., 2004; Hontela et al., 1995; Larose et al., 2008; Levesque et al., 2002; Nero et al., 2006a, Nero et al., 2006b; Peters et al., 2007; van den Heuvel et al., 1999a; van den Heuvel et al., 1999b). They are a desired model as they are relatively sedentary in nature and reflect the status of the water bodies in their habitat (Aalto and Newsome, 1990; Giguere et al., 2004; Hontela et al., 1995; Levesque et al., 2002).

The importance of yellow perch is reflected in the publications made to date utilizing these organisms as model systems (Audet and Couture, 2003; Giguere et al., 2004; Giguere et al., 2005; Hontela et al., 1995; Larose et al., 2008; Levesque et al., 2002; Nero et al., 2006; Peters et al., 2007; Pyle and Wood, 2008; van den Heuvel et al., 1999a; van den Heuvel et al., 1999b). Cell cultures derived from yellow perch have been used to assist in gaining a better understanding of physiological, virological or toxicological events. In the past, yellow perch cell cultures have included skeletal muscle cultures that contained stem-like cells (Dodson et al., 2008), isolated adrenocortical cells used to study the uptake and toxicity of cadmium (Lacroix and Hontela., 2004; Raynal et al., 2005) and cultured oocytes used for the evaluation of steroidogenic chemicals (DeManno and Goetz, 1987; Goetz and Bergman, 1978a, Goetz and Bergman, 1978b).

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To the best of our knowledge, a continuous yellow perch cell line has not been reported. A yellow perch cell line could facilitate virological, physiological, aquacultural and toxicological studies. Successful aquaculture requires knowledge about factors such as general species adaptability, their life history as well as health and disease controls (Manci, 2001). Utilization of a cell line could help scientists understand initial basic criteria for sustaining and increasing *Perca flavescens* aquaculture. In general, fish species are prone to affliction by diseases and intracellular pathogens which can be problematic for commercial fishing and in aquaculture. The study of yellow perch cell lines could provide insight as to how disease causing pathogens affect fish cells thus leading to a better understanding of how these pathogens could compromise fish health, growth and survival. As well, a cell line could have a beneficial application in toxicology by being more time efficient for testing the effects of contaminants using cell viability assays (Castano et al., 2003; Bols et al., 2005). These assays can be rapidly performed with the ability for easy exposure and inexpensive cost if cell culture equipment is available (Castano et al., 2003; Bols et al., 2005).As well, the assays would only require minimal amounts of contaminants (100 µl per well in a 96-well plate) The objective of this thesis was to develop and characterize, YPF5, a yellow perch cell line and evaluate for toxicological applications.

4.1. Characterization of YPF5

The elongated slender morphology of YPF5 suggests that these cells could be considered fibroblastic-like. However, experiments were done to further confirm the hypothesis that YPF5 is mostly of fibroblast origin. YPF5 cells were positive for immuno

expression of vimentin (Figure 3.18), which can be a marker for fibroblast cells (Challa and Stefanovic, 2011). Another cell line, GFSk-S1, a fibroblastic goldfish skin (Lee et al., 1997) was tested for vimentin expression in conjunction with YPF5 and was also determined to express vimentin (Figure 3.18). However, in contrast Lin et al., 2013 characterized a new koi fin cell line using immunocytochemistry and determined that the koi cells did not express vimentin and therefore characterized it as exhibiting epithelial morphology (Lin et al., 2013). YPF5 was also immunopositive for collagen type 1 expression (Figure 3.17). Fibroblasts are largely responsible for the production of collagen fibers and the establishment of extracellular collagen matrix in the connective tissue (Alberts et al., 2010; Chan et al., 1990). As well, collagen type I is the form of collagen that is predominantly expressed in fibroblasts found in the dermal connective tissue, especially in fish fins (Chan et al., 1990, Duran et al., 2011) which further confirm the fibroblastic origin of YPF5. Furthermore, it has been shown that under the stimulatory influence of ascorbic acid, fibroblast cells increase collagen synthesis, collagen secretion and the formation of collagen matrix in the extracellular space (Chan et al., 1990). In the cytoplasm, procollagen exists as a precursor that undergoes post-translational processes to produce collagen and eventually the fibril formation (Chan et al., 1990). Indeed, with exposure to ascorbic acid, YPF5 cells assembled procollagen into fibers (Figure 3.17). This result correlates with similar findings with human and goldfish skin fibroblasts by Chan et al. (1990) and Lee et al. (1997), respectively.

Species authentication of a cell line is an important aspect of cell line characterization. The classical approach is karyotyping and this method is used in many

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papers that characterize a newly developed cell line (Lakra et al., 2010; Swaminathan, 2010; Wharton et al., 1977). In this study, karyotyping determined that YPF5 had a modal chromosome number of 48. The karyotyping result was the same diploid chromosome number for *P. flavescens* as previously reported by Danzmann, 1979. It should be noted that other fish species possess diploid chromosome numbers of 48. Another more novel way to species-authenticate a cell line is by DNA barcoding. With this technique species-specific genetic markers such as mitochondrial cytochrome oxidase c subunit 1 are used and amplified via polymerase chain reaction (Ivanova et al., 2007; Ward et al., 2005). In the past, Kawano et al. (2010), Sansom et al. (2013) and Vo et al. (in press) have utilized the concept of DNA barcoding with fish cell lines.   By using this approach, YPF5 was confirmed (100% sequence identity) to have been derived from *P. flavescens*.

Temperatures for fish cell line proliferation are known to encompass the range of the *in vivo* fish species (Bols, 1992). Lannan (1994) stated that the temperature ranges for cold-water fish cell lines are between 4 - 24 °C but are 15 – 37 °C for cell lines of fish that inhabit warmer waters. Growth studies are commonly conducted on newly reported cell lines as an aspect of characterization to determine the optimal growth temperatures (Babu et al., 2011; Lakra et al., 2010; Lin et al., 2013; Zhao et al., 2006).

P. flavescens is known to be a cool-water fish with a temperature preference between 21 - 24 °C (NRCAC, 2006). Studies have shown that optimal temperatures for yellow perch differ depending on life stage (Heidinger and Kayes, 1993) and the age of

the fish is a factor (Ferguson, 1958; McCauley and Read, 1973). The results of the temperature study of YPF5 cells indicated that proliferation was affected by various temperatures. Temperatures of 20, 26 and 29 ° C allowed for an increase in cell proliferation but the temperatures 4, 14 and 37 ° C caused a decrease in proliferation. McCauley and Read's (1973) findings indicate that adult yellow perch selected temperatures between 17.6-20.1° C while juveniles preferred slightly warmer temperatures at 20-23.3 ° C. Ferguson (1958) used yellow perch fingerlings for his lab experiments and found that 24.2 ° C is the temperature at which they would congregate.   Reported lethal temperatures for yellow perch were 29.2 ° C for juveniles and 33° C or higher for adults (Hokanson, 1977). 26 ° C is the temperature for optimal growth of YPF5; however, the cells were maintained at either room temperature (approximately 22 °) or 18 ° C to reduce cell culture maintenance costs.

In vertebrates, alkaline phosphatase (AP) is highly expressed by embryonic stem cells (Wakamatsu et al., 1994; Sun et al., 1995), primordial stem cells (Resnick et al., 1992; Deniz Koç and Yüce, 2012), testicular stem cells (Hong et al., 2004), leukocytes (Meseguer et al., 1994) and osteogenic cells (Witten et al., 1997). While the high AP expression is a marker of stem or progenitor cells, it can also be an indicator of osteoblast differentiation (Yohay et al., 1994). The AP results indicated that GFSk-S1 was negative for alkaline phosphatase activity while ZEB2J (previously shown as AP positive by Xing et al., 2008) and YPF5 were both positive. Confluent monolayers of YPF5 had approximately 52% of the cells appearing histochemically positive for alkaline phosphatase activity. This result implies a few possible explanations. YPF5 could be

osteoblast-like cells, AP-positive mesenchymal progenitor cells or mesenchymal cells that could undergo AP-associated osteogenic differentiation.

In 1961, Hayflick and Moorhead published that human cell cultures can replicate only a finite number of times before they enter senescence (Hayflick and Moorhead, 1961; Hayflick, 1984; Shay and Wright, 2000). Senescence-associated β-galactosidase (SA-β-gal) expression indicates that cells could be undergoing senescence (Kruz et al., 2000). If cells express a blue colouration after being treated with X-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside) then senescence-associated β-galactosidase is present and cells are undergoing senescence (Debacq-Chainiaux et al., 2009). Out of the cell cultures tested for SA-β-gal expression, Eel brain culture demonstrated cells positive for senescence since approximately 88% of the culture expressed a blue colouration. YPF5 presented very minimal blue reaction, suggesting that SA-β-gal was probably not present in the culture. The lack of SA-β-gal activity further suggests that the cells have not senesced and could potentially become an immortal cell line.

Previous studies have indicated that exposure to hydrocortisone (cortisol) has caused fibroblastic cells to appear morphologically epithelial-like (Lee et al., 1986). A study by Lee et al (1986), using a fibroblastic cell line called RTG-2 (Rainbow Trout Gonadal) and cortisol concentrations ranging from 5-2000ng/mL, indicated a morphological change in cells to be more epithelial-like. When RTG-2 cells were exposed to cortisol, the morphological change occurred both in the presence and absence of serum (Lee et al., 1986). These changes became more evident with time and cells lost

their typical "whorl" shape at confluency and became more flattened (Lee et al., 1986).

YPF5 demonstrated many of the same characteristics when exposed to cortisol. When

comparing the images of the YPF5 control (no addition of cortisol) to those of YPF5 (with

10-1000ng/mL of cortisol in media) (Figure 3.5), it was evident that the control

illustrates the before mentioned whorls while the cortisol exposed cells all appeared

more flattened (and epithelial-like). This was especially obvious after 3 days. Studies

have also investigated the effect of cortisol on cell proliferation and discovered that cell

confluency has an influence (Lee et al., 1986). Cortisol appeared to have an effect on

proliferation of cells which are confluent versus under confluent in the fibroblastic RTG-

2 culture (Lee et al., 1986). However, cortisol did not have an effect on CHSE-214

proliferation, an epithelial-like Chinook salmon embryo cell line (Lee et al., 1986). When

looking at the effect on YPF5 proliferation, cortisol concentrations appeared to have an

effect in comparison to the control only on Day 3 ($p<0.05*$). However, on Day 7 there

was no significant difference between YPF5 growth with cortisol concentrations versus

no cortisol addition (Figure 3.12) .RU 38486 (RU486) is a synthetic antiglucocorticoid

and a high affinity glucocorticoid antagonist (Lee and Bols, 1989a; Lee and Bols, 1989b).

RU 486 has been shown to prevent the inhibition of proliferation usually seen by the

effects of cortisol as well as cell morphology changes (Lee and Bols, 1989a; Lee and Bols,

1989b). The effect of RU486 on cell morphology only was tested with YPF5 and it was

determined it had no morphological effect by itself (Figure 3.6). Then cortisol

concentrations with the addition of RU 486 were tested and similar findings resulted as

no epithelial-like morphological changes were present when 100ng/mL of RU 486 were

added in conjunction to control, 10 and 100ng/mL concentrations of cortisol. However, as time increased and at the high concentrations of cortisol (such as 1000ng/mL) morphological changes could be seen (Figure 3.7)

4.2 Toxicology Applications using YPF5

4. 2.1 Cell viability assays

Cell viability assays using cell lines can be used to evaluate the cytotoxicity of various environmental contaminants. Previously, RTgill-W1 (a rainbow trout gill cell line) was used to investigate the possibility of testing industrial effluent (Dayeh et al., 2002). In addition, Dayeh et al. (2005), Castano et al. (1995), Tan et al. (2008) and Babich et al. (1986) were a few of the studies involved in using a variety of cell lines for the testing of mining effluent or heavy metals. A more recent study by Sansom et al. (2013) used six different cell lines to test the toxicity of oil sands processed affected waters and naphthenic acids.

Evaluating cytotoxicity using fish cell lines is accomplished by measuring different parameters such as cell membrane integrity, metabolic impairment and lysosomal damage using three fluorometric dyes; CFDA-AM, Alamar Blue and Neutral red (Bols et al., 2005; Dayeh et al., 2013). A fluorometric plate reader is used, at specific wavelengths depending on the dyes, and a measurement of cell viability in Relative Fluorescent Units (RFUs) expressed as a percentage of the control is obtained (Bols et al., 2005; Dayeh et al., 2013). The following assays; Alamar Blue, CFDA-AM and Neutral Red assays (for NH_4Cl only) were used throughout this thesis to determine the cytotoxicity of environmental contaminants in YPF5.

4. 2.2 Environmental contaminants: Copper Sulphate, Ammonium Chloride, Crude Naphthenic Acid

YPF5 cells exposed to the environmental contaminants copper sulphate, ammonium chloride and Naphthenic Acid showed a decrease in cell viability as the contaminant concentrations increased. This effect was measured with Alamar Blue and CFDA-AM fluorometric dyes. As seen in Table 4, the average Alamar Blue and CFDA-AM EC50 values in µg/mL for each contaminant were similar, indicating the usefulness of the double viability assay (AB and CFDA in conjunction) for rapid assessment of toxicant effects.

Copper is a compound that can be found in metal-contaminated waters *Perca flavescens* are known to inhabit (Pyle and Wood, 2008). Studies have been conducted using both yellow perch and rainbow trout to compare the copper tolerance of each fish (Taylor et al., 2003; Pyle and Wood, 2008). These studies looked at endpoints such as Lethal Concentrations 96-h/48-h LC50s or time-to-death ET50 (Taylor et al., 2003; Pyle and Wood, 2008). It was found that yellow perch is significantly more tolerant to copper in water in comparison to rainbow trout (Taylor et al., 2003; Pyle and Wood, 2008). A study by Taylor et al. (2003) indicated that rainbow trout and yellow perch have similar mechanism for copper toxicity, however, results of a 96-h LC50 test in hard water reported mortality for rainbow trout at 1.05µmol/L and yellow perch mortality occurred at 4.16µmol/L. Similar patterns were found while using soft water and a 48-h LC50 value of 0.14 µmol/L for rainbow trout and 0.53 µmol/L for yellow perch were reported (Taylor et al., 2003). A study by Sansom et al. (2013) compared the 24-h EC50 values for copper sulphate of six different fish cell lines (rainbow trout liver, rainbow trout gill,

fathead minnow testis, fathead minnow liver, Goldfish Skin and Walleye Fin). These results ranged from 3.5-8.3 μg/mL for copper sulphate while the reported 24-h EC50 value for YPF5 is between 2.18-2.25 μg/mL. This suggests that the YPF5 cell line is more sensitive to copper than the previously reported cell lines, including cell lines derived from rainbow trout. When comparing the 24-h EC50 value of YPF5 (2.18-2.25 μg/mL) with the 96-h LC50 value of yellow perch (4.16μmol/L), the time interval should be noted. Preliminary experiments using 48, 72 and 96 hr AB and CFDA-AM assays were conducted. The EC50 for 72 hr exposure is 0.9018 μg/mL for AB, 0.888 for CFDA-AM μg/mL and the 96 hr is 0.5081 μg/mL and 0.4706 μg/mL. As the time interval increased, the EC50 of copper sulphate on YPF5 decreased.

The hot water extraction process used for extracting bitumen may lead to a concentrated level of Naphthenic Acids, the toxic component in oil sands process affected waters (Allen, 2008a; Herman et al., 1994; Holowenko et al., 2002; Nero et al, 2006b; Rogers et al., 2002a; Rogers et al., 2002b; Young et al., 2007). Typical NA concentrations in the oil sands industry are between 80-100mg/L (Allen, 2008a; Grant et al., 2008; Holowenko et al., 2002; Rogers et al., 2002b) and in comparison, local rivers usually have less than 1 mg/L concentration of NA (Grant et al., 2008). Nero et al (2006b) conducted a study using yellow perch to determine Naphthenic Acid effects at the organismal level. Two types of Naphthenic Acid mixtures were used, a commercial type and a type extracted from the oil sands process-affected water (Nero et al., 2006b). Results from this study state that in less than 96hr after being exposed to 6.8mg/L of extracted Naphthenic Acid and 3.6mg/L of commercial NA, there was 100% mortality in

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yellow perch (Nero et al., 2006b). Fathead minnow have a 96-h LC50 value of 5.6mg/L as reported in the American Petroleum Institute Petroleum HPV testing group (2012). Sansom et al. (2013) utilized fish cell lines and reported a 24-h Crude Naphthenic Acid EC50 range between 45.26 -146.3µg/mL for Alamar Blue values and between 158.8-682.7µg/mL for CFDA-AM. These ranges were considerably higher than the YPF5 24h EC50 values which were 34.90 µg/mL Alamar Blue and 40.27 µg/mL for CFDA-AM (Table 4).

Research is ongoing into finding ways to mitigate the toxicity of Naphthenic Acids since reclamation is an important issue being studied by oil sands producers (Allen, 2008a). A potential remediation strategy involving the use of adsorbents (activated carbon, clays, and zeolites) has been investigated (Allen, 2008b). Activated carbon treatment has been used with limited success as this treatment does not produce consistent results and it was effective at removing naphthenic acids from processed waters but no other toxic pollutants such as benzene, toluene etc. (Allen, 2008b). Other industries have used adsorbents for remediation. Clay has gained some success in remediating metal contaminants by acting as an adsorbent (García-Sánchezet al., 1999; Quintelas et al., 2011). A common clay called bentonite had previously been used in the oil industry to absorb oil and grease as well as animal waste (WHO, 2005). A modification of bentonite has been shown to decrease the toxicity of organic compounds such as benzene, toluene etc. through binding (Allen, 2008b). Evaluation at the cellular level could provide preliminary data at a fraction of the cost needed for whole animal testing. Bentonite was added to the Crude Naphthenic Acid

concentrations to determine if there was a remediation effect when exposed to YPF5 cells. When comparing the average EC_{50} values of Crude Naphthenic Acid alone versus Crude Naphthenic Acid with bentonite, there was a statistically significant difference ($p<0.05*, p < 0.01**$) (Figure 3.23). This indicated that bentonite did provide a remediating effect. Appendix D Figure 6.5 showed that in general, the most statistically significant bentonite remediating effect was found at CrNA concentrations of 25 - 100μg/mL.

YPF5 was also used to test the toxicity of another environmental contaminant, ammonium chloride. This contaminant was the only one that appeared to require high concentrations for a toxic effect. Appendix D Figure 6.3 indicates that even at 1000μg/mL NH_4Cl did not decrease cell viability as dramatically as the other contaminants (making the calculated EC50 more variable). However, concentrations from 250-1000μg/mL were considered significantly different from the control. The average 24-h EC50 value calculated using YPF5 was 793.77μg/mL for Alamar Blue and 718μg/mL for CFDA-AM (Table 4). According to Espey (2003, dissertation), yellow perch 96-h LC50 value varies between 0.66-1.02mg/L for ammonia, depending on the temperature the fish were kept at.

4. 2.3 Oil Sands Processed Water Samples

The OSPW samples used in this thesis were # 1, 2, 3, 4, 5, 12, 14, 15 and 16 from Sansom et al (2013), originally obtained from Syncrude Canada, Ltd. As these samples were used in previous work the quantities available for each sample varied. These 9 samples were chosen based on their cell viability results (toxic or non-toxic) in Sansom

et al. (2013). The cell viability results of YPF5 exposed to the OSPW samples show a somewhat similar pattern to Sansom et al. (2013). However, the toxic samples #12 and #16 did not appear to have as much of an effect on YPF5 as compared with other cell lines (Figure 3.27) (Sansom et al., 2013). As well, the average RFUs from these two samples (#12 and 16) were not statistically significant from the control RFUs. As mentioned earlier, NA is believed to be the cause of toxicity in OSPWs and Table 5 presents the NA composition in the 9 different OSPW samples (Sansom et al., 2013 data obtained from Dr. Mike MacKinnon) used.   Samples #12 and 16 possessed the highest concentration of NA (44.22 and 65.63μg/mL respectively) and should hypothetically be the more toxic samples tested, causing a decrease in the Average Relative Fluorescent Units for cell viability. For YPF5, this was true; however, as mentioned earlier, YPF5 appeared less sensitive to sample #12 and #16 in comparison to other cell lines from Sansom et al. (2013). In general, for the 9 OSPW samples used with YPF5, the correlation coefficient^2 (R^2) for cell viability and NA concentration was only 0.1958 for Alamar Blue and 0.3020 for CFDA-AM. The most likely explanation for this relationship is that the Naphthenic Acid content in the OSPW samples had become degraded with age. The biodegradation of Naphthenic Acids has been investigated in the past (Clemente and Fedorak, 2005).

Remediation methods were tested *in vitro* using these OSPW samples to determine if either the addition of absorbent material (bentonite) or photodegradation with UV provided any ameliorating effects in terms of cell viability. Figure 3.26 show the results for Alamar Blue and CFDA-AM respectively when YPF5 cells were exposed to

OSPW samples alone and with the addition of bentonite or with UV radiation. From these graphs, there is indication that in some samples there were differences when using remediation techniques (in comparison to OSPW samples alone). However, due to the variation in the 3 trials the results were not significantly different.

5.1 Conclusions and Future Directions

In summary, YPF5 appears to be the first continuous yellow perch cell line and was derived from the caudal fin of an adult yellow perch. These cells have been authenticated as being derived from *Perca flavescens* through DNA barcoding, and karyotyping results indicated a diploid chromosome number of 48 which was reported by Danzmann (1979) (yellow perch are one of the fish species with 2n=48). YPF5 has been maintained and grown for over two years, passaged 67 times (to date) and possesses fibroblastic properties (morphology, vimentin expression, collagen secretion). These cells have been successfully cryopreserved for future use and they continue to grow after thawing. These cells have responded to environmental contaminants, hormones and vitamins in a dose dependent manner and had comparable results to literature data when exposed to cortisol and ascorbic acid but were less sensitive than copper sulphate and ammonium chloride data. These cells also provided a similar response to naphthenic acids and OSPW samples as other established fish cell lines. The evaluation of two remediation strategies i*n vitro*, bentonite adsorption and UV exposure, show promise but should be studied further.

There is still more work that should go into characterizing this novel cell line and future experiments could focus on the ability of the cells to produce and secrete collagen. Fibroproliferative disorders have been noted *in vivo* with toxicant exposures and it would be of interest to study if the addition of toxicants have any effect in collagen secretion *in vitro*. Additionally, since cortisol is well known to modulate collagen secretion, this could be investigated with YPF5. Further investigations using ascorbic acid could be done by evaluating if non-lethal concentrations of naphthenic acid, OSPW, copper and cortisol prevent collagen matrix from forming. YPF5 could also be used for further studies evaluating the effects of OSPWs and Naphthenic Acids. In addition, as yellow perch are sedentary and may live in metal contaminated waters, the YPF5 cell line could be used in evaluating such environments. In North American waterways (including Lake Ontario), there have been reports of a microsporidian parasite species, Heterosporis, found in the muscle of yellow perch (Sutherland, 2002; GLFC, 2010). Despite Heterosporis' inability to infect humans, this parasite affects the texture and quality of the fish fillets which could eventually make yellow perch unappealing for sports and commercial fishing and if fish readily become infected, this could potential decrease the desire for yellow perch aquaculture (Sutherland, 2002; GLFC, 2010). Viruses such as viral hemorrhagic septicemia (VHSV) have been reported to infect yellow perch (Kane-Sutton et al., 2010). The YPF5 cell line could be useful as a cost effective tool to help increase the productivity and quality of yellow perch aquaculture and in the investigation of viruses and or pathogens that are a concern in the whole organism.

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Appendix

Appendix A: Protocol for Primary Culturing and the Solutions

50mL of 10X DPBS Solution

DPBS = Dulbeccos' Phosphate Buffered Saline Solution

P/S = Penicillin Streptomycin

Dissect fish and cut organs into pieces

Add 500uL or more of antibiotics

1L of L-15 Media with % FBS and 1% P/S

450mL of Distilled Water

Rinse organ pieces with 1X DPBS 3-4 times

Aliquot 50mL of L-15 Media test tubes

500mL of 1X DPBs

Aliquot 1X DPBS into 50mL test tubes

Place washed organ tissue into individual flasks

Add 1 mL of media to each individual flask to allow attachment

- Change media daily for approximately 1 week
- Once cells attached use ~2-3 mL media
- Observe for growth

1.5mL P/S

1 X DPBS with 3% P/S

Figure 6.1: Schematic representation of method used for primary culturing of Yellow Perch #1-5. Two solutions, DPBS and L-15 media with additional antibiotics were required to be made before the actual processing of fish.

DPBS = Dulbeccos' Phosphate Buffered Saline Solution

P/S = Penicillin Streptomycin

50mL of 10X DPBS Solution

450mL of Autoclaved MilliQ water

500mL of 1X DPBs

Aliquot 1X DPBS into 50mL test tubes

1.5mL P/S

1 X DPBS with 0.3% P/S

Dissect fish and cut organs into pieces

Rinse organ pieces with 1X DPBS 3-4 times

Place washed organ tissue into individual flasks

Add 500uL or more of antibiotics

1L of L-15 Media with % FBS and 1% P/S

Aliquot 50mL of L-15 Media test tubes

Add 1 mL of media to each individual flask to allow attachment

- Change media daily for approximately 1 week
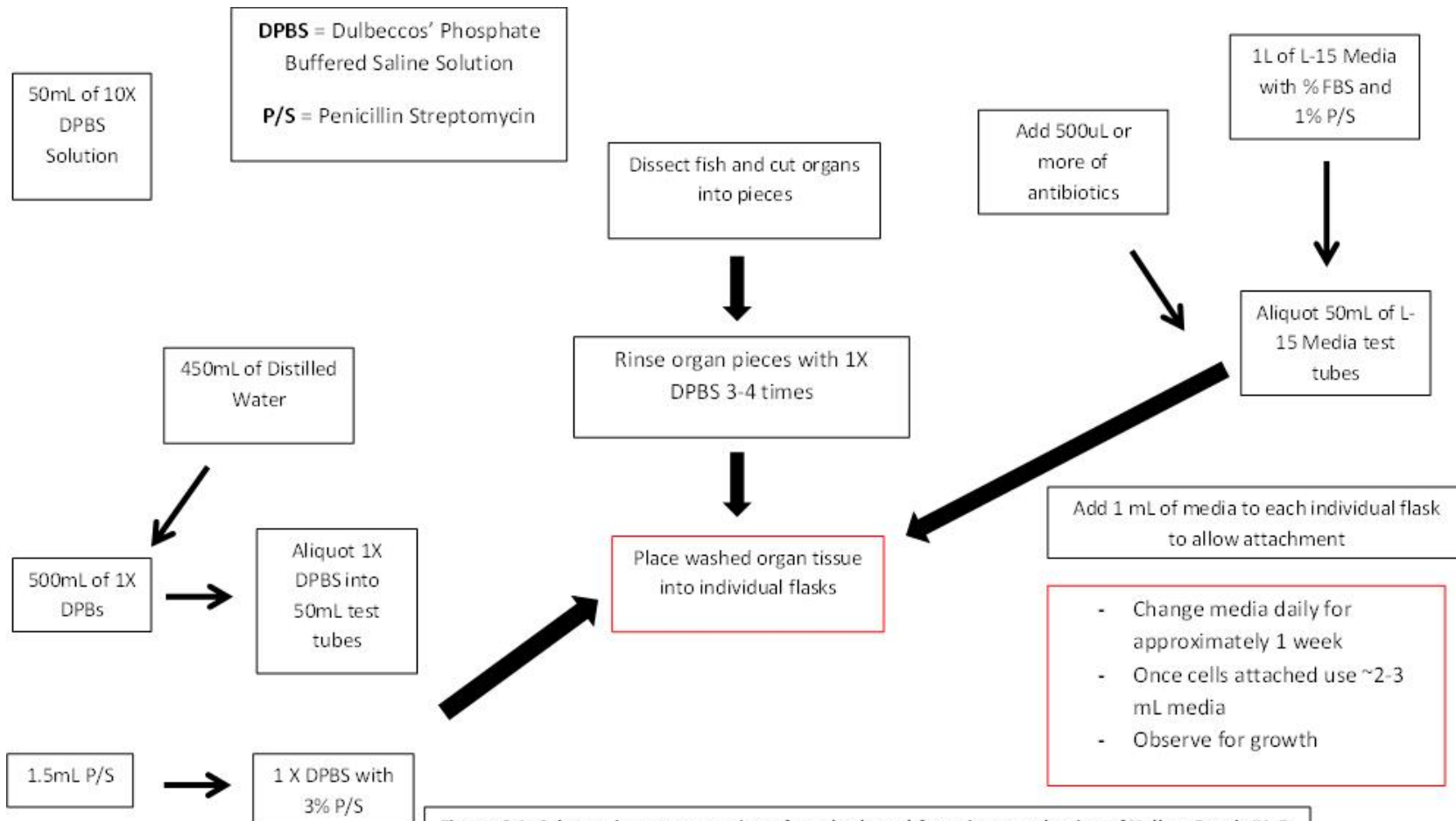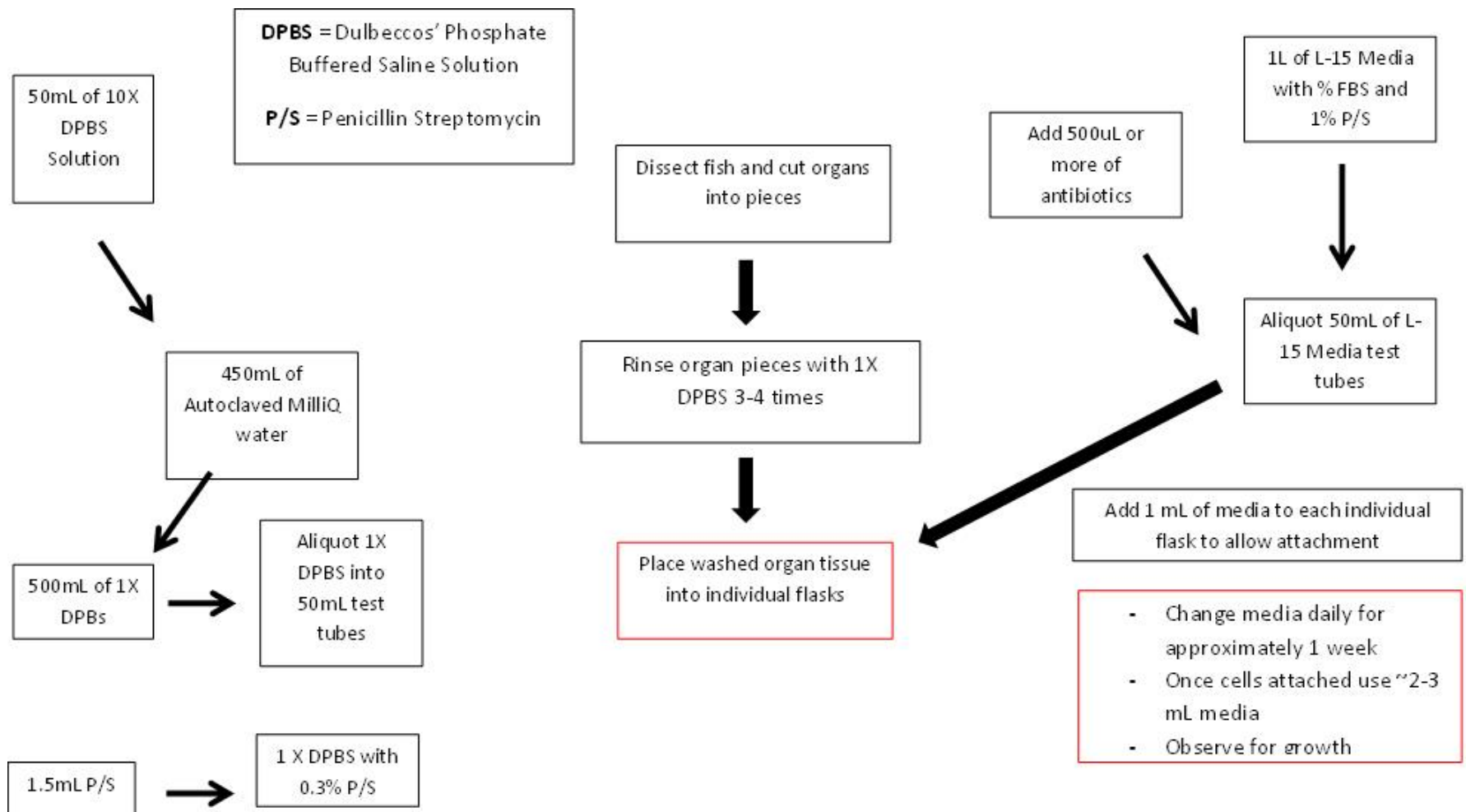- Once cells attached use ~2-3 mL media
- Observe for growth

Figure 6.2: Schematic representation of method used for primary culturing of Yellow Perch #6-8. Two solutions, DPBS and L-15 media with additional antibiotics were required to be made before the actual processing of fish. Please note that the DPBS solution contained only 0.3% P/S. The correct concentration should be 3% as illustrated in Figure 6.1

Appendix B: Protocol for L-15ex and 5X L-15ex

L15ex
Add the following solutions to 1000mL cell culture grade distilled autoclaved water.
Add aseptically

- 68mL solution A
- 11.4mL solution B
- 34mL solution C
- 11.4mL sodium pyruvate
- 11.4mL galactose

 Store at room temperature

For 5X L15ex
Add the following solutions to 200mL cell culture grade distilled autoclaved water.
Add aseptically

- 68mL solution A
- 11.4mL solution B
- 34mL solution C
- 11.4mL sodium pyruvate
- 11.4mL galactose

Solution A: Add the following to 600mL MilliQ Water
80g NaCl   BDH 0286
4g KCl   BDH 108343
2g $MgSO_4$ Caledon 4860-1
2g $MgCl_2$ BDH 108349

- Autoclave
- Store at room temp

Solution B: Add the following to 100mL MilliQ Water
1.4g $CaCl_2$ BDH 107204

- Autoclave
- Store at room temp

Solution C: Add the following to 300mL of MilliQ water
1.9g $Na2HPO_4$ Sigma S-5136
0.6g KH_2PO_4 BDH 108275

- Autoclave
- Store at room temp

Sodium pyruvate: Add the following to 300mL of MilliQ water
5.5g Sodium Pyruvate   Alfa Aesar B14T013

- Filer sterilize (0.2um)
- Dispense in 12mL amount
- Store at -20C

Galactose: Add the following to 100mL of MilliQ Water
9g galactose   Sigma G5388

- Filter sterilize (0.2um)
- Dispense in 12mL amount
- Store at -20C

Appendix C: Cytotoxicity Assay Protocols (Alamar Blue, CFDA-AM, Neutral Red)

The cytotoxicity assay protocols for Alamar Blue, CFDA-AM and Neutral red were adapted from Dayeh et al., 2013.

Alamar Blue Protocol

1. Turn on the laminar flow hood and thoroughly clean all surfaces with 70% ethanol

2. *Prepare the 5% Alamar Blue working solution in L-15 ex by adding 525.5μL of Alamar Blue (Invitrogen DAL 1100) from the refrigerator to 10mL of L-15ex solution. Please note this should be done aseptically in the flow hood and in the dark to prevent photo degradation of the dye.*

3. Remove the exposure concentrations from the 96-well plate by inverting it on a paper towel.

4. Rinse each well with 200μL of L-15ex and remove this washing solution onto another paper towel.

5. Add 100μL of the Alamar Blue Solution to each well of the 96 –well plate using a repeater pipette.

6. Incubate plate(s) in the dark for 1hour at room temperature.

7. After the incubation time the plates can be read on the fluorescent SpectraMAX reader. The plate reader should be set at excitation and emission wavelengths of AB 530 and 590nm respectively.

CFDA-AM Protocol

1. Turn on the laminar flow hood and thoroughly clean all surfaces with 70% ethanol

2. A 4mM stock solution of CFDA-AM should be prepared by dissolving in sterile DMSO and aliquoted and kept in the dark and in the freezer to avoid photo degradation.

3. Prepare the CFDA-AM working solution in L-15 ex by adding 10.4µL of CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester) (Sigma) from the freezer to 10mL of L-15ex solution. *Please note that this should be done aseptically in the flow hood and in the dark to prevent photo degradation of the dye*.

4. Remove the exposure concentrations from the 96-well plate by inverting it on a paper towel.

5. Rinse each well with 200µL of L-15ex and remove this washing solution onto another paper towel.

6. Add 100µL of the CFDA-AM Solution to each well of the 96 –well plate using a repeater pipette.

7. Incubate plate(s) in the dark for 1hour at room temperature.

8. After the incubation time the plates can be read on the fluorescent SpectraMAX reader. The plate reader should be set at excitation and emission wavelengths of CFDA-AM 485 and 530nm respectively.

Neutral Red Protocol

1. Turn on the laminar flow hood and thoroughly clean all surfaces with 70% ethanol

2. Prepare Neutral Red working solution in L-15 ex by diluting the neutral red stock solution (33μg/mL of Sigma N2889 Neutral Red) 1:100.

3. Remove the exposure concentrations from the 96-well plate by inverting it on a paper towel.

4. Rinse each well with 200μL of L-15ex and remove this washing solution onto another paper towel.

5. Add 100μL of the Neutral Red Working Solution to each well of the 96 –well plate using a repeater pipette.

6. Incubate plate(s) in the dark for 1hour at room temperature.

7. Remove the working solution by inverting the plate onto paper towels and rinse each well with 100 μL of Neutral Red Fixative Solution (0.5% (v/v) formaldehyde and 1% (w/v) $CaCl_2$ in MilliQ water. Store this solution in the dark).

8. Add 100 μL of Neutral Red Extraction Solution (1% (v/v) acetic acid and 50% (v/v) ethanol in MilliQ water. Store solution in dark) and let sit for approximately 10 minutes.

9. Then measure the fluorescence using the SpectraMAX reader. The plate reader should be set at excitation and emission wavelengths of NR as 530 and 645nm respectively.
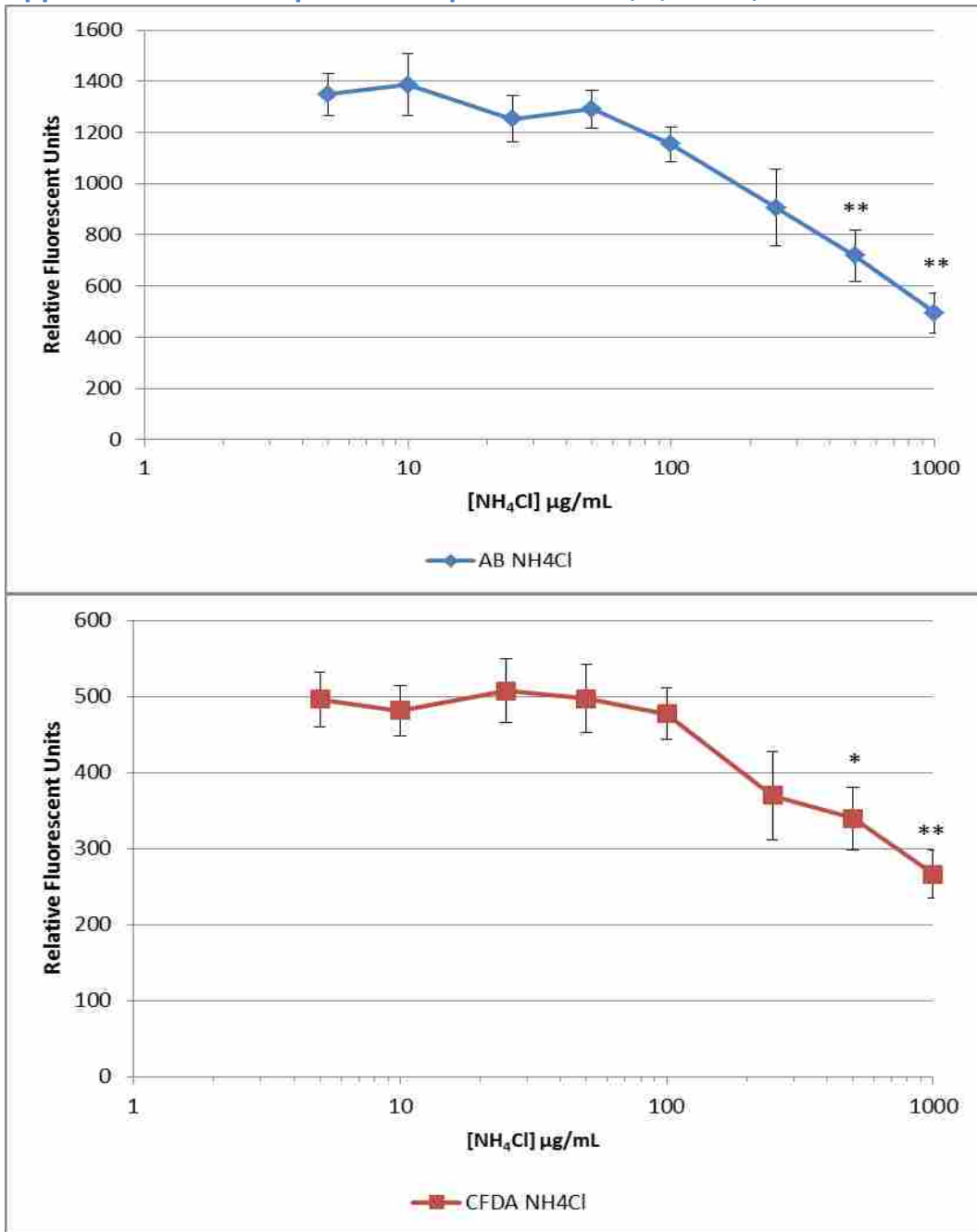
Appendix D: YPF5 Response to exposure to NH$_4$Cl, CuSO$_4$ and CrNA

Figure 6.3: Effects of NH$_4$Cl on YPF5 viability. Representative dose response curve for YPF5 exposed to NH$_4$Cl at the indicated concentrations for 24h. Cell viability assays **A)** Alamar Blue and **B)** CFDA-AM were conducted in 96 well plates. Following a 24h attachment cells were exposed with increasing concentrations of NH$_4$Cl. After an additional 24hrs, indicator dyes were added and RFUs were read using a fluorometric plate reader. Relative Fluorescent Units (RFUs) are shown on the Y axis of this graph and concentrations of NH$_4$Cl. in µg/mL on the X axis. Error bars are standard deviations. n= 4-6 well replicates. Asterisks indicate significant differences of mean RFU values compared to control (p<0.05 *, p<0.01 **)
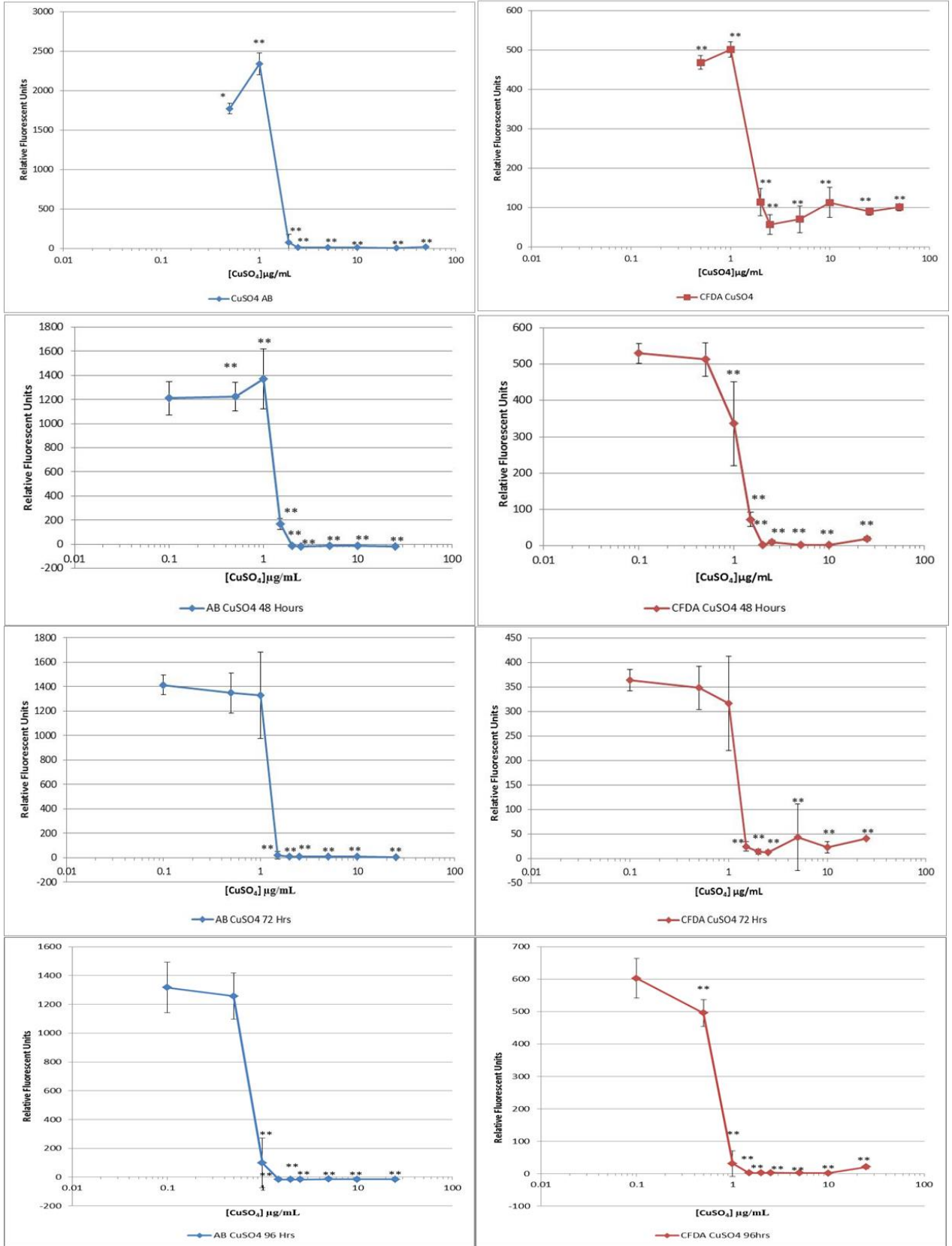
Figure 6.4: Effects of CuSO$_4$ on YPF5 viability measured by 24h, 48h, 72h and 96 h Alamar Blue and CFDA assays. Representative dose response curves for YPF5 exposed to CuSO4 at indicated concentrations for 24h, 48h, 72h and 96h. Cell viability assays (panels A, C, E, G) Alamar Blue and (panels B, D, F, H) CFDA-AM were conducted in 96 well plates. Following a 24h attachment cells were exposed with increasing concentrations of CuSO$_4$. After an additional 24hrs **(panels A, B)**, 48hrs **(panels C, D)**, 72hrs **(panels E, F)** or 96hrs **(panels G, H),** AB and CFDA viability assays were performed and Relative Fluorescent Units (RFUs) were read using a fluorometric plate reader. Plotted values are Average RFUs and standard deviations are indicated as error bars. Nominal concentrations of CuSO$_4$ in µg/mL were tested at n= 4-6 well replicates. Asterisks indicate significant differences of mean RFU values compared to control ($p < 0.05$ *, $p < 0.01$ **)
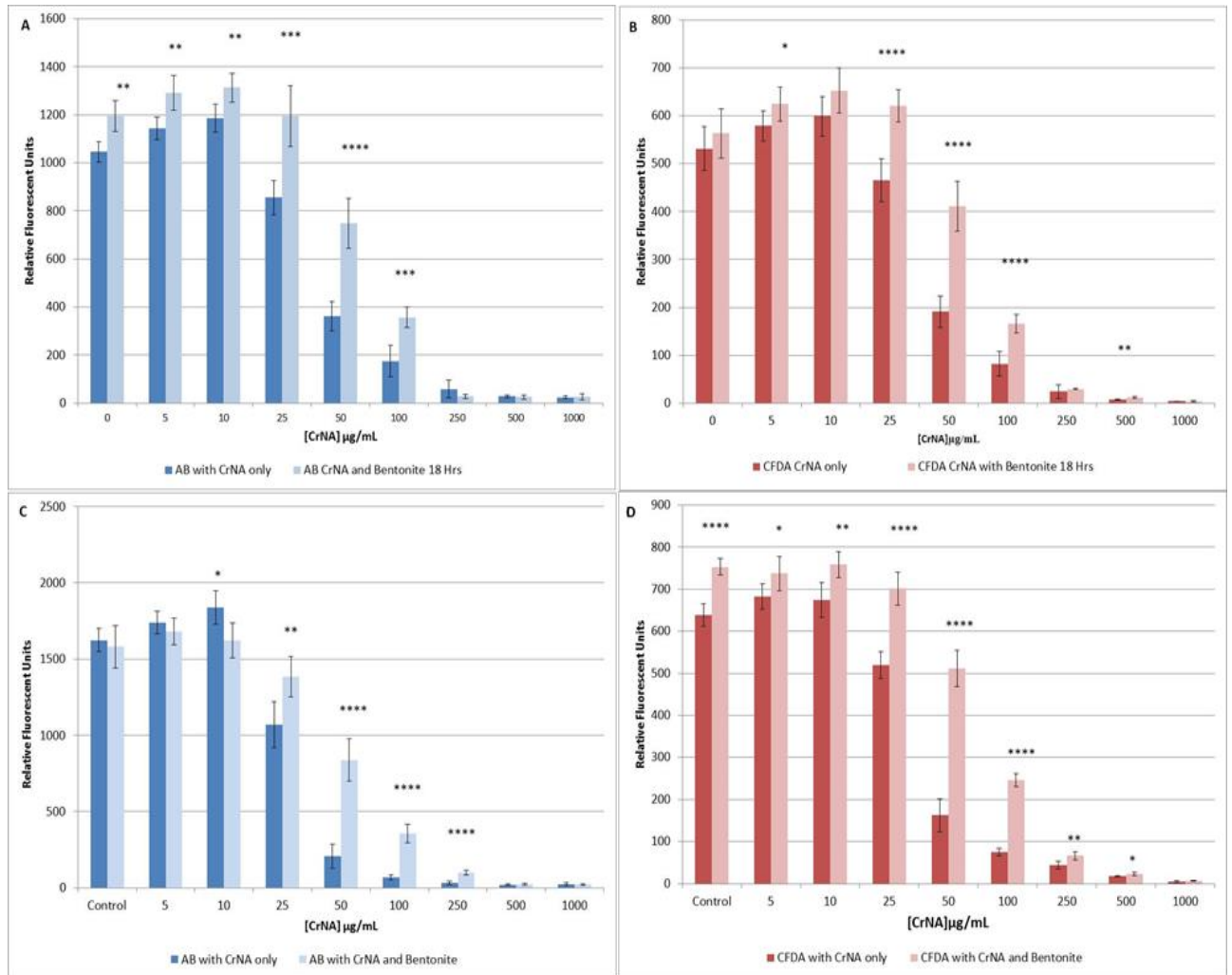
Figure 6.5: Comparison of CrNA with and without bentonite pretreatment (18hr and 48hr). YPF5 cells were exposed to CrNA with and without bentonite pre-treatment at the indicated concentrations for 24h. Cell viability assays **(panels A,C)** Alamar Blue and **(panels B, D)** CFDA-AM were conducted in 96 well plates. Following a 24h attachment cells were exposed with increasing concentrations of CrNA with or without bentonite pre-treatment. Bentonite was added to the CrNA concentrations **(A-B)** 18hrs or **(C-D)** 48hrs prior to exposure. After an additional 24hrs, indicator dyes were added and RFUs were read using a fluorometric plate reader. Average Relative Fluorescent Units (RFUs) are shown on the Y axis of this graph and concentrations of CrNA in µg/mL on the X axis. Error bars indicate standard deviation. n= 4-6 well replicates. Asterisks indicate significant differences between CrNA only and CrNA with the addition of bentonite (p<0.05 *, p<0.01 **, p<0.001***, p<0.0001****)
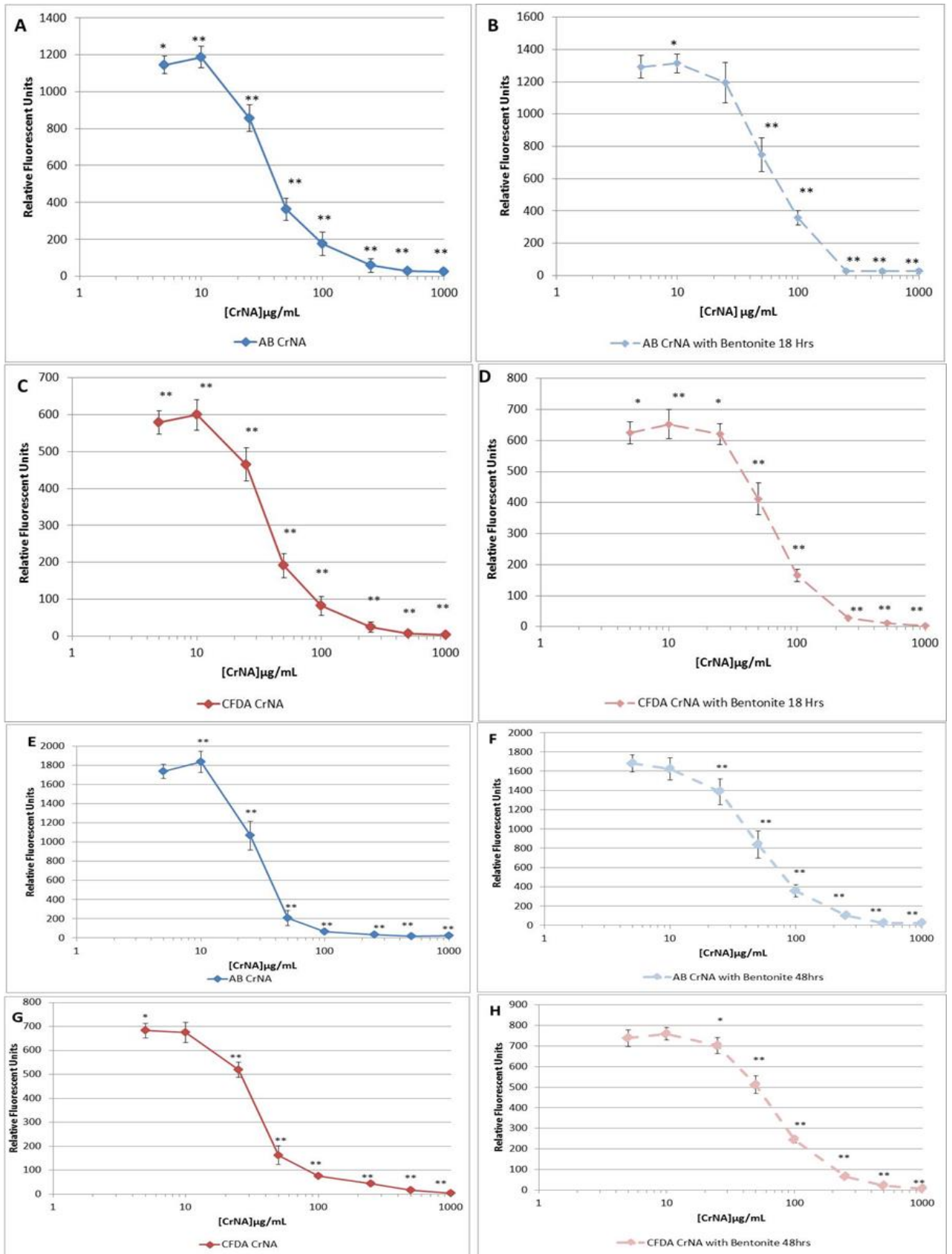
Figure 6.6: Effects of CrNA with and without Bentonite pretreatment on YPF5 viability.YPF5 were exposed to **(panels A, C, E, G)** CrNA with no bentonite and **(panels B, D, F, H)** CrNA with bentonite at the indicated concentrations for 24h. Cell viability assays **(panels A, B, E, F)** Alamar Blue and **(panels C, D, G, H)** CFDA-AM were conducted in 96 well plates. Following a 24h attachment cells were exposed with increasing concentrations of CrNA with or without bentonite. Bentonite was added to the CrNA concentrations **(panels B, D)** 18hrs or **(panels F, H)** 48hrs prior to exposure. After an additional 24hrs, indicator dyes were added and RFUs were read using a fluorometric plate reader. Average Relative Fluorescent Units (RFUs) are shown on the Y axis of this graph and concentrations of NH_4Cl. in µg/mL on the X axis. n= 4-6 well replicates. Asterisks indicate significant differences of mean RFU values compared to control (p<0.05 *, p<0.01 **)

Appendix E: Coulter Counting Protocol

1) Remove media from well with micropipette

2) Wash well with 1mL of media and remove

3) Add approximately 500 µl of TE to the well. Wait until cells detach (may need to put plate on a shaker to help facilitate detachment)

4) Add 500 µl of media to the well while pipetting up and down to ensure cells are detached

5) Obtain a cuvette and fill with 9mL of isotonic IsoFlow Sheath Fluid solution (Coulter)

6) Remove the 1mL of cell solution with a micropipette and add to the cuvette. It is necessary to ensure that cuvettes are labelled properly so samples aren't mixed up!

7) Insert cuvette into the aperture of the Coluter Counter.

8) Set the upper and lower limit for particle size count to be 10-35µm for YPF5

9) For each well repeat Steps 1-8 and use a new cuvette each time.