Utilization of Alternative Testing Methods in the Evaluation of Pulp and Paper Mill Effluent Toxicity

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3 Acknowledgements

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4 Abstract

Pulp mills are required to perform routine regulatory testing to ensure the effluent they release from their water treatment plants is not harmful to humans and the environment. The key components within the effluent that cause toxicity include factors such as total suspended solids, chemical oxygen demand, concentration of resin and fatty acids and the colour of the effluent. In December 2013 a pulp mill in Terrace Bay, ON experienced three failures when their effluent caused 90 to 100% mortality to tested rainbow trout. In this study, the objective was to determine the potential cause of this failure. Prior to this failure, mechanical issues resulted in an increase in pulp fibre with in the mills secondary treatment system. Since mortality was observed in trout tests and not *Daphnia* tests, the increase in fibre was suggested as a likely cause. Acute toxicity screenings were conducted using effluent that had been spiked with sludge obtained from the primary clarifier, mimicking concentrations observed during the failure. There was 10% mortality in one of the untreated samples and no mortality occurred in any of the spiked samples. The lack of fibre induced mortality combined with increased levels of sulphur suggested volatile sulphur compounds as a potential cause for the failure. Given their volatile nature, current test methods are not suitable for testing these compounds especially at low concentrations. Another objective of this study was to determine if the toxicity of effluent could be measured using a crystal violet staining (CVS) assay applied to piscine cell lines as an alternative to *in vivo* methods. Cell lines derived from rainbow trout liver (RTL-W1) and gills (RTgill-W1), as well as fat head minnow liver (FHM-L) were given acute exposures to NaCl and N-NO_3, and chronic exposures to NaCl. Calculated LC_{50} and IC_{25} values were compared to literature values and their associated ±3 standard deviation control limits. All three cell lines had their calculated acute NaCl LC_{50} values fall within the ±3 SD limits of the literature LC_{50} of 8.92g/L, signifying the potential for these cell lines to be used as an alternative to *in vivo* studies. The FHM-L and RTL-W1 LC_{50}s for N-NO_3 were both outside of the ±3 SD warning limits and thus the cell lines are not a suitable alternative for *in vivo* studies of N-NO_3. The chronic tests for RTL-W1

and FHM-L produced IC_{25} values for NaCl within the ±3 SD warning limits of the literature value. The RTgill-W1 IC_{25} value however was outside the warning limits and thus is not suitable for use as an *in vitro* alternative. The CVS assay is a promising alternative to *in vivo* test results, however given that only 70% of the assays conducted were capable of having an LC_{50} or IC_{25} value derived from them a single assay is currently not powerful enough to accurately represent the *in vivo* results. Replicate assays are required to ensure accuracy of results when conducting *in vivo* comparisons.

5 Literature Review

5.1 Pulp and Paper Mill Effluents

Pulp and paper mill effluent contains a wide array of chemicals, which is usually a mixture of inorganic salts and organic material, the majority of which is of the high molecular weight variety (Hardy, 2002). There have been over 500 different organic compounds identified in pulp mill effluents (Hardy, 2002), and it has best been described as a "Pandora's Box" of waste chemicals (Cheng, 2010).

5.1.1 Components of Wood

The main components of wood are cellulose, hemicellulose, lignin, and extractives; and the relative amount of each component is dependent on the species of tree (Table 1), as well as the age of the tree from which it comes (Lafleur, 1996).

Table 1. Typical composition of North American wood species (%) (Lafleur, 1996)

Component	Percent Composition	
	Hardwoods	**Softwoods**
Cellulose	42 – 47%	51 – 55%
Hemicellulose	20 – 30%	15 – 20%
Lignin	16 – 25%	23 – 33%
Extractives	0.2 – 3.5%	0.5 – 7%

On a molecular level, cellulose is a linear polymer that is connected by 1,4'-$_{\beta}$-linkages (Bierman, 1996; Lafleur, 1996). The number of units that make up each polymer is >10,000 in unaltered wood, however it can be <1000 in highly bleached kraft pulp (Biermann, 1996). Cellulose is a solid white

material that exists as either a crystalline or an amorphous state. Typically cellulose is about 50-70% crystalline in wood, and forms the structure of wood fibre (Biermann, 1996).

Hemicelluloses are solid white material that are fibrous in nature and are rarely crystalline (Biermann, 1996). They typically form the "flesh" that fills out the fibre and are used to strengthen paper and pulp (Biermann, 1996). Hemicelluloses are a class of polymer sugars, which are typically monosaccharides (Biermann, 1996). They are also much more susceptible to chemical degradation than cellulose (Biermann, 1996). Hemicelluloses are soluble in 18.5% NaOH, as well as dilute alkali at high temperatures (e.g. kraft cooking) (Biermann, 1996).

Lignin is a complex polymer that consists of three phenylpropane units and has an amorphous three dimensional structure (Biermann, 1996). Lignin is the adhesive that holds wood fibres together and during chemical pulping its removal allows the fibres to be separated (Biermann, 1996). There are two main lignin monomers that are found in wood, hardwoods contain both coniferyl alcohol (50-75%) and sinapyl alcohol (25-50%), while softwoods containing only coniferyl alcohol (Biermann, 1996).

Extractives are diverse in nature; however they are all soluble inorganic solvents or water (Biermann, 1996). Extractives include terpenes, phenolic compounds and fatty acids. These extractives can be present in both wood and bark (Biermann, 1996).

5.1.2 The Pulp Making Process

Wood pulp is a mass of wood fibres that is often dispersed in water (Hardy, 2002). These fibres are created through the fiberization of wood, which can be achieved through mechanical, chemi-mechanical, semi-mechanical or chemical pulping (Biermann, 1996). The goal of the pulping process is to liberate fibres that can be dispersed in water through either a chemical of physical process (Hardy, 2002). There are four main processes of pulping: stone groundwood pulping, thermomechanical pulping, repulping wastepaper, and the kraft process of pulping (Hardy, 2002).

Stone groundwood pulping (SGW) involves grinding logs against an abrasive surface (Biermann, 1996). The fibres are released from the wood and washed away with water. The pulp slurry then passes through a screen before being used to make paper (Biermann, 1996).

Thermomechanical pulping (TMP) involves the steaming of wood chips under pressure before and during the fiberization process, using disk refining. The fibres produced during this process have a higher tear strength and freeness, as well as pulp that is brighter than SGW pulp (Biermann, 1996).

Repulping wastepaper involves breaking up wastepaper and separating ink and coating particles from the fibre through the addition of water, chemicals, and mechanical action. The ink and coating particles are separated using a number of different screening, floatation, and cleaning steps which leave the pulp suitable for reuse in papermaking (Biermann, 1996).

The kraft process involves the cooking of wood chips in liquor composed of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) (Biermann, 1996). The alkaline conditions breakdown the lignins and the sodium salts that are created are soluble in the cooking liquor (Biermann, 1996). The pulp then passes through a screen and the brown stock washers which removes any residual liquor. Liquor can then be concentrated and incinerated to leave a residue that is dissolved and then recausticized for reuse (Biermann, 1996). This process results in strong darkly coloured fibre which is then bleached.

5.1.3 Composition of Effluent

Pulp and paper effluent can have a very diverse composition, some of which are toxic to aquatic life and some of which are harmless. Unbleached mill effluents have been found to contain resin acids, soaps, fatty acids, diterpene alcohols and phytosterols. Chlorine bleached effluent also contains chlorinated phenols, chlorinated acids, alcohols, aldehydes, keytones, sugars and aliphatic and aromatic hydrocarbons (Government of Canada, 1991). Toxicity of effluent can vary depending on whether hardwood or softwoods are being used to produce the paper. It has been found that softwood effluent

is more toxic than effluent produced from hardwoods due to the higher production of phenolic by-products (Government of Canada, 1991). Production method also influences the toxicity of the effluent.

Effluents produced from bleach plants often have the largest source of organic pollutants found in kraft mills. Effluents from softwood bleaching have a much higher colour and chemical oxygen demand (COD) loading than those from hardwood bleaching (Cabrera, 2017). During the bleaching process chromophores are removed to yield brighter pulp (Cabrera, 2017).

There are several methods to reduce pollution in kraft mills and these include extensive use of water recirculation, construction of systems for recovery of accidental spills, reducing the kappa number prior to bleaching and better washing, oxygen delignification, as well as extended delignification (Hardy, 2002).

During production the paper machine generates a large amount of water, which has high levels of total suspended solids (TSS) called white water and most of the water is recovered and used for the dilution of thick stocks (Hardy, 2002). Effluents released from the paper machine may contain fibre or fibre fragments, as well as chemicals used in the machine which can include roll release agents, defoamers, biocides and surfactants for cleaning fabric (Biermann, 1996).

5.1.3.1 Suspended Solids

TSS is a measurement of the mass of solids per volume of water. TSS measurement involves filtering a sample of known volume through a glass fibre filter. After filtration the filter is dried and the mass of the sample determined. It is extremely important to control and remove TSS since many toxic compounds are hydrophobic and found in suspended solids.

5.1.3.2 Chemical Oxygen Demand

Chemical oxygen demand (COD) is a measure of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant (Greenburg et al.,

1992). COD can be empirically related to biological oxygen demand (BOD), organic carbon or organic matter and is used for monitoring and control after the correlation has been established (Greenburg et al., 1992). Since COD reduces the dissolved oxygen levels within the effluent, reducing the amount of COD has been associated with removing many of the hazardous chemicals responsible for the increased COD.

5.1.3.3 Biochemical Oxygen Demand

The determination of BOD is an empirical test that is used to determine the relative oxygen requirements of wastewaters, effluents and polluted waters (Greenburg et al., 1992). BOD measurement is commonly used to measure waster loadings in treatment plants and to evaluate the BOD-removal efficiency of such treatment systems (Greenburg et al., 1992). BOD is tested my measuring the oxygen utilized during a specific incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulfides and ferrous iron(Greenburg et al., 1992).

5.1.3.4 Colour

Lignin, lignin derivatives and polymerized tannins are the primary cause of colour in pulp and paper mill effluent. The compounds are released via the pulping, bleaching, and recovery sections of production (Ali and Sreekrishnan, 2001). Lignin and its derivatives have carbon-to-carbon biphenyl linkages which offer resistance to degradation. These double bonds conjugate with an aromatic ring, quinone methides and quinone groups to cause the colour of the solution (Ali and Sreekrishnan, 2001) Lignin molecules have demonstrated self-condensation, particularly in acidic media, which is responsible for their resistance to degradation by simple molecular species (Ali and Sreekrishnan, 2001)

Colour can be measured using several different methods including the visual comparison method, the colorimetric method, the tri-stimulus filter method, and the spectroscopic method (typically used in pulp and paper) (Cook, 2000). Before colour can be measured the turbidity of the

sample must be removed via filtration or centrifugation (Cook, 2000). Colour is also highly pH-dependent, so the sample needs to be adjusted to a standard pH of 7.6 to ensure proper quality control (Cook, 2000).

Colour used to not be an important factor when evaluating toxicity and pollution; however it has now been found to not only be an aesthetic problem but also a problem capable of causing changes in algal and plant productivity due to reduced penetration of solar radiation. These changes are capable of causing imbalance in water ecosystems.

It is not very common to have regulations that control colour; these types of regulations would only be present in areas with clear water or water that has a low flow rate. Many rivers however have a significant natural colouring and as such the effect of colour loading caused by the effluent isn't as much of a concern (Hardy, 2002).

5.1.3.5 Resin and Fatty Acids

Resin acids are tricyclic diterpenes that occur naturally within the resin of tree wood and bark and transferred to water and sewage during the pulping process (Ali and Sreekrishnan, 2001). Resin acids are weak hydrophobic acids that can be toxic to fish at concentrations of 200-800µg/L and have been found at concentrations as high as several hundred part per million within pulp and paper mill effluent; even though their solubility ranges from 3-6 mg/L (Ali and Sreekrishnan, 2001). The pH of the wastewater has also been found to affect the toxicity and solubility causing 96h LC_{50}s to range from 0.4 to 1.7 mg/L for rainbow trout. Resin acids have also been found to be predominantly adsorbed to fine suspended matter in wastewater, which has led to the development of detoxification method where fine particles are removed prior to anaerobic treatment.

Resin acids and unsaturated fatty acids (16-C and 18-C), such as oleic acids, linoleic acid and linolenic acid from softwood pulp and paper mills are toxic to fish; especially salmonids (Ali and Sreekrishnan, 2001).

It has been found that chemical pulping will result in a lower concentration of resin acids in effluent than what is found in mechanical pulping. This is due to chemical pulping effluents producing less lignin and hemicellulose than mechanical and chemi-mechanical pulping (Biermann, 1996). With chemical pulping there are methods to recover chemical by-products from the pulping process and use them for other purposes.

A common method of chemical recovery is through the recovery of weak black liquor (WBL). Weak black liquor is a very alkaline solution (pH =11.5-13.5) that is comprised of inorganic cooking elements and degraded dissolved wood substances (Ragsdale, 2011). The organic components in WBL include: sodium salts of polysachharinnic acids, resin acids, fatty acids and alkali lignin (Ragsdale, 2011). During WBL recovery, the WBL is concentrated via evaporation and then burned in the recovery boiler to produce heat and green liquor, which is then converted into white liquor (a sodium-based alkaline solution used for digestion of wood chips during the cooking process) (Ragsdale, 2011). Chemical recovery prevents most of the resin acids from escaping with the rest of the process effluent.

Resin acids are divided into pimaric-type resin acids (isopimaric, sandaracopimaric and pimaric acid) and abietic-type resin acids (palustric, neoabietic, levopimaric and abietic acid); with pimaric- type acids being more toxic and less soluble than abietic-type acids (Leiviska et al., 2009).

Resin Acids have been found to contribute up to 70% of the total effluent toxicity and can cause many effects such as lethality, chronic sub-lethal toxicity, and bioaccumulation (Li et al., 1996). Li et al. (1996) conducted a review of the 96h LC50 of resin acids for rainbow trout and found that it was between 0.2 and 1.7ppm.

Fatty acids are also toxic to fish, with unsaturated fatty acids being more toxic than saturated; however both are less toxic than resin acids (Leiviska et al. 2009). Fatty acids are the hydrolysis products of esters (fats and waxes) that are formed during pulping (Makris, 2003).

5.1.3.6 Sterols

Another common wood extractive are sterols, which have been found to disturb the hormonal action within aquatic organisms. Plant sterol structure resembles that steroid hormones within vertebrates and thus can affect development, growth and reproduction. These types of long-term effects can even occur at low concentrations, with phytosterol disturbing fish growth at concentrations of 10 µg/L (Kostamo et al., 2004). β-sitosterol has been found to be the dominating sterol in effluents from bleached kraft mills (Leiviska et al., 2009); and can be found both in free form and as fatty acid esters (Lafleur, 1996). Secondary treatment systems such as aerated lagoons and activated sludge plants are capable of removing most wood extractives from the effluent; however they do not completely eliminate them (Servos, 1996).

5.1.4 Treatment of Pulp and Paper Effluent

There are typically a number of different stages involved in effluent treatment, including primary treatment which removes suspend solids, secondary treatment which uses microbes to degrade organic matter, and tertiary treatment which removes colour.

Primary treatment involves the use of clarifiers to remove suspended solids and this is typically done through sedimentation, which is a process where suspended solids are allowed to settle to the bottom. A large auger (squeegee) spins and pushes the solids out of the bottom of the clarifier in the form of sludge. In many pulp and paper mills the sludge is then sent from the clarifier to the recovery boiler to burn as fuel.

Secondary effluent treatment involves the removal of organic material through use of an aerated stabilization basin (ASB) or an activated sludge plant. These treatment methods are also for the

control of organic and inorganic matter that is discharged. These materials are measured in the form of adsorbable organic halides (AOX), COD, and other specific compounds (Stromberg et al., 1996). AOX are formed from reactions between chlorine and lignin or other organic material (Cheng, 2010). This removal of organics during the biological treatment process is due to microbial degradation but to small extent; it is also due to adsorption and air stripping (LaFleur, 1996)

5.1.4.1 Environmental Concerns Historically Related to Pulp and Paper Effluents

Suspended solids in pulp and paper mill effluents caused detrimental effects to the environment between the 1950s and the late 1970s. The solids would settle to the bottom of the receiving waters and form a fibrous mat that destroyed normal bottom ecology. The benthic organisms would consume excessive amounts of oxygen, which would create an anaerobic environment, which was also acutely toxic to fish (McMaster et al. 2003). The focus shifted to identification of the toxic chemicals contained within pulp & paper effluent during the 1970s and early 80s; with special attention being paid to toxicity associated with resin and fatty acids as well as chlorinated phenolics (McMaster et al. 2003).

Secondary treatment and the move to elemental chlorine free bleaching became more common in the 1990s (Stromberg et al., 1996). This resulted in low BOD_5, COD, TSS and chlorinated organics in pulp and paper mill effluents (Stromberg et al., 1996). It has been determined that chlorinated chemicals cannot completely account for the toxicity of pulp mill effluent (Servos, 1996). The role of natural wood extractives in toxicity is becoming more recognized and secondary treatment has become very effective at removing the majority of these compounds and thus toxicity is a minor concern (LaFleur, 1996). One popular method of secondary treatment is the use of an aerated stabilization basin.

5.1.4.1.1 Aerated Stabilization Basins

Aerated stabilization basins (ASBs) are aerated lagoons which are often used for the biotreatment of pulp and paper mill effluent. Due to their lower nutrient and solids management requirements ASBs have lower operating costs than activated sludge systems and many have offered

long periods of trouble free service (Mahmood and Paice, 2006). Poor ASB performance is mainly caused by excess solids accumulation, and high nutrient and total suspended solids (TSS) discharge (Mahmood and Paice, 2006).

ASBs are complex systems comprised of aerobic, anoxic, and anaerobic zones. For successful effluent treatment ASBs must be able to convert BOD to biosolids and CO_2 or methane; and remove biosolids by aerobic digestion and benthal stabilization (Mahmood and Paice, 2006). The design of the ASB as well as the placement of the aerators causes variation in benthal feedback to the water column from anoxic-anaerobic benthal zones between different ASBs (Mahmood and Paice, 2006).

Pulp and paper ASBs have complex microbial communities with the primary colonizers and BOD degraders being aerobic, anoxic, and anaerobic bacteria. Algal communities are common in ASB for municipal effluents, providing oxygen during daylight hours; however they are rare in pulp and paper as there is little light penetration in the dark effluents (Mahmood and Paice, 2006). This issue is further antagonized by the deliberate mixing of aerators leading to increased turbidity by suspended solids. Higher TSS is always a negative feature due to it either being biodegradable, contributing to a higher BOD, or it being inert requiring dredging or being discharged into receiving water (Mahmood and Paice, 2006). Excessive solids accumulation and efficient nutrient management are the two issues of greatest concern to ASBs and are strongly influenced by BOD and TSS loading. Thus BOD and TSS loadings are key factors in designing and operating a successful ASB.

Primary Clarifiers are popular method for solids removal owing to their smaller footprint, capability of removing settled solids, and lack of dependency on dredging. It has been suggested that subsurface aerations systems can create a “pseudo-activated sludge” system within the ASB, with mixed liqueur suspended solids near the discharged end where the solids rapidly accumulate (Mahmood and Paice, 2006). ASBs with very high mixing and aeration intensity within the first cell have been shown to

perform worse than ASBs with evenly mixed aeration (Mahmood and Paice, 2006). Benthal surface area has been found to be equally as important as ASB total volume, as the larger settling area reduces dredging frequency (Mahmood and Paice, 2006).

Total reduced sulphur (TRS) compounds are a common compounds found within the solids of the ASB. They are a common contaminant within the pulp and paper industry and include compounds such as hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH), dimethyl sulphide (DMS), and dimethyl disulphide (DMDS) (Iliuta and Larachi, 2007a; Iliuta and Larachi, 2007b). Determining the specific TRS compound located within emissions is difficult due to the highly reactive nature of the compounds, which can result in severe losses during sampling and analysis. In order to minimize losses, materials that are inert towards sulphur compounds must be used (Wardencki, 1998).

Many factors can directly or indirectly affect the emissions of sulphur compounds within wastewater. Given the complex nature of a wastewater treatment system factors such as temperature, pH, dissolved oxygen (DO), COD, BOD, distribution of the concentration of sulphide and microbial behaviour can all cause variations in sulphur emissions (Liang, 2008).

Sulphates are biologically reduced by sulphate reducing bacteria (SRB) and factors such as DO, temperature and pH affect the growth of SRB. Wastewater has a critical DO concentration of 0.1-1.0 mg/L, below which sulphate reduction can occur (Liang, 2008). Sulphate reduction typically increases with temperature, with the optimum temperature range being 28^{o}C to 32^{o}C (Liang, 2008). Typically SRB exist at pH ranges from 5.5 to 9, however sulphate reduction has also been observed at a pH as low as 3.3 (Liang, 2008).

Methylation of H_2S forms organic sulphur compounds such as methyl mercaptan and dimethyl sulphide (Figure 1). The oxidation of methyl mercaptan leads to dimethyl disulphide (Higgins et al., 2006)

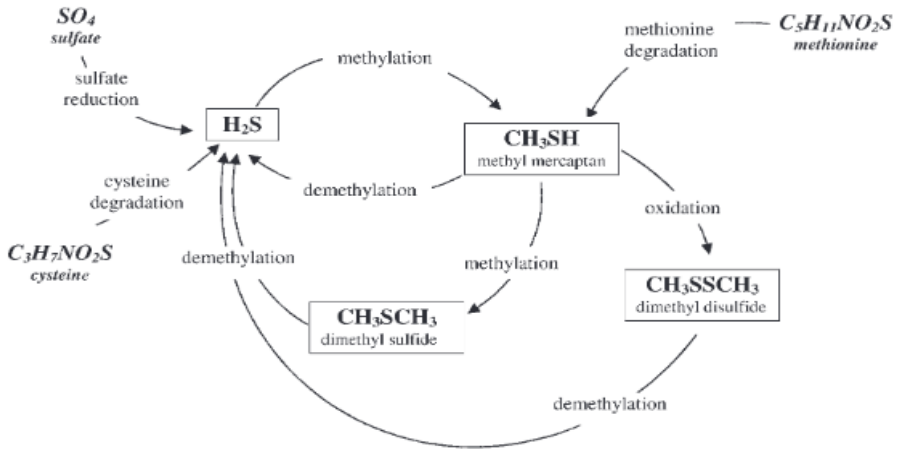

Figure 1. Cyclic pathway proposed to describe the production and transformations of volatile organic sulphur compounds and hydrogen sulfides (Higgins et al., 2006)

Studies have suggested that much of the hydrogen sulphide entering the ASB may be consumed by aerobic chemical and bacterial oxidation, precipitation, and assimilatory reactions that break down H_2S and incorporate it into biomass (Figure 1) (Liang, 2008)

5.2 Aquatic Toxicology

Studying the toxicity of effluents, or aquatic toxicology, is the qualitative or quantitative study of adverse or toxic effects of chemicals and other anthropogenic materials on aquatic organisms (Rand and Petrocelli, 1985). Toxic effects can include both lethal (mortality) and sub-lethal effects. Sub-lethal effects include changes in growth, development, reproduction, pharmacokinetic responses, pathology, biochemistry, physiology, and organism behaviour (Rand and Petrocelli, 1985). These effects can be expressed using quantifiable data such as percentage of hatching eggs, changes in length and weight, percent inhibition of enzymes, tumour incidence, number of skeletal abnormalities and number of organisms killed (Rand and Petrocelli, 1985).

Aquatic toxicology has evolved into a multidisciplinary field that includes many aspects of other basic sciences. It is important to understand the chemical, physical and biological factors that influence the environmental concentrations of chemicals. This data enables the understanding of how toxic agents behave in the environment and how the environment influences these agents, which enables the estimation of potential exposure of aquatic organisms. Physiology and biochemistry knowledge is also important in order to understand how toxic agents can affect organisms.

Toxicity is the relative property of a chemicals ability to have a harmful effect on an organism and is a function of the concentration of the chemical and the duration of the exposure period (Rand and Petrocelli, 1985). Toxicity is used to describe toxicants, which are the agents that cause the adverse response (Rand and Petrocelli, 1985).

The concentration, transportation, transformation, and disposition of chemicals in the environment are primarily controlled by (Rand and Petrocelli, 1985):

1. The physical and chemical properties of the compound,
2. The physical, chemical and biological properties of the ecosystem,
3. The source and flow rate of the chemical into the environment.

5.2.1 Factors Influencing Toxicity

5.2.1.1 Exposure

Exposure is important in determining the toxicity of a toxicant and is influenced by the kind, duration and frequency of exposure, as well as the concentration of the chemical. The solubility of the chemical also plays an important role. Water-soluble chemicals are more readily available for uptake than water-insoluble chemicals which are tightly bound to sediment and suspended particles. As water-insoluble chemicals gradually disassociate from the suspended particles they can enter via skin exposure

and the gills (Rand and Petrocelli, 1985). Chemicals located in food can enter via the digestive tract during digestion. The route of chemical exposure can affect kinetic factors such as absorption, distribution, biotransformation and excretion of chemicals (Rand and Petrocelli, 1985).

The duration of the exposure can be classified as acute (short-term) or chronic (long-term) exposure. Acute exposures involve a single exposure or multiple exposures within a short period of time (hours-days). Chemicals are rapidly absorbed and cause immediate or slightly delayed effects. Chronic exposure involve exposure to low concentrations of chemical over a long period of time (weeks, months, years), chemicals are delivered continuously or at a periodic interval. Chronic tests generally cover the entire reproductive life-cycle; however some test can only cover a portion of the life-cycle (usually sensitive stages of development) and are referred to as sub-chronic tests. Sub-chronic tests are also referred to as early life stage, critical life stage, embryo-larval, or egg-fry tests (Rand and Petrocelli, 1985).

Frequency can influence toxicity, for example an acute exposure from a single concentration may cause an effect, but two successive exposures cumulatively equal to the first exposure may cause no effect (Rand and Petrocelli, 1985). This difference can be attributed to the ability of the organism to metabolise the chemical with the rate of metabolism determining how much chemical from the initial exposure remains in the organism during the second exposure (Rand and Petrocelli, 1985).

5.2.1.2 Organismal Factors

The susceptibility of an organism to toxicants differs between different species. Accessibility of the chemical can attribute to this difference, with some organisms being able to block out the host media for short periods of time (e.g. clams ability to undergo anaerobic metabolism) (Rand and Petrocelli, 1985). The rate and pattern of metabolism, as well as the rate of excretion is also a cause of this difference. Organisms with a high rate of metabolism and excretion are capable of removing

chemicals from their bodies before it can reach a concentration high enough to cause an effect (Rand and Petrocelli, 1985). Genetic differences between populations can also attribute to this difference due to some populations being more genetically equipped to handle higher levels of certain chemicals. Differences in diet composition and bioaccumulation are also capable of causing in differences in the susceptibility of species to toxicants. The differences may also occur between different aged organisms, with younger organisms being shown to be more susceptible to toxicants than adults. Finally, the overall health of the organism can cause a large difference in response. Regulatory tests are generally performed using healthy organisms; however unhealthy organisms may experience a different response to the toxicant.

5.2.1.3 Chemical Factors

The toxicity associated with a specific toxicant can be influenced by chemical factors in the environment, as well as chemical factors related to the toxicant itself. In the environment factors such as pH, DO, temperature, and dissolved solids in the water media can influence the bioavailability of toxicants in the water. The composition of the specific chemical is also important to the toxicity of the chemical. Impurities in the chemical can help to reduce the toxicity or the impurities themselves may be more toxic than the actual chemical. Thus laboratory tests on a pure chemical may not always exhibit the same response as those seen in the environment. Physical and chemical properties of the toxicant such as solubility, pH, vapor pressure, etc. are also important factors to consider. Another factor to consider is the selectivity of the chemicals mode of action. Some chemicals can be nonselective in their mode of action and are able to cause harmful effects to all types of cells and tissues (Rand and Petrocelli, 1985). However other chemicals may be so specific in their mode of actions, that only specific cells are harmed while all others function correctly (Rand and Petrocelli, 1985). The selectivity in mode of action can also be species-specific which can attribute to differences between different species, as well as between different genetic groups.

5.2.2 Common Toxicity Testing Methods

5.2.2.1 Acute Toxicity Testing

Acute aquatic tests primarily determine the concentration at which lethality occurs within a sample after a short period of exposure. This is due to the ease in measurement of death and the criterion for death being lack of movement (especially gills in fish, and heart/lungs for invertebrates). For fish a 96-hr exposure period is commonly used as this is sufficient time to allow for the chemicals mode of action. Following the exposure period a 50% response is calculated as this the easiest measurement to determine and this is recorded as an LC_{50} value. In some cases where death does not occur an EC_{50} (effective concentration) value is recorded and this is commonly used with micro invertebrates (e.g. daphnia). EC_{50} values in daphnia are used when organisms are immobile; however their heart and gills continue to function. The results of acute tests are generally calculated as an all-or-nothing response (Pass/Fail) where the percentage of organisms killed is recorded or they are calculated as an LC_{50}/EC_{50} where the median concentration that causes 50% mortality is recorded, along with its 95% confidence limits. In Canada the two species commonly used for acute aquatic tests are Rainbow Trout (*Oncorhynchus mykiss*) and *Daphnia magna*.

5.2.2.2 Chronic Toxicity Testing

Chronic toxicity tests are used to examine sub-lethal effects of chemicals and involve a longer period of exposure. The specific criterion measured during testing varies depending on the species being used and in Canada, the two most commonly used species are Fathead Minnows (*Pimephales promelas*) and *Ceriodaphnia dubia.* In Canada, chronic tests using Fathead Minnows are early life-stage tests. This test uses growth as a criterion for measuring toxicity, with tests evaluating changes in length and mean dry weight of <24hr old larvae over a seven day period. *C. dubia* tests are performed for the duration of their reproductive cycle. Neonates which are <24hrs old are evaluate for mortality, time to first brood, as well as number of broods produced throughout a seven day period. In both case the data

is recorded as an IC_{50} (inhibitory concentration), which is the concentration at which the observed criterion is inhibit in 50% of the population. This data is recorded along with its 95% confidence limits.

Due to the unspecific nature of these standardized test methods, the cause of toxicity can be difficult to determine. Thus more detailed tests have been designed to outline the specific contaminant that is causing toxicity. The most common of example of such a test is the Toxicity Identification Evaluation (TIE) &/or Toxicity Reduction Evaluation (TRE).

5.2.3 Toxicity Identification Evaluation

The toxicity identification evaluation (TIE), sometimes known as the toxicity reduction evaluation (TRE) is a test that is conducted to determine the toxic components of a substance. The TIE test involves three distinct phases and each phase is dependant of the result of the phase prior. Phase 1 involves characterizing the effluent and determining the group the toxicant belongs to. Toxicants are characterized based on six main properties determined by whether they are: chelatable, filterable, reducible, a non-polar organic, or pH sensitive. Phase 2 involves the identification of the specific toxicant; this is an easier process due to the results of the initial phase. The final phase, phase 3, involves confirming the specific toxicant in question.

5.2.3.1 Phase 1: Characterization

Phase 1 uses several distinct processes that are typically spread out over two days. The initial toxicity test is performed on the first day and this test is used to determine if the sample is sufficiently toxic to perform a TIE (Norberg-King et al., 1991). This test provides an estimate on the 24-hour LC_{50}, which allows the determination of the appropriate exposure series for the rest of the phase 1 tests (Norberg-King et al., 1991).

The baseline toxicity test is performed next and this test is used to compare to all the characterization tests. This also checks whether toxicity has changed from the initial test. If there are

any manipulations after the phase 1, they will have a companion baseline test (Norberg-King et al., 1991).

The pH adjustment test occurs to determine the effect that pH has on the toxicity of the sample. Changes in pH can affect the solubility, polarity, volatility and speciation of compounds. Aliquots of effluent are adjusted to pH3 and pH 11; portions of these adjusted samples are saved for subsequent manipulations (Norberg-King et al., 1991). These manipulations include aeration, filtration, and solid phase extraction and portion of the adjusted samples are adjusted back to initial pH (pHi) for testing (Norberg-King et al., 1991).

The aeration test is used to evaluate the role of volatile, sublatable, or oxidizable compounds; as pH has been known to affect the volatility of compounds, e.g. ammonia. Sample aliquots of pH 3, pHi, and pH 11 are exposed to moderate aeration for an hour (Norberg-King et al., 1991). The pH 3 and pH 11 samples are then adjusted back to pHi and used to determine if there is a reduction in toxicity (Norberg-King et al., 1991).

Filtration provides information on toxicants associated with filterable material. A pH shift may affect the solubility of the toxicants. The filtration step also provides a comparison for the C_{18} solid phase extraction test.

C_{18} solid phase extraction (SPE) filters out portions of the sample by pumping the sample through C_{18} columns (Norberg-King et al., 1991). The tube acts as a sorbent to retain analytes of interest or interferences (Norberg-King et al., 1991). This retention of analytes can occur through a variety of different actions and in addition to the different kinds of sorbents make SPE a powerful technique for identifying contaminants within a sample (Otles and Kartal, 2016).

Oxidant reduction sodium thiosulfate evaluates the toxicity due to oxidants. This can either be done using a gradient approach where one concentration of effluent is used with a series of thiosulfate concentrations, or using a matrix approach (Norberg-King et al., 1991). The matrix approach involves using a 3 x 3 matrix of thiosulfate versus effluent concentration (Norberg-King et al., 1991).

EDTA chelation test involves the evaluation of the contribution of cationic metals. This process forms a chelate which renders the metal biologically unavailable and this process can be performed using either a gradient of a matrix approach (Norberg-King et al., 1991). This process however does not chelate anionic metals such as selenium, arsenic, and chromate (Norberg-King et al., 1991).

Once these tests have been performed it is possible to move onto phase 2 and identify the toxicant within the sample.

5.2.3.2 Phase 2: Identification

Some species of toxicants are easy to analyze, while others are more difficult and this had led to a variety of techniques being used. Species such as ammonia, chlorine, metals, and ions can be analyzed relatively easily using techniques such as potentiometric titration, ICP-MS and specific ion electrodes (Durhan et al., 1993). Non-polar organics, as well as samples that contain multiple toxicants can be harder to analyze (Durhan et al., 1993). Non-polar organics are typically analyzed using GC/MS, HPLC, SPE, ELISA, TDS- ion chromatography (Durhan et al., 1993).

The data from phase 1 is useful in narrowing down the tests required for phase 2. For example if EDTA reduced toxicity, then measure the levels of copper, lead, cadmium, nickel, and zinc; however, if sodium thiosulfate reduced toxicity then measure copper, cadmium and silver.

These types of tests can be conducted for all of the categories that were tested in phase 1. Once the toxicants have been identified, then phase 3 can be performed.

5.2.3.3 Phase 3: Confirmation

Phase 3 of the TIE test is often included in the results of phase 2. This phase involves confirming the results of phase 2 and is simply performed by evaluating the evidence that was found in phase 2, as well as ensure that strict quality assurance and quality control standards were met.

5.3 Alternative Techniques in Toxicity Testing

Currently the average acute toxicity test requires 25L of effluent, as well as 60 fish. The average chronic toxicity test uses 10.5L of effluent and 240 fish, this is test is often repeated up to 10 times. This means that potentially 2400 fish and 100L of effluent can be used in a chronic toxicity testing. Tanneberger et al. (2013) stated that an average of three million fish are used annually in North America for whole effluent testing. Effluent release is also variable and episodic which results in frequent testing to ensure that any toxic episodes are captured. Thus new methods of toxicity testing are required to decreases the amount of effluent being used, as well as the number of animals required for regulatory toxicity tests.

A variety of different alternatives have been developed. Some of which include quantitative structure-activity relationships (QSAR), thresholds of toxicological concern (TCC), the use of fish embryos as a surrogate life stage, and cell lines developed from fish. Alternative methods have been traditionally only been developed for acute toxicity tests, however recent advances have led to the development of alternatives for chronic toxicity testing.

5.3.1 Quantitative Structure-Activity Relationships

QSAR models are a no-testing method that is based on the similarity principle and assume that physical, chemical and biological properties of a contaminant are related to its geometric of electronic properties (Lillicrap et al. 2016). Factors such as the log octanol-water coefficient or knowledge of the chemicals 2D &3D structure can be used to predict (eco)toxicological endpoints (Lillicrap et al. 2016). The model uses information acquired from databases of known properties & biomathematical

approaches. QSAR models require three main components: a high quality data set with experimentally measured biological activities, a dataset exhibiting the structure-related properties for the chemical structure, and a characterization of the relationship (Lillicrap et al. 2016). The models generated are typically mechanistic (use prior knowledge of the mechanism of the studied activity) or descriptive (use algorithms to select most relevant date for prediction) (Lillicrap et al. 2016). Given that these models are based strictly on tested data only the most reliable studies should be used for developing the model. Models developed without proper validation of the study data will suffer from poor predictive capacity. Because the models use information from databases the two biggest sources of error result from input error and poor data points (Lillicrap et al. 2016). These factors can result in a valid QSAR that actually deviates from the tested data.

QSAR models have been in development for approximately 20 years and have been used for a variety of different applications including rapid screening approaches, risk assessment, regulatory applications and drug discovery (Lillicrap et al. 2016). Using QSAR models new substances can be rapidly screened for their persistent, bioaccumulative and toxic (PBT) properties and their carcinogenic, mutagenic, or reproductively toxic (CMR) properties (Lillicrap et al. 2016). QSARS can also be used to identify flawed studies as well as aiding in increasing the accuracy of lab results.

The Organization for Economic Co-operation and Development (OECD) published five rules to ensure the validity of QSAR models, including the requirement for defined endpoints; the use of unambiguous algorithms; a defined domain of applicability; an appropriate measure of goodness of fit, robustness & predictivity; and mechanistic interpretation (Lillicrap et al. 2016). These rules OECD rules have been integrated into the Registration, Evaluation, Authorisation and Restriction of Chemicals [REACH].

5.3.2 Thresholds for Toxicological Concern

Thresholds for toxicological concern (TTC) are a well-established method for evaluating safety to humans based on indirect food contact. TTC's use existing data rather than requiring testing to determine exposure concentrations below which there is little risk for toxicity (Lillicrap et al. 2016). TTCs have been explored by several groups and summarize the wealth of information as predicted no-observed effect concentration (NOEC) in the form of statistical distributions (Lillicrap et al. 2016). Ecological (eco-)TTCs can be developed to allow prediction of toxicity for untested chemicals based on structural attribute, mode of action, or functional use (Lillicrap et al. 2016). TTCs can also be used to assess the chemicals at early tiers of risk assessment and can provide hazard perspective on chemicals that lack QSAR models (Lillicrap et al. 2016).

5.3.3 Fish Embryo Test

The fish embryo test (FET) is used in place of testing on juvenile fish, as in many locations embryos are not a protected life stage and thus are not subject to the same regulations as fish. In Germany a 48hr post-fertilization zebra embryo test has replaced the fish test for assessing the toxicity of effluent. This embryo test has also been adopted into OECD as a new test guideline and acts as a promising alternative due to its similar sensitivity to acute fish toxicity (Braunbeck et al., 2005; Lillicrap et al. 2016). While most embryo tests use zerbrafish embryos, Braunbeck et al. (2005) had success with fathead minnow and medaka embryos. Zebrafish however have more consistent spawning behaviours and thus are easier to control the timing of the tests. The consistency also enables tests to commence at an earlier life stage due to all embryos being fertilized at the same time. Sensitivity of embryo test to contaminants could be affected by not only differences in species but differences in chorion permeability and should be considered in the development of guidelines (Lillicrap et al. 2016) .

It has been found that the chorion in FHM was thicker and had narrower pores than that of zebrafish embryos, which implies that FHM chorion is potentially less permeable than that of zebrafish

(Lillicrap et al. 2016). Lillicrap et al. (2016) confirmed these differences through visual examination using scanning electron microscopy. It has been hypothesized that different species could exhibit different toxicant sensitivities at different stages of embryogenesis, as is observed in other life stages. Concerns have been raised however regarding the chorion as a barrier and the metabolic capacity of embryos versus that of larval and juvenile fish. It has been found that large molecules such as quaternary ammoniums do not pass through the chorion and thus the FET has been expanded from 48h to 96h in to include the hatching process and enable the capture of non-permeable molecules (Lillicrap et al. 2016).

Differences in metabolic activity between embryos could underestimate the toxicity of certain substances (e.g. allyl alcohol). Fish embryos may also be more sensitive than juvenile fish to certain chemicals and thus a balanced opinion is needed to determine the suitability of embryos for testing (Lillicrap et al. 2016). Belanger et al. (2013) conducted a review of embryo tests and found that the tests had a slope of 1.0 with an intercept near the origin, demonstrating the close relationship between fish and embryo tests. Embryo testing may also be useful in the studying of adverse outcome pathways (AOP) due to 90% of the genome being active during embryogenesis (Lillicrap et al. 2016). FET testing used during mode-of-action assessments could also enhance predictions in chronic ecotoxicological effects (Lillicrap et al. 2016).

5.3.4 Use of Cell Cultures in Toxicity Testing

Currently it has been suggested to use cell cultures as a replacement for whole animal toxicity testing. The use of cell cultures in toxicity testing has been proposed due to the process having several advantages: (1) they provide the ability to control the environment of the cellular phenomena being studied, (2) the results can be achieved more rapidly and cost effectively, (3) cells can be easily exposed to toxicants, (4) there is a reduced volume of effluent required for this type of testing, and (5) can compare the sensitivity of multiple species to toxicants under equivalent conditions (Bols et al., 2005; Schirmer,2006; Cheng, 2010).

Cell cultures are typically divided into two types: primary cell cultures and cell lines. A primary cell culture is a group of tissue cells that is taken directly from an animal and then cultured in lab. A cell line however is a culture of cells that has been created from the proliferation of a primary cell culture. Continuous cell lines became available approximately 50 years ago and most recently fish cells lines have been used to evaluate mode of action (Lillicrap et al. 2016). Due to the abundance and diversity of fish species, piscine cell lines were developed and used as indicators for the toxicity of individual compounds. The underlying theory for the using cell lines in testing is that interactions of a toxicant with the cells of an organism is a precursor to changes in tissue or organ function that will ultimately affect the function of the whole organism (Schirmer, 2006). All aspects of cellular activities can be evaluated when using cell cultures, such as cytotoxicity, cell viability, genotoxicity, as well as the effects on cell-specific functions and parameters (Bols et al. 2005). Fish cell lines have been used to evaluate xenobiotic metabolism, DNA damage, membrane transport, oxidative stress, and specialized processes (e.g. phagocytosis and synthesis of specific proteins) (Lillicrap et al. 2016). Fish cell lines have also been used for both toxicity and cytotoxicity testing and provide reliable information in surveillance programs that target environmental chemicals (e.g. fish gill cells used to distinguish between acutely toxic and non-toxic pulp and paper effluent) (Lillicrap et al. 2016).

Tanneberger et al. (2013) demonstrated a nearly 1:1 correlation of acute fish toxicity data and cytotoxicity assays for a variety of chemicals. This fish gill cell line assay has been subjected to international round robin testing to demonstrate its robustness and interlaboratory reproducibility and is also being evaluated as a potential new standard method within the International Organization for Standardization (Lillicrap et al. 2016).

Baron et al. (2012) developed a rainbow trout primary hepatocyte spheroidal cultures system that is capable of being maintained for one month. This cell culture model allows the examination of

active transport processes through the gill epithelium. Cell lines are also being developed in conjunction with advanced technologies such as fluidic biochips for rapid evaluation of water safety (Lillicrap et al. 2016).

There have however been several problems encountered when using cell cultures. One problem is that a single culture only has a limited number of target sites in comparison to the whole organism and thus it alone will not be able to represent the diversity in target sites found in the organism (Schirmer, 2006). Currently the selection of cell cultures used during in vitro/in vivo comparisons of fish have been based on the availability of cell cultures more than on the specific function or target site (Schirmer, 2006). An exception to this trend however is found in the use of primary hepatocytes and gills of rainbow trout. This is because these groups representing major target sites for chemical uptake, as well as freshly isolated cells still possessing most of their differentiated functions (Schirmer, 2006). However, during acute toxicity testing it has been found that there is a weaker correlation than that of cell lines (Schirmer, 2006). This was especially true for hepatocytes and this may be a reflection of the variability intrinsic to freshly isolated cells (Schirmer, 2006). It has been found that higher concentrations of chemical are required in the cell cultures than in the fish in order to detect a toxic response (Schirmer, 2006). Few studies have reported cases where the cell lines were significantly more sensitive than fish (Schirmer, 2006). This lower sensitivity of cell lines is a major obstacle that must be overcome in order to make cell cultures a viable replacement for whole organism testing (Schirmer, 2006).

Shirmer (2006) suggested several ideas for improving the in vitro approach to toxicity testing. One such suggestion involves the selection of appropriately suited cells, culture environment, endpoint measurements, as well as the means to account for the chemical concentrations that are bioavailable to

the cultured cells. She also suggested assessing the advantages and limitations of the different methods of applying samples to cultured cells.

5.3.4.1 Factors Influencing Tests on Cell Cultures

5.3.4.1.1 Effect of Bioavailability of Chemicals on Testing

The bioavailability of test chemicals is likely the most important factor to consider when attempting to improve the use of cell cultures in toxicity testing. This because bioavailability has the potential to influence of the other factors, for example if fewer chemicals are available for uptake then exposure periods may need to be increased in order to account for this effect.

When a chemical is added to the test system it can be influenced by a number of different processes, such as: evaporation, degradation of test chemicals, adsorption of chemicals to the culturing vessel, and binding to the growth media (Figure 2) (Groothuis et al., 2015). These factors can influence the concentration of chemical that is actually available for uptake by the cells. Once this chemical has been taken in by the cell, it can then be influenced by further processes such as: becoming bound to the cell membrane, binding to internal constituents, metabolism of the cell (Figure 2) (Groothuis et al., 2015). These processes leave an even smaller concentration that is available to actually bind with the cellular target site and cause an effect and thus an important factor is the test assay setup.

One factor to consider when setting up the assay is the influence the addition of growth serum can have on the availability of the test chemical. Gülden and Seibert (2003) found that chemicals such as pentachlorophenol, 2,4,5-trichlorophenol, hexachlorophene and nonylpheneol can bind to serum albumin to extents of more than 95, 68, 62, and 44% respectively. This demonstrates that addition of serum can greatly influence the bioavailability of test chemicals. Test assay setup also greatly influences the dose metric used in the test.

5.3.4.1.2 Selection of Appropriate Dose Metrics for Use in Testing

Ultimately the reliability and accuracy of *in vitro-in vivo* extrapolations (IVIVE) is dependant the reliability of the *in vitro* model and this model can be unreliable if an improper dose metric is selected. A dose metric (*i.e.* exposure metric) can be defined as the measure of dose, which is a specified quantity of test chemical within a test system or (part of an) organism (Schirmer, 2006). Currently quantification of chemical toxicity is generally based on measurements of external exposure; however, internal concentrations of chemicals are more suitable for interpreting and extrapolating toxicological effects (Stadnicka et al.2012). However, there are many other dose metrics that commonly exist in toxicology (Table 2) Theoretically the most relevant dose metric for *in vitro* response would be the biologically effective dose (BED) which is the concentration at the site of action because this dose is most closely related to the initial molecular changes caused by the chemical. Paustenbach (2000) has defined BED as "the amount that actually reaches cells, sites, or membranes where adverse effects occur, [this] may represent only a fraction of the delivered dose, but is obviously the best one for predicting adverse effects." However, as stated by Groothus et al. (2015), such a dose cannot be practically measured in most cases and often surrogates are used instead. Such as the internal cell, organ, or organism concentration, the freely available concentration and the nominal or total concentration within the exposure media.

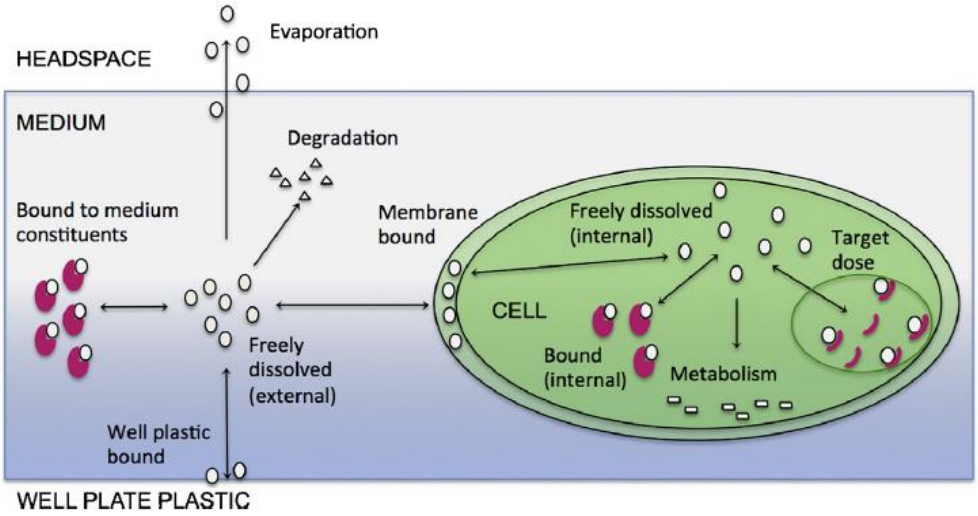

Figure 2. An illustration of the different processes influencing the bioavailability of a chemical in a typical *in vitro* cytotoxicity assay (Groothuis et al. 2015).

According to Groothuis et al. (2015) total internal concentration could be measured using critical cell burdens, analogous to critical body residues (CBR), as well as the concentration of test chemicals found in or on cells at a the point in time that causes a perturbation to a toxicity pathway. Also, if data about the partitioning and uptake rate into cells is available then this could be used to model internal concentrations. Currently however, the extraction of tissues or cells for concentration measurements remains delicate work and as such very few groups have tried it.

Table 2. Glossary of common dose metrics used in toxicology (Groothuis et al., 2015**).**

Dose Metric	Definition
Nominal Concentration	Total amount of chemical divided by the volume of exposure media to which the chemical is added (e.g. µmol/L media)
Total Concentration	Analytically measured concentrations in exposure media (e.g. µmol/L media). Includes chemicals freely available in media and those bound to media constituents
Freely Available/ Free Concentration	The unbound concentration of a test chemical within exposure media (e.g. µmol/L media)
Total Internal Concentration	Concentration of test chemical within the cells (e.g. µmol/10^6 cells)
Cytoplasm concentration and Membrane Concentration	Concentration in the cytoplasm of cells, can be freely available or bound to constituents within the cytoplasm (e.g. µmol/L cytoplasm). Membrane concentration is the concentration associated with the membrane fraction of cells
Target Concentration/ Biologically Effective Dose (BED)	Dose found at the target site in cells or tissues that causes a (toxicological) effect (e.g. µmol/ µmol receptor
Area Under the Curve (AUC)	Any of the above-mentioned dose metrics integrated over time (e.g. µmol/L x min)
Time Weighted Average (TWA)	Any of the above-mentioned dose metrics averaged over time (e.g. average µmol/L)
Biokinetic/toxicodynamic modelling (BK/TD)	Any of the above-mentioned dose metrics modelled over time

Free concentration appears to currently be the most proficient dose metric to use for estimating BED. Studies have shown free concentration to be a more consistent dose metric to use in place of nominal concentration, as it is not dependent on the test assay setup. Studies have also shown that nominal concentration can underestimate the toxic potency of test chemicals (Schirmer, 2006). This concept was outlined in a study conducted by Gülden and Seibert (2005), where the authors calculated the nominal concentration (EC_{50}) and the free cytotoxic concentration (EC_{50u}) for a number of organic

chemicals with a wide range of cytotoxic potency. These values were then compared with LC_{50} values from acute fish toxicity assays and the results showed that EC_{50u} and LC_{50} corresponded better than EC_{50} and LC_{50} values (Gülden and Seibert, 2005). This indicates that the variation between *in vivo* and *in vitro* test assays can be explained, at least in part, by the difference in bioavailability. It is important that the difference between EC_{50} and EC_{50u} was greater for chemicals with a higher cytotoxic potency (Gülden and Seibert, 2005). The authors suggested that for chemicals with higher cytotoxic potency, *in vitro* determination of protein binding parameters is essential.

Currently, equilibrium dialysis, ultrafiltration, and centrifugation can be used to measure free concentration, as well as binding affinities of test chemicals to (extra) cellular matrices (Groothuis et al., 2015). Studies have also shown that solid-phase microextraction (SPME) can be used as a relatively simple technique for measuring the binding affinities of test chemicals to serum constituents (Groothuis et al., 2015). These methods do however have some drawbacks, equilibrium dialysis, ultrafication, and centrifugations are difficult to use for hydrophobic test chemicals and may not be compatible with *in vitro* matrices (Groothuis et al., 2015). Also, SPME use directly in the assay is limited by the compatibility of the test chemical with the fibre coating and kinetics behind the chemical uptake into the fibre (Groothuis et al., 2015). Also, chemicals that are very hydrophobic will partition slowly into the fibre and thus the exposure period of the cell assay may not be long enough for equilibrium to be reached between fibre and culture media (Groothuis et al., 2015). For these reasons modelling the free concentration of a test compound *in vitro* would be less cumbersome than measuring it.

The development of serum-free culture media has allowed for dose metrics such as total concentration to be a more feasible measurement of BED, as in serum-free culture media this value would be representative of the freely available concentration. This metric can be easily measured through common analytical techniques. Serum-free media, such as Leibovitz's L-15/ex media, are also

capable of coming in a dried powder form and this allows for easy use with effluents and test samples. The powder can be mixed with the effluent to enable exposure to 100% effluent.

5.3.4.1.3 Selection of Cell Species

As previously stated, the species of cells used in testing can greatly influence the overall result of the test. Currently, IVIVE studies are using both mammalian and fish cell lines as well as fish embryos. The FET does have several advantages however it still requires the use of recently fertilized fish embryos and as such still requires live animals to produce these embryos. Tests using primary cell cultures and cell lines have been rigorously studied and have demonstrated several advantages as well as disadvantages. There is still a debate as to whether to use mammalian or fish cells in testing. Both cell species have demonstrated a similar relationship between the results they express and those exhibited by *in vivo* tests, as well as both groups have advantages and disadvantages.

Mammalian cell lines have been characterized extensively due to their similarity to human cells and many different cell lines from a variety of different mammalian species and tissue origins have been established. Fish cell lines however were originally developed based on availability, with little regard for cell function or tissue origin. Currently groups such as Bols et al. (1994) and Vo et al. (2013) have been working to fill this gap by developing new cell lines for testing. One major advantage of fish cell lines is the ability to be cultured under similar test conditions to those experienced by live fish *in vivo*. Typically mammalian cells need to be cultured at temperatures of around 35^{o}C; while fish cell lines can be cultured at temperatures between 18^{o}C and 30^{o}C, which is more typical of the conditions experienced by the fish during *in vivo* testing. One of the biggest advantages of fish cell lines is that they can be cultured in 100% effluent. Most cell cultures will require effluent to be diluted with culture media containing serum to allow for the right consistency of media for cell growth. Fish cells however are capable of growing in liquid media and as such serum-free powders such as L-15/ex can be mixed with the test effluent to allow for exposure to 100% effluent.

5.3.4.2 Fish Cell Lines Developed

The first permanent fish cell line developed was a rainbow trout gonadal cell line (RTG-2) that was developed in in 1962 and since then there has been a variety of different cell lines developed from various species and sources. Lakra et al. 2011 conducted a review of cell lines developed from fish between 1994 and 2010. In total there were 47 cell lines developed from 19 different species of fresh water fish (Table 3) and 51 cell lines developed from 22 species of marine fish (Table 4). Rainbow trout had the largest number cell lines derived with 21 distinct cell lines.

Given that rainbow trout is the one of the most common species used for toxicity testing, cell lines developed from rainbow trout would be one of the most effective alternatives to live fish. The cell lines developed from rainbow trout are derived from a variety of tissue including: gill, liver, spleen, skin, pituitary gland, and pronephros (Lakra et al., 2011). The rainbow trout liver cell line (RTL-W1) was established in 1984 from an adult male fish liver and has undergone more than 200 passages (Cheng, 2010). Another cell line used in testing is the rainbow trout gill cell line (RTgill-W1) which was developed from gill epithelial cells (Bols et al. 1994).

Another important species for ecotoxicological testing is the fathead minnow (*Pimephales promelas*), which is a fish species that has been utilized for acute toxicity testing and is a staple for chronic toxicity testing in North America. The development of a cell line has provided the opportunity to study interactions of effluents with fish while requiring minimal animal usage. The cell line FHM-L has been derived from an adult male fathead minnow liver and was developed by Dr. Lucy Lee in collaboration with the Dr. David Law lab and is the first immortal cell line that has been developed for fatheads.

Table 3. Catalogue of freshwater piscine cell lines developed between 1994 and 2010 (Lakra et al. 2011)

Common Name	Species Name	Number of Cell Lines Derived
Rainbow Trout	*Oncorhynchus mykiss*	21
Mrigal	*Cirrhinus mirgala*	1
Goldfish	*Carassius sp.*	1
Japanese Eel	*Anguilla japonica*	1
Rohu	*Labeo rohita*	4
Goldfish	*Carasssius auratus*	3
Barb	*Puntius schwanenfeldi*	1
Snakehead	*Ophicepalus striatus*	2
African Catfish	*Clarias gariepinus*	1
Snakehead	*Channa striatus*	1
White Sturgeon	*Acipenser transmontanus*	1
White Bass	*Morone chrysops*	1
Xipmorphus	*Xipmorphus sp.*	1
Golden mahseer	*Tor putitara*	1
Siberian Sturgeon	*Acipenser baerii*	1
Catla	*Catla catla*	3
Chinese Sturgeon	*Acipenser sinensis*	1
Mandarin Fish	*Siniperca chuatsi*	1
Pearl Spot	*Etroplus suratensis*	1

Table 4. Catalogue of Marine piscine cell lines developed between 1994 and 2010 (Lakra et al. 2011)

Common Name	Scientific Name	Number of Cell Lines Derived
Coho Salmon	Oncorhynchus kisutch	3
Atlantic Salmon	Salmo Salar	3
Spot Croaker	Leiostomus xanthurus	3
Flounder	Paralichthys olivaceus	1
Gilt-head Seabream	Sparus aurata	3
Sea Perch	Lateolabrax japonicas	2
Read Seabream	Pagrosomus major	3
Orange-spotted Grouper	Epinephelus coioides	5
Sea Trout	Salmo trutta	1
Atlantic Salmon	Salmo salar	1
Yellow Grouper	Epinephelus awoara	5
Japanese Flounder	Paralichthys olivaceus	2
Japanese Plichard	Sardinops sagax neopilchardus	2
Sea perch	Lateolabrax japonicas	1
White Sturgeon	Acipenser transmontanus	3
Turbot	Scophthalmus maximus	2
Blue Fin Trevally	Caranx melampygus	2
Haddock	Melanohrammus aeglefinus	1
Red-Spotted Grouper	Epinephalus akaara	3
Rock Fish Grouper	Epinephelus quoyanus	3
Half- Smooth Tongue Sole	Cynoglossus semilacvis	1
Atlantic Cod	Gadus morhua	1

5.3.4.3 In vitro Cytotoxicity Assays

The development of more cell lines for testing has also lead to a variety of methods for conducting cytotoxicity assays, with most methods involving the use of a microtiter plate and a type of cellular dye. The classic method of testing is a dye exclusion method that involves testing cell membrane integrity via its ability to prevent large molecules such as dyes from entering the cell. The trypan blue exclusion technique is common; however it can be tricky to perform as the results must be scored under a light microscope (Bols, et al. 2005). This method however has been used in tests which measure the capability of the plasma membrane to retain a marker molecule (Bols, et al. 2005). One of the earlier markers considered for use in fish was the intracellular enzyme lactate dehydrogenase (LDH), however LDH studies in mammals have demonstrated complications caused by several factors including: the protein synthesis rate for LDH production, the metabolic turnover rate of LDH in the cell, and the degradation of LDH in the extracellular medium (Putnam et al., 2002).

Fluorescent dye markers have also be utilized as methods for determining cytotoxicity. Common fluorescent dyes include 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), alamar Blue (rezurin, AB) and neutral red (NR). CFDA-AM is a virtually non-fluorescent indicator dye that acts as an esterase substrate capable of entering living cells (Schirmer et al. 1997). Cells with their plasma membranes intact maintain a cytoplasmic milieu which supports esterase activity and resulting in the production of a fluorescent product (Schirmer et al. 1997). Thus CFDA-AM can be used as an indirect measurement of plasma membrane integrity. Alamar blue is an indicator dye that measures cellular activity. The dye is taken up by the cells where oxidoreductases and the mitochondrial electron transport chain convert it into its reduced form which is more fluorescent than the oxidized form (Schirmer et al. 1997). For both CFDA-AM and AB assays a reduction in fluorescent readings is a measure of cytotoxicity. AB reduction can also be applied repeatedly to cultures over a period of time to evaluate recovery from metabolic impairment (Bols et al. 2005). NR is also used to measure membrane integrity in fish cells; however it is also capable of detecting injuring to specific lysosomes.

Neutral red (3-amino-7dimethylamino-2-methylphenazine hydrochloride) can be measured spectrophotometrically or fluorometrically and although it specifically accumulates in lysosomes, NR retention is dependent on having an intact plasma membrane, adequate energy metabolisms and functioning lysosomes (Bols et al. 2005). While the NR assay likely detects impairment of all 3 parameters similar to other viability assays, Schirmer et al. (1998) found that UV radiation in the presence of either acenaphthylene, acenaphthene, or phenanthrene caused phototoxicity to RTgill-W1 which was detectable by NR and not the other dyes, which suggests that lysosomal damage occurs before the loss of cell viability.

Metabolic impairment can also be evaluated through measuring ATP content or the cells ability to reduce 3-(4,5-dimethylthizol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (Bols et al. 2005). ATP can

be measured within cultures after exposure to excotoxicants (e.g. heavy metals and phenols). Less direct measurement such as MTT assays have demonstrated success with cell lines such as PLHC-1, however it has been found that MTT did not work well with some fish cell lines. This is possibly due to low succinate dehydrogenase activity in the mitochondria (Bols et al., 2005).

Another dye that has been used for cytotoxicity testing is crystal violet staining solution (CV), which stains cell cultures within a multi-well plate and then absorbance is measured to determine cell viability. The absorbance of stained cells does not always represent the viable number of stained cells, due to the un-specific nature of the stain which stains all cells present regardless of viability. Thus CV should be applied as part of a battery of tests to ensure accuracy of results. Chiba et al. (1998) utilized CV as part of a combination assay (NMC-assay) that also utilized MTT and NR staining methods and could be applied in succession to the same plate of cells. Feoktistova et al. (2016) however will be able to develop a consistent protocol for determining viability of cultured cells using CV. Adherent cells detach from cell culture plates during cell death and this characteristic is used for indirect quantification of cell death and for the determination of differences in proliferation following stimulation with toxic agents.

6 Investigation into Toxicity Occurrences at a Pulp and Paper Mill

6.1 Introduction

Pulp and paper mill effluent has several specific factors of concern during the evaluation of toxicity. These factors include: TSS which can act as a binding substrate for hydrophobic chemicals as well as cause physical toxicity due to fibres causing blockages in the gills of fish; COD which lowers the amount of dissolved oxygen within the effluent; effluent colour which limits the ability of sun light to penetrate the effluent; and resin and fatty acids (RFAs) which are one of the most common causes of toxicity.

In December of 2013, a mill in Terrace Bay, ON experienced three toxicity failures between the dates of December 2nd and December 11th (December 2, 9 and 11th). Samples collected from the outlet of the aerated stabilization basin (ASB) were sent to the Aquatic Toxicity Research Centre at Lakehead University in Thunder Bay, ON for 96 hour acute pass/fail toxicity testing using Rainbow Trout and 48 hour acute pass/fail testing using *D. magna*. The sample collected on December 11th was used to conduct 96 hour Trout and 48 hour *D. magna* multi-concentration tests. The sample collected on December 2nd exhibited 100% mortality in the trout tests and the samples collected on the 9th and 11th exhibited 90% mortality in the trout tests. The December 2nd sample exhibited 100% mortality at the 24 hour mark of the test. The December 9th sample exhibited 70% mortality at the 24 hour mark of the test and then 90% mortality at the 48 hour mark of the test. Finally the December 11th sample exhibited 40% mortality at the 24 hours mark of the test and 90% mortality at the 48 hour mark of the test. These results demonstrate a trend of reduced toxicity to trout in the effluent leaving the ASB outlet. There was no mortality or stress observed in the daphnia tests that were conducted.

Typically mortality in the trout tests occurs at the same time as mortality in the daphnia, as daphnia has been found to demonstrate similar toxicity to those of other model organisms including vertebrates (Guilhermino et al., 2000; Radix et al., 2000); however in this instance there was no

mortality in the daphnia tests conducted. This suggested that a high concentration of suspended fibre was the likely cause of mortality. It is possible for fibre to clog the gills of the fish causing mortality, without mortality occurring in *D. magna*. Fine fibre can cause mortality in the *D. magna* if it is able to get trapped underneath the exoskeleton; however larger fibres cannot fit under the exoskeleton and thus won't cause mortality.

The theory of increased suspended solids being the likely cause of mortality was supported by occurrences in the wastewater treatment facility prior to the failure. At the time of the failure one of the primary clarifiers was shut down which led to increase in pulp fibre (clarifier sludge) in the anaerobic zone of the ASB. Fibre in the anaerobic zone of the ASB is decomposed releasing chemicals compounds that were bound to the fibre.

The objective of this project was to determine the effects of the decomposition of excess amounts of fibre in the anaerobic zone of the ASB on the toxicity of effluent. It was hypothesized that as the toxicity of the effluent would increase as the amount of decomposed fibre in the ASB increased.

6.1.1 The Study Site

The Study site was at AV Terrace Bay, a kraft pulp and paper mill located in Terrace Bay, Ontario. Currently it has a water treatment system containing two primary clarifiers and an aerated stabilization.

In 1994 AVTB made updates to their wastewater treatment system to meet the requirements of Section 53 of the Ontario Water Resources Act (Appendix. A) The treatment system consists of two primary clarifiers and a three cell ASB (Figure 3).

In May of 2014 an assessment of the ASB was conducted (Figure 3) (TGCL, 2014). During the assessment the minimum dissolved oxygen increased from 0.00mg/L in cell 1 to a maximum of 7.79 mg/L in cell 3. There was neutral pH (7.28-7.93) in all 3 cells, with the outlet flow of cell 3 having a pH range of 7.53 to 7.83 (TGCL, 2014). The minimum pH of 2.3 was found in the Acid Sewer sample and the

maximum pH of 10.23 was found in the Clarifier Inlet sample. The Acid Sewer sample was found to have the lowest concentration of TRS compounds, while the ASB inlet sample was found to have the highest concentration, with relatively consistent concentrations being found throughout the ASB. The assessment also identified a layer of mud (fine sediment) accumulation 0.5-0.8m in depth at the bottom of the ASB (TGCL, 2014). This mud accumulation reduces the hydraulic retention time (HRT) with in the ASB, which was confirmed using a lithium tracer study. The assessment demonstrated a calculated HRT of 10.9 days, which is much shorter than the theoretical HRT of 14.2 days (TGCL, 2014). The assessment also demonstrated significant short circuiting, with effluent being capable of passing from cell 1 inlet to cell 3 outlet in as little as 4 days (TGCL, 2014).

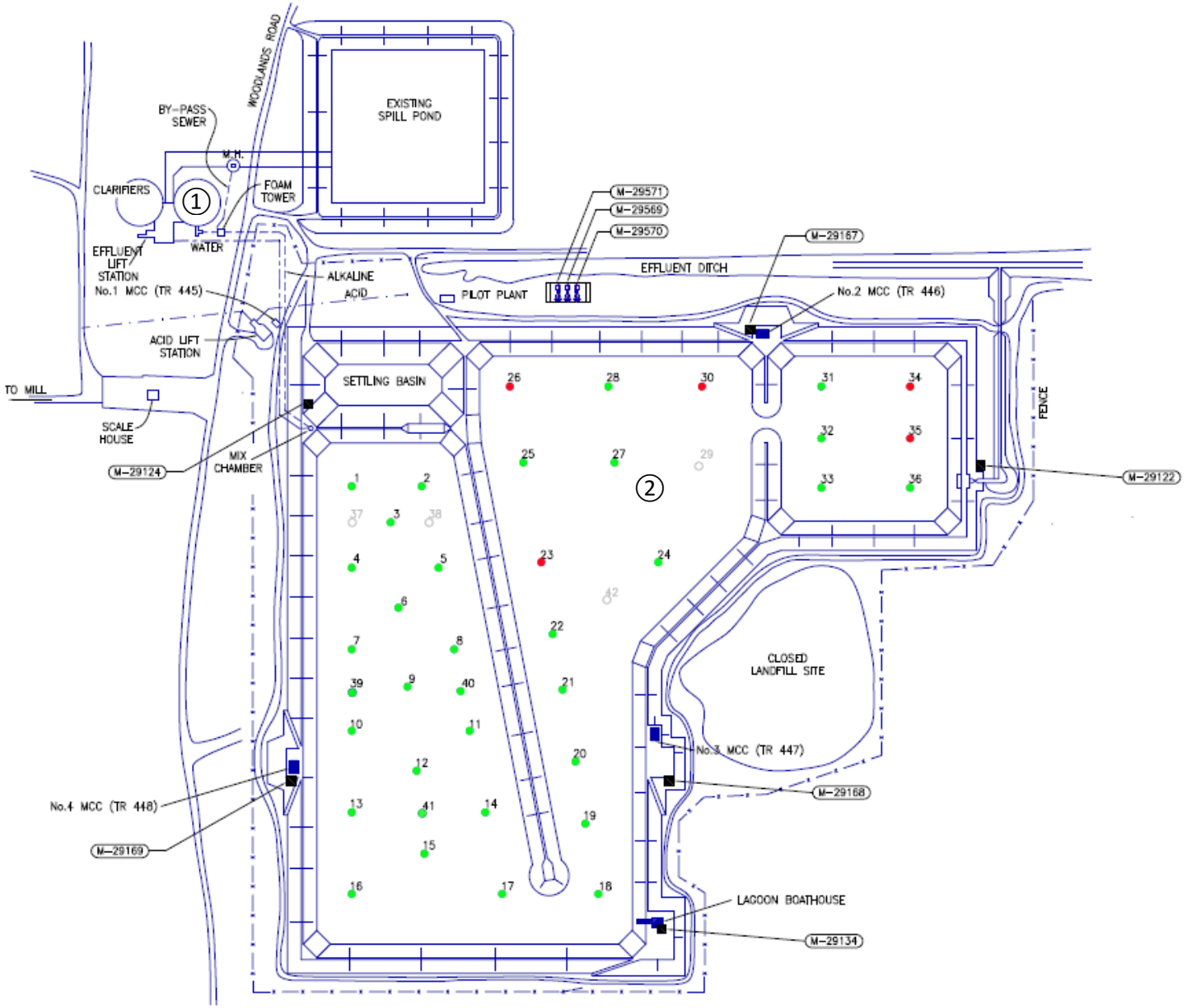

Figure 3. Water treatment system from AV Terrace Bay containing two primary clarifiers (1) and an aerated stabilization basisn with sub-surface aerators (2).

6.2 Methods

6.2.1 Historical Data

AVTB supplied their daily effluent monitoring for the year of 2013 and the data during the time of the incident was compared to the data for the rest of the year. Specifically the outlet BOD, pH and TSS were looked at during the time of the incident and the inlet BOD, pH and TSS were looked at for the 14 days prior to the incident. The outlet BOD at the time of the incident ranged from 46 to 96 mg/L with a mean of 60.6 mg/L, which is well within the yearly range of 12.5 to 310 mg/L (Table 5). The outlet pH ranged from 7.3 to 8.2 with a mean of 7.7 which is within the yearly range of 6.3 to 9.3 (Table 5). Finally the outlet TSS ranged from 70 to 104 mg/L with a mean of 82.6 mg/L, which is within the range for the rest of the year (13-170 mg/L) however it is above the annual mean of 51.4 mg/L (Table 5). This demonstrates that while outlet TSS was within the typical yearly range it did increase during the time of the incident.

Table 5. Comparison of 2013 yearly chemical data to those observed during the incident (12 days at the start of December 2013). Data includes average values for biological oxygen demand (BOD) in mg/L, pH, total suspended solids (TSS) in mg/L, and temperature (^{o}C).

Parameter	**Source**			
	ASB Inlet		ASB Outlet	
	Year (2013)	Incident	Year (2013)	Incident
BOD (mg/L)	252	212	52.88	60.6
pH	7.57	7.48	7.63	7.7
TSS (mg/L)	63.16	53	51.37	82.6
Temp. (^{o}C)	40.3	35.7	18.4	11.8

6.2.2 Sample Collection

Effluent samples were collected from the ASB inlet line leaving the primary clarifiers at AVTB. A sample of the partially treated effluent was sent to the Lakehead University Environmental Lab (LUEL) for chemical testing. Dried treatment bacteria were also obtained from AVTB. Typically the dried bacteria is activated in water and then added in with the ASB inlet supply to supplement or increase the bacteria level found within the ASB. The dried bacteria was added to water at a concentration of 37.7g/L to 1 L of water and left on a stir plate to mix for a period of 5 hours. After the mixing period the bacteria mixture was divided among 4 replicates of partially treated effluent with 250mL of bacteria mixture being added to 500mL of the partially treated effluent. The 4 effluent and bacteria mixtures were covered and left at room temperature to aerate with two replicates being left for 4 days and two for 14 days. These aeration times represent the minimum and maximum retention time within the ASB at AVTB, with the covering turning the mixtures into a more anaerobic environment. Following the aeration periods the samples were sent to LUEL for chemical testing of the effluent.

Upon examination of the historical data and the results of the treatment tests, it was determined that the TSS increase was a likely cause for mortality in the trout tests. To confirm this acute toxicity screenings were conducted on samples created in lab. Acute toxicity screenings are rapid acute toxicity tests that utilize fewer organisms and smaller volume of effluent. The samples used in the screening were collected from the ASB inlet (effluent that has only received primary treatment) and the ASB outlet (effluent that has received both primary and secondary treatment). A sample of pulp clarifier sludge (solids removed during primary treatment) was also collected from the clarifiers within AV Terrace Bay.

100L of effluent collected from the ASB inlet was divided into 4 replicates containing 25L of effluent. A sub sample of effluent was sent to LUEL for chemical analysis to determine the initial TSS within the untreated effluent (17mg/L) (Table 7). Acute toxicity tests were conducted on the four replicates, with 10 fish being used for each replicate. Two replicates were left unaltered, with TSS of

17mg/L and the other two had pulp clarifier sludge added in order to increase the TSS from 17 mg/L to 75mg/L and 105 mg/L respectively. Following the screening there was 10% mortality observed in one of the 17mg/L and the other 17mg/L showed 1 distressed organism. There was no mortality observed in the 75mg/L or the 105mg/L samples.

A further 50L (2 x 25L pails) of effluent was collected from the ASB inlet and 50L (2 x 25L pails) was collected from the ASB outlet. These samples were divided into 4 replicates containing 10L of effluent. 100g of pulp clarifier sludge was added to 2 replicates from the ASB outlet and 2 replicates from the ASB inlet (Table 7). The other two replicates were left as collected to represent "control" samples from those locations. Following the addition of the sludge, all replicates were sealed with no head space and left in closed buckets in order to create anaerobic environments which facilitate the breakdown of the sludge and suspended solids releasing bound contaminants. One control replicate and one sludge replicate from both the inlet and the outlet were left sealed for four days and then other control and sludge from the inlet and the outlet were left sealed for 14 days in order to mimic the minimum and maximum retention time within the ASB of AVTB (Table 6). Once the replicates had been left for the appropriate time period a one litre sub-sample was collected and sent to LUEL for analysis, the remainder of the sample was divided into two four litre replicates and an acute toxicity screening was conducted using 3 fish/4L of sample.

Table 6. Test setup for testing the effects of incubation time on the toxicity of effluent caused by the breakdown of pulp clarifier sludge in an anaerobic environment. The effluent was collected from the inlet and outlet sources of the aerated stabilization basin from a kraft pulp mill.

Incubation Time Period	**Control**		**Sludge (Effluent + 100g of clarifier Sludge)**	
4 Day	Inlet (2 X 5L)	Outlet (2 X 5L)	Inlet (2 X 5L)	Outlet (2 X 5L)
14 Day	Inlet (2 X 5L)	Outlet (2 X 5L)	Inlet (2 X 5L)	Outlet (2 X 5L)

6.3 Results

The analysis of the four and 14 day treated effluent demonstrated a five times increase in TSS from untreated effluent to the four day treated effluent and a 10x increase in TSS from the untreated effluent to the 14day treated effluent (Table 8). Sulfur compound also compounds also demonstrated increases two to four times higher than the value measured in untreated effluent (Table 7). There was also an increase in conductivity; however this increase is associated with the increase in the level of suspended solids.

Table 7. Evaluation of the chemical levels observed during the recreation of the bacterial treatment of effluent occurring in the aerated stabilization basin (ASB) of a kraft pulp and paper mill. Effluent was exposed to bacteria that are used to supplement the bacterial colonies within the ASB for periods of either 4 days or 14 days, which were the minimum and maximum retention times of effluent with the ASB. Concentrations consist of the average values of two replicates of each treatment.

Chemical	Untreated	4 Day Treatment	14 Day Treatment
Conductivity (mg/L)	1381	3430	5145
Dissolved Organic Carbon (mg/L)	202	485	721
N-NH4+NH3 (mg/L)	0.09	17	168
Nitrite NO2-N (IC) (mg/L)	not tested	<0.009 mg/L	2.95*
Nitrate NO3-N [IC] (mg/L)	not tested	0.328	3.49
Sulfur (mg/L)	64	211	155
Sulphate (SO4) [IC] (mg/L)	159	514	634
Total Dissolved Solids (mg/L)	1187	3130	4407
Total Nitrogen (mg/L)	0.178	39.5	333.9
Total Phosphorous (mg/L)	0.54	140	100
Total Suspended Solids (mg/L)	17	83	183

*1 of the 2 replicates was below the detection limit

There was no mortality or stressed organisms observed in any of the screening tests. There were also no significant differences in the initial chemical parameters measured in the sludge and control replicates. It is interesting to note that three of the four sludge samples showed lower DO than their control counter parts (Table 8). The only exception was the 14 day inlet sample, where the control had a DO of 0.5mg/L while the sludge had a DO of 1.9mg/L (Table 8). The analysis conducted by LUEL also demonstrated no significant difference in the solids content between all of the control and sludge

replicates regardless of the time spent sealed. The inlet samples demonstrated approximately half as much total sulfur as the outlet samples. This information combined with the chemical of analysis of the clarifier sludge revealing high concentrations of total recoverable sulphur (> 1000 µg/g) led to the conclusion that sulphur compounds were likely related to the cause of mortality. The excess clarifier pulp fibre entering the ASB during the incident would have increased the amount of sulphur being released into the ASB via the breakdown of fibre.

Table 8. Chemical parameter measured in the ASB inlet and outlet samples after 4 and 14 day exposures. Initial parameters were measured in control and sludge samples, with measurements taking place prior to the aeration period at the start of the test.

	Inlet				Outlet			
	4 Day		14 Day		4 Day		14 Day	
Parameter	Control	Sludge	Control	Sludge	Control	Sludge	Control	Sludge
Temperature (°C)	15.3	15.1	14.8	15.2	14.7	14.7	14.5	14.6
pH	6.89	6.61	6.79	6.63	6.98	6.97	6.91	7.14
Dissolved Oxygen (mg/L)	6.4	4	0.5	1.9	6.6	3.7	3.6	3.3
Condctivity (µS/cm)	1223	1201	1173	1164	1544	1497	1442	1420

6.4 Discussion

The results indicate that mortalities in the rainbow trout where likely caused by the breakdown of excess fibre from the clarifiers within the ASB, specifically in the anaerobic zone of the ASB. During the incident the breakdown of the excess fibre likely led to an increase in the BOD within the ASB and a study conducted by Liang (2008) demonstrated that an increase in the degradation of organic materials leads to an increase production of reduced sulphur compounds. Liang (2008) also found that dimethyl sulphide (DMS) and other total reduced sulphur compounds were positively correlated with TSS. The higher TSS at the time of the incident likely would have led to an increase in the TRS compounds. Other TRS compounds included hydrogen sulphide (H_2S), dimethyl disulphide (DMDS), and methyl mercaptan (CH_3SH, MM). The ASB does contain sulphate reducing bacteria (SRB) and other bacteria that can alter the sulphur compounds within the effluent; however these bacteria typically have an optimum

temperature range of 28°C – 32°C and the critical DO concentration for SRBs is 0.1-1.0 mg/L (Liang, 2008). The incident occurred in December causing the average effluent temperature to drop to 12.5°C, which is significantly lower than the optimum temperature range. Oxygen solubility is also increased at lower temperatures which would have likely led to an increase in dissolved oxygen during the incident. These factors facilitate an increase in the concentration of TRS compounds within the ASB.

It was concluded that the increase in TRS compounds was cause of the mortality in the effluent; specifically the compounds DMDS and MM. MM and DMDS are extremely common compounds found within pulp and paper effluent and have been found to have boiling points of 5.8°C and 118°C respectively (Iliuta and Larachi, 2007a). Given that the average effluent temperature was 12.5°C the DMDS compounds would have remained in solution and then MM compounds would have stayed in solution longer and not been lost to evaporation as rapidly as at warmer temperatures. The effluent would have stayed below 16°C from sample collection until completion of the acute toxicity test, as samples must be kept between 1°C and 8°C during transport and storage prior to testing and a temperature of 15 ± 1°C is required for the trout tests (EPS 1/RM/09). The daphnia tests however are conducted at a temperature of 20°C ± 2°C, which would have increased the rate at which MM evaporates out of solution (EPS 1/RM/11). MM has also been found to easily react with oxidants to from DMDS, which means that the 90 minute aeration time (@ 6.5 ± 1.0 mL/min /L) prior to testing (EPS 1/RM/09), would have caused a conversion from MM to DMDS, as opposed to causing the loss of the sulphur compounds.

TRS compound such as H_2S, MM and DMDS are highly toxic and corrosive gases. For example at 20 µL/L H_2S is capable is capable of irritating the eyes and respiratory tract. Thirty minutes of exposure to H_2S at 500 µL/L can cause severe sickness, whereas 30 minutes of exposure at 1000 µL/L can cause death (Iliuta and Larachi, 2007b). It also appears that MM and DMDS are capable of eliciting similar

toxic effects to H_2S; however they require slightly higher concentrations to do so (Iliuta and Larachi, 2007b).

The results of the tests point to total reduced sulphur compounds as the likely cause of the toxicity failure, however their volatile nature can make testing using traditional methods difficult especially at lower concentrations that require longer exposure periods. Traditional *in vivo* methods require the tests be conducted within specific temperature ranges as well as other factors such as exposure to light and the addition to oxygen, which can alter the test chemical. These concerns have led for a need for alternative testing methods that allow for more flexible testing methods which enable more controlled chemical exposures.

7 Evaluating Cell Cultures for Use in Pulp and Paper Toxicity Testing

7.1 Introduction

Alternative testing methods will not only assist in the testing of volatile chemicals, but they are also able to supplement or replace testing for all toxicity tests, especially in the area of regulatory testing. Currently researchers are attempting to develop more modern and efficient techniques for regulatory toxicity testing. In Canada, regulatory testing is mandatory for all major industries. This testing ensures that effluent being released by industry is not harmful to the environment in which it is being released.

Cell cultures are one of the more promising alternatives as they can be conducted in rapid succession and allow for more controlled exposures. The limited effluent required as well as the smaller testing and area allow for more tests to be conducted while minimizing waste. One type of cytotoxicity assay is the crystal violet staining method and while the unspecific nature of the CVS has made it more suited for use in a battery of tests; Feoktistova et al. (2016) developed a CVS assay that was used for testing cell viability of mammalian cell lines following exposure to pharmaceuticals. The objective of this study was to determine if the CVS assay developed by Feoktistova et al. (2016) could be applied to piscine cell lines as an alternative for testing toxicity of pulp and paper effluents. Additionally this study aimed to increase the amount of available test data utilizing cell lines derived from fathead minnows.

Laboratories that conduct *in vivo* studies are required to perform routine reference toxicant testing to ensure that the batch of fish being used for testing are at an acceptable standard. Reference toxicant tests expose a random subset of the batch of fish to a standardized chemical and measure the response of the organism following exposure (EPS 1/RM/13). The exposure concentrations cover a range that will cause the desired effect (e.g. LC_{50}, IC_{25}, etc.) (EPS 1/RM/13). The results of these tests are recorded graphically and used to generate a Shewhart chart for quality control (QC) (Koutras et al., 2007). The mean result is marked and warning limits are placed at ± 2 SDs from that mean result. The

results of future reference toxicants are added to the QC chart and as long as the result is within the warning limit then the batch is acceptable for use in toxicity testing (EPS 1/RM/46). The mean and warning limits of these QC charts are constantly being updated with new data to ensure that the chart accurately represent the consistency of results achieved by the lab (EPS 1/RM/46). In addition the warning limits on the QC charts there is also a control limit at ± 3 SDs. If the result of the reference toxicant falls between the ±2 SD warning limit and the ±3 SD control limit then batch may still be used for testing; however QA/QC requirements state that the result must include an action plan outlining why such a large deviation occurred and how it will be corrected (EPS 1/RM/46). If the result falls outside of the ±3 SD control limit then the results fail and the batch cannot be used for testing until a passing reference toxicant test has been conducted on the batch.

7.2 Methods

Tests were conducted using cell lines derived from fathead minnow liver (FHM-L), rainbow trout gills (RTgill-W1), and rainbow trout livers (RTL-W1) that had been previously acquired by Dr. David Law and had been stored at -80^{o}C since 2009. The FHM-L, RTgill-W1 and RTL –W1 cell lines were grown in 25cm^2 or 50cm^2 flasks containing Leibovitz's L15 media with 1% (v/v) 100x penicillin/streptomycin and 5% (v/v) fetal bovine serum (FBS). Flasks of cells were incubated at 27^{o}C and 22^{o}C (both rainbow trout cell lines) respectively until the cells gained confluence.

Subcultivation occurred when cells reached 80-90% confluence. Cells were detached from the flask using a 5minute exposure of TrypLE and then L-15 media was added to deactivate the TrypLE. The cell solution was centrifuged at 1,000 x g for 7 minutes. Supernatant fluid was and then the pellet was resuspended in 10mL of fresh media and passaged into 1 or 2 sterile flasks. All cell culture reagents were tissue culture grade, autoclaved or filter sterilized prior to use.

Prior to the testing period, the cells were removed from the growth surface using TrypLE, centrifuged and resuspended in L15media with FBS. Cell Density was calculated using a hemocytometer and then cells were seeded in 96-well plates at a density of 32,000 cells/cm^2. The cells were then incubated for 96hours to gain confluence.

7.2.1 Cell Exposure Period

Plates containing confluent cells were loaded with varying concentrations of test solutions at 200 µL per well. Test solution concentrations were calculated by using the literature value for *in vivo* LC_{50} or IC_{25} to develop a 50% dilution series that included test concentrations above and below the literature value. Plates were exposed to the test solution and incubated at their respective incubation temperatures. The exposure period varied based on the type of test being performed (acute or chronic) and following the exposure period cells were washed 3x with distilled de-ionized water (DDW). Cell viability was calculated using the crystal violent staining (CVS) assay developed by Feoktistova et al. (2016). Cells were stained with 50 µL of 0.5% crystal violet and incubated for 20 minutes at room temperature. Following the staining period plates were washed 3x with DDW and left to dry for 24 hrs at room temperature. Once dry, 200 µL of methanol was added and cells were incubated for 20 minutes at room temperature to suspend the dye. Following dye suspension optical density (OD) was measured in triplicate at 570nm using a microtiter plate reader. The absorbance values were then used to calculate the percent viability of the cells using the following equation:

$$\%viability = \frac{Test\ concentration\ OD_{570}}{Control\ OD_{570}} * 100$$

The absorbance of the control concentrations is set to 100% viability and is used as the standard to confirm viability of the test concentrations. The % viability for each concentration of the tests was used to calculate the LC_{50} and IC_{25} values.

Chemical toxicity data was obtained from the ECOTOX database. The ECOTOXicology (ECOTOX) knowledgebase is a source for locating single chemical toxicity data for aquatic life, terrestrial plants and wildlife. ECOTOX was created and is maintained by the US.EPA's National Health and Environmental Effects Research Laboratory's (NHEERL's) Mid-Continent Ecology Division (MED).

Literature data for the standardized chemicals was used to calculate a mean result (i.e. LC_{50}), as well as its associated warning and control limits in order to generate a QC chart representing *in vivo* data. The results from all of the cytotoxicity assays were then plotted on these control charts enabling the determination of the viability of the assay as an alternative.

Acute tests had an exposure period of 24 hours (1/4 of the 96hr *in vivo* test time) and were conducted using either sodium chloride (NaCl) or sodium nitrate ($NaNO_3$). Test solutions were divided into 5 concentrations and a control (Table 9), with 14 replicates per concentration. The chronic tests had an exposure period of 42 hrs (1/4 of the 7 day *in vivo* test time) and were conducted using NaCl.

Table 9. Dilution series for both the acute and chronic tests conducted using NaCl (g/L) and $NaNO_3$ (mg/L). The stock solution (#5) was prepared and the solutions 1 through 4 were created by diluting the #5 solution with culture media.

Test Solution #	Concentration of NaCl (g/L)	Concentration of $NaNO_3$ (mg/L)
5 (100%)	13.33	2700
4 (50%)	6.67	1350
3 (25%)	3.34	675
2 (12.5%)	1.67	338
1 (6.25%)	0.835	169
Control (0%)	0	0

7.3 Results

7.3.1 Cell Viability Assay Results

The cell viability assays were conducted for all 3 cell lines and demonstrated variable results. During the acute NaCl tests the RTL-W1 cell line demonstrated an average cell viability of 76.57% in response to 0.68 g/L of NaCl; however the cell viability of individual tests ranged from 60.06% viability to 87.00% viability. In response to 10.77 g/L NaCl the average viability for RTL-W1 was 66.43% with individual test values ranging from 18.63% viability to 100% viability. This discrepancy between individual test values was also observed in tests conducted with the other cell lines. RTgill-W1 demonstrated an average viability of 83.33% in response to 0.66 g/L NaCl with a range of 49.89% viability to 100% viability; while the FHM-L cell line demonstrated an average viability of 85.25% viability in response to 0.68 g/L NaCl with a range of 32.29% viability to 100% viability. This variability in results between individual tests was also observed in the acute $N\text{-}NO_3$ results and the chronic NaCl results (Table 10).

7.3.2 Acute Test Results

Acute NaCl tests were conducted using FHM-L, RTL-W1 and RTgill-W1 and the cell viability was used to calculate the LC_{50} for these tests. However not all tests were able to have an LC_{50} derived from them. FHM-L had 71.4% of the tests conducted produced quantifiable results, while 75% of RTL-W1 tests and 66.7% of RTgill-W1 tests produced quantifiable results (Table 11). The FHM-L cell line had a tested LC_{50} value of 5.25g/L NaCl and ranged from 4.46 g/L – 5.97 g/L NaCl. The *in vivo* literature LC_{50} for fathead minnows was 7.25 g/L NaCl, with a warning limit of 4.04-10.45 g/L NaCl (± 2SD) (Figure 4). The RTL-W1 cell line had a tested LC_{50} value of 1.62 g/L NaCl and ranged from 0.244 g/L – 3.981 g/L NaCl (Figure 4). The RTgill-W1 cell line had a tested LC_{50} of 11.58 g/L NaCl and ranged from 10.31 g/L – 12.84 g/L NaCl (Figure 4). The *in vivo* literature LC_{50} for rainbow trout was 8.92 g/L NaCl, with a warning limit of 0 – 20.23 g/L NaCl (± 2SD). Since the tested LC_{50} values fall within the warning limits for the *in vivo* tests these values can be seen as an acceptable method for conducting NaCl tests (Figure 4).

Table 10. The **c**ell viability measurements for RTL-W1, RTgill-W1 and FHM-L in response to the lowest and highest test concentration following acute and chronic exposures to NaCl (g/L) and acute exposures to N-NO_3 (mg/L).

Test Type	Tested Chemical	Cell Type	Test Concentration	Average Cell Viability Measure	Minimum Cell Viability Measured	Maximum Cell Viability Measured
Acute	NaCl	RTL-W1	0.68 g/L	76.57 %	60.06 %	87.99 %
			10.77 g/L	66.43%	18.63%	100%
		RTgill-W1	0.66 g/L	83.33%	49.98%	100%
			10.62 g/L	53.61%	49.29%	56.86%
		FHM-L	0.68 g/L	85.25%	32.29%	100%
			10.86 g/L	35.76%	10.76%	70.20%
	N-NO_3	RTL-W1	212 mg/L	71.42%	54.00%	83.59%
			3406 mg/L	90.37%	79.37%	100%
		FHM-L	212 mg/L	80.16%	53.73%	100%
			3406 mg/L	47.52%	24.67%	62.56%
Chronic	NaCl	RTL-W1	0.42 g/L	97.70%	90.80%	100%
			6.67 g/L	85.70%	50.70%	100%
		RTgill-W1	0.52 g/L	94.19%	59.35%	100%
			8.26 g/L	79.33%	52.71%	91.19%
		FHM-L	068 g/L	76.57%	60.06%	87.99%
			10.77 g/L	66.43%	18.63%	100%

Table 11. Outline of the number and percentage of tests that had quantifiable LC_{50} and Ic_{25} values for cell lines experiencing acute and chronic exposures to NaCl and N-NO_3.

Test Type Conducted	Chemical Tested	Cell Line Used	# of Tests Conducted (n)	# of Tests with Quantifiable LC_{50} Values (n)	Percentage of Tests with Quantifiable LC_{50} Values (%)
Acute	NaCl	RTgill-W1	3	2	66.7
		RTL	4	3	75
		FHML	7	5	71.4
	N-NO_3	FHML	4	3	75
		RTL	4	3	75
Chronic	NaCl	FHML	4	3	75
		RTgill-W1	7	3	43
		RTL	4	4	100 (50[a])

[a] 50% of the IC_{25} values generated were below the -3 standard deviation control limit.

Acute N-NO_3 tests were conducted using both FHM-L and RTL-W1, with 75% of the tests conducted for both cell lines producing quantifiable results (Table 11). The FHM-L and RTL-W1 produced tested LC_{50}s of 2666 mg/L (2423-2872) and 26.25 mg/L (6.62-239.32) respectively (Figure 5). The *in vivo* literature LC_{50} was 1341 mg/L with warning limits of 844.96 – 1837 mg/L. Both the FHM-L and RTL-W1 tested LC_{50} values were not only outside of the ±2 standard deviation (SD) warning limits but also the ±3 SD control limit, signifying that currently the test method cannot be used for conducting tests with N-NO_3 (Figure 5).

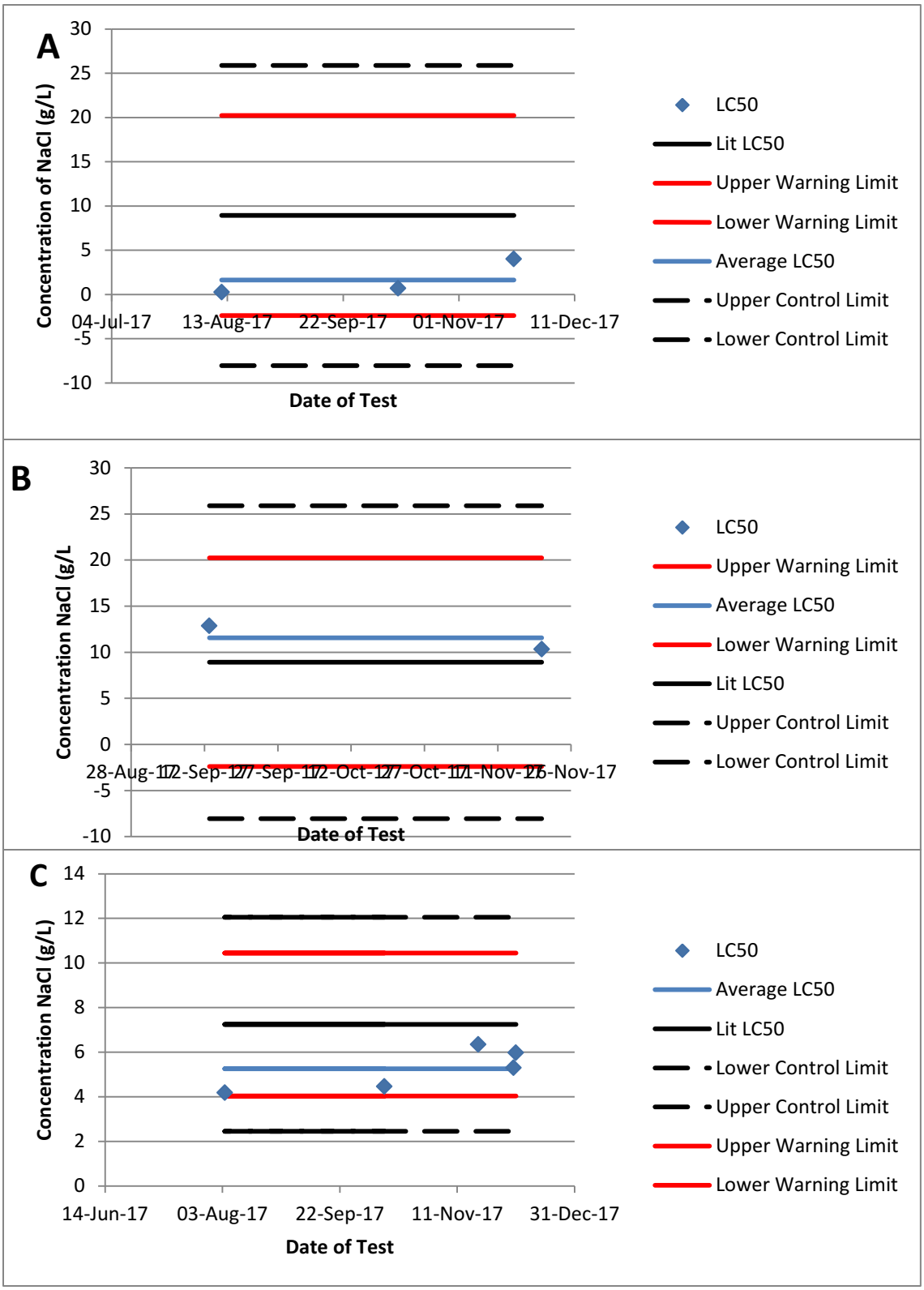

Figure 4. Comparison of literature derived LC_{50} values to those calculated from test exposures to NaCl using 3 cell lines. Tests were conducted using RTL-W1 (A), RTgill-W1 (B), and FHM-L (C) and were plotted alongside the literature average LC_{50} as well as its respective warning (±2 SD) and control (±3 SD) limits.

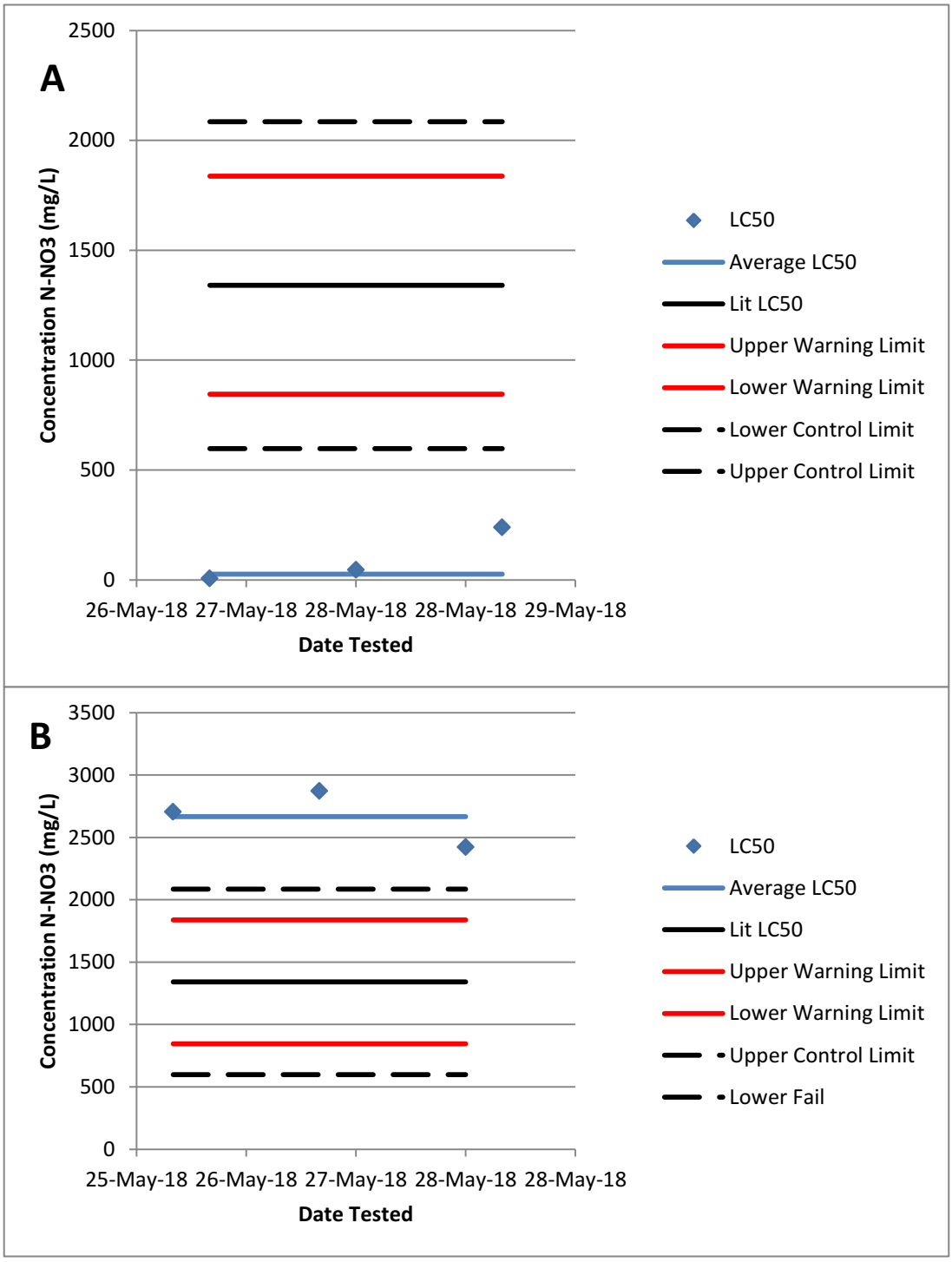

Figure 5. Comparison of literature derived LC_{50} values to those calculated from test exposures to N-NO_3 using 2 cell lines. Tests were conducted using RTL-W1 (A) and FHM-L (B) and were plotted alongside the literature average LC_{50} as well as its respective warning (±2 SD) and control (±3 SD) limits.

7.3.3 Chronic Test Results

Chronic NaCl tests were conducted using FHM-L, RTL-W1, and RTgill-W1l and had 75%, 100% and 43% of the tests conducted produce quantifiable results (Table 11). It is important to note that while 100% of the RTL-W1 tests conducted produced quantifiable results, 50% of the generated IC_{25}S were below the -3 SD control limit; however both the IC_{25} generated using all of the data (1.60 g/L) and the IC_{25} generated using only the data within the control limits (2.4 g/L) were within the control the warning limits outlined in the literature. As only an IC_{25} value (1.17 g/L NaCl) and not the associated warning limits could be derived from the literature for rainbow trout, the RTL-W1 and the RTgill-W1 test data were compared to both rainbow trout and fathead minnow *in vivo* data. The FHM-L had a tested IC_{25} of 2.49 g/L NaCl and ranged from 0.081 g/L to 5.896 g/L (Figure 6). The *in vivo* fathead minnow data demonstrated an IC_{25} value of 1.77 g/L NaCl with warning limits of 0.64 – 2.9 g/L NaCl. The RTL-W1 cell line had a tested IC_{25} of 1.60 g/L NaCl and ranged from 0.001 – 2.428 g/L NaCl (Figure 6). The RTgill-W1 cell line had a tested IC_{25} value of 3.61 g/L NaCl and ranged from 1.14 – 7.32 g/L NaCl (Figure 6). The FHM-L and RTL-W1 cell lines had IC_{25}s within the control limits for fathead minnows; however the RTgill-W1 cell line IC_{25} was just above the upper control limit (3.47 g/L NaCl). While the RTgill-W1 line is outside of the control limits and thus does not provide reliable test data, both the RTL-W1 and FHM-L IC_{25}s were inside the warning limits signifying their ability to be used as an alternative to chronic toxicity testing for NaCl.

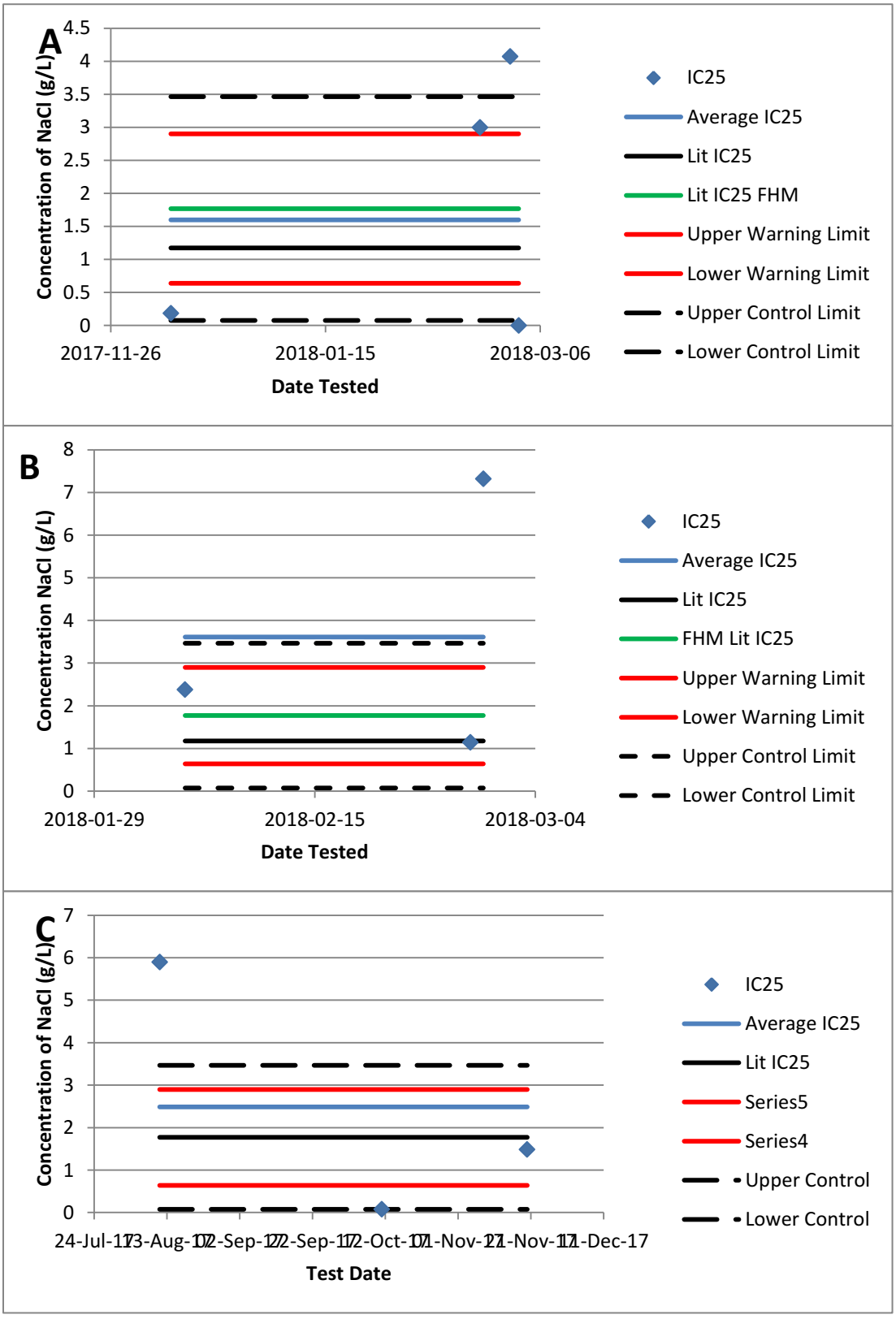

Figure 6. Comparison of literature derived IC_{25} values to those calculated from test exposures to NaCl using 3 cell lines. Tests were conducted using RTL-W1 (A), RTgill-W1 (B), and FHM-L (C) and were plotted alongside the literature average IC_{25} as well as its respective warning (±2 SD) and control (±3 SD) limits. Since only a single IC_{25} value was able to be derived from the literature, the rainbow trout cell lines were compared to the rainbow trout IC_{25} and the fathead minnow IC_{25} and its associated control limits.

7.4 Discussion

The results of the cell viability assays demonstrated large inter-test variability. With standard deviations for acute NaCl test ranging from ±12.65% viability for the RTgill-W1 line to ±24.34% viability for FHM-L cell line and for the acute N-NO_3 the standard deviation ranged from ±13.97% viability for the RTL-W1 cell line to ±17.65% for the FHM-L cell line. Chronic NaCl tests had cell viability that ranged from ±13.80% viability for the RTgill-W1 cell line to ±19.75% viability for the FHM-L. Given that tests with <50% viability would be treated as a failing test; deviations of this margin can cause large discrepancies in results (EPS 1/RM/13). Thus currently a single assay is not powerful enough to accurately represent the results exhibited *in vivo*. However given the small volume of effluent required for this method, 17mL as opposed to 20-25L for the *in vivo* method, numerous tests can be performed while still using only a small amount of sample.

The viabilities were used to calculate lethal (LC) and inhibitory (IC) concentrations in order to enable to the comparison of the *in vitro* results to those that are generated *in vivo*. These comparisons enable the evaluation of the effectiveness of the CVS assay as an *in vitro* alternative and were conducted using standardized quality control methods used during *in vivo* testing (EPS 1/RM/46).

For the acute NaCl tests the FHM-L results had an average LC_{50} of 7.25 g/L and the results of each test fell between the literature LC_{50} of 8.92 g/L and the lower warning limit of 4.04 g/L (Figure 4). The acute NaCl tests for RTgill-W1 had a tested average LC_{50} of 11.58 g/L and all of the test results fell between the literature LC_{50} of 8.92 g/L and the upper warning limit of 20.23 g/L (Figure 4). While the RTL- W1 line had a tested averaged of 1.62 g/L and all of the tests results were between the literature average and the lower warning limit of -2.39g/L NaCl (Figure 4). RTL-W1 however had two tests with an LC_{50} of < 1.0g/L and one with an LC_{50} of 3.9g/L (Figure 4). Demonstrating that while the test method is capable of accurately representing the results exhibited *in vivo* the testing power of an individual test is

not strong enough to accurately represent *in vivo* results 100% of the time and thus several replicates should be performed.

Acute tests with $N\text{-}NO_3$ had less promising results however. RTL-W1 had a tested LC_{50} of 26.25 mg/L $N\text{-}NO_3$, which falls below both the literature average of 1341mg/L $N\text{-}NO_3$ and the lower control limit of 596.94 mg/L $N\text{-}NO_3$ (Figure 5). While the FHM-L had a tested average of 2666.76mg/L which was fell above both the literature average of 1341mg/L $N\text{-}NO_3$ and the upper control limit of 2085.06 mg/L $N\text{-}NO_3$ (Figure 5). The $N\text{-}NO_3$ toxicity date was not available on the ECOTOX database or for Rainbow Trout, thus both cell lines were compared to fathead minnow toxicity data collected by Scott and Crunkilton (2000), with warning and control limits being derived from the LC_{50} data published in their article. Since all of the RTL-W1 results fell below the lower control limit and all of the FHM-L data fell above the upper control limit, neither the RTL-W1 nor the FHM-L cell line results are capable of predicting the *in vivo* response of fathead minnows to acute $N\text{-}NO_3$ exposure.

With regards to the chronic test results, the FHM-L line had an average IC_{25} of 2.49g/L NaCl, which is between the literature average of 1.77g/L and the literature upper warning limit of 2.90g/L (Figure 6). It is important to note that one of the test results was just inside the lower control limit of 0.075g/L and another was outside of the upper control limit of 3.47g/L. In the ECOTOX database there was a single result for chronic NaCl exposure to rainbow trout, thus RTgill-W1 and RTL-W1 were compared to both the rainbow trout IC_{25} value (1.17g/L NaCl) and the fathead minnow IC_{25} value (1.77g/L NaCl) and its associated control limits (Figure 6). The RTgill-W1 cell line had a tested average $IC_{25}$of 3.61g/L NaCl which fell above the upper control limit for fathead minnows of 3.47g/L (Figure 6). It should be noted that one of the tests had an IC_{25} value of 7.3g/L NaCl and with this outlier removed the average IC_{25} of the remaining tests is 1.76g/L NaCl (Figure 6). The RTL-W1 cell line however had a tested average IC_{25} of 1.60g/L NaCl which fell between the rainbow trout average of 1.17g/L NaCl and the

upper warning limit for fathead minnows (2.4g/L) (Figure 6. While the average is value is well within the acceptable control limits, 50% of the tested IC_{25}values were below the 0.075g/L lower control limit for fathead minnows. The chronic NaCl results further support the conclusion that the CVS assay using fish cell lines is capable of representing *in vivo* responses of fish; however the power of individual tests is limited due to the range of variation between individual tests.

CVS assays are an unspecific assay with CV staining both viable and non-viable cells and while the CVS method being utilized is designed in such a way that non-viable cells would be lost during the washing process due to their lack of adherence, it is still possible that non-viable cells may still adhere to the bottom of the plate during the assay. Chiba et al. (1998) found that results of CVS assays are variable when cells are grown uniformly on the bottom of the plate due to both viable and non-viable cells being present. It has been recommended that the CVS staining assay be utilized as part of a battery of tests using multiple dyes to accurately access different factors associated with cell death (Chiba et al. 1998). Another solution for the CVS assay variability would be to increase the number of replicates, as more this would give a greater understanding of the cellular response to the stimulus.

The method developed by Feoktistova et al. (2016) was designed for evaluating the response of mammalian cell lines to pharmaceutical compounds. Specifically in the method they outline the example of exposing an aneuploid immortal keratinocyte cell line (HaCaT) to 2-20 µg of poly [I:C]. The application of this method to fish cell lines enables rapid assessment of environmental contaminants with minimal waste production. The variability in results is a concern however increasing the number of replicates conducted can increase the accuracy of the reported result. One major area of concern is the type of cell line the assay is being applied to. Schirmer (2006) conducted a review of the literature on *in vitro* assays conducted using piscine cell lines and one the studies found that rainbow trout liver (R1) was most sensitive to toxicants when tested using a CVS assay and in a number of cases it was more sensitive

than fish. These findings were supported by the CVS assay for $N\text{-}NO_3$ where RTL-W1 was found to have responses at concentrations well below the lower control limit.

Moving forward the CVS assay method developed by Feoktistova et al. (2016) shows some promise for use with piscine cell lines. A more detailed study needs to be conducting utilizing multiple cell lines as well as a larger range of contaminants in order to properly access the capabilities of this method. This study used cell lines that were available however prior to their use in the project the cell lines being tested had been stored in a -80^{o}C freezer for a period of 6-9 years, which may have impacted the long term viability of the cell line. Specifically issues arose with growth and viability of the RTgill-W1 cell line, which is why it was not utilized during any of the $N\text{-}NO_3$ testing. The FHM-L cell line produced some of the more promising results throughout the project and was also the most prolific and consistent of the three cell lines being tested. Since few studies have been published using the FHM-L cell line, further studies applying this cell line to a variety of different chemicals and test methods would further improve its use as an *in vitro* alternative.

8 Conclusions

Current testing methods are most effective means of determining effluent toxicity, however they are costly and time intensive, and do not provide enough information to determine what is actually happening to the organism. The biological requirements of the test organisms also make it difficult to accurately test many compounds, especially low doses of volatile chemicals. Thus alternatives methods are required to not only reduce and replace the use of animals in testing, but to also enable more advanced studies of complex compounds such as volatile chemicals. *In vitro* assays utilizing cells cultures have been demonstrated to be on promising alternative. Cell cultures allow for more controlled testing environments, with factors such as light and oxygen being able to be controlled more closely that with whole organisms. Volatile chemicals can also be more accurately tested with individual wells being able

to be sealed to form a closed system. Kramer et al. (2010) also had success with the use of polydimethylsiloxane (PDMS) sheets, which could be loaded with test chemicals and place in the test well to enable continuous release of chemical. The system was able to reach equilibrium within 24 hours and the sheets could maintain media concentration for >72hours. This system worked even for very hydrophobic chemicals such as benzo(*a*)pyrene. While cell cultures have shown promise as an alternative, refinement is still needed before they can be utilized in the assessment of failures such as those that occurred at AVTB.

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10 Appendix A

In 1994 many pulp and paper mills had to upgrade their waste water treatment plants to meet the requirements of Section 53 of the Ontario Water Resources Act. In order to meet these requirements AVTB upgraded their water treatment system to include the following components:

To their Collection and Transmission System:

- One alkaline sewer effluent collection system consisting of a collection sump, screens and three pumps for collection and transfer of combined alkaline sewer effluent to the primary clarifiers;
- One acid sewer effluent collection system consisting of an approximately 250 meter long, 915 millimeter diameter polyethylene pipe for transfer of acidic effluent from bleach plant to the acidic effluent lift station;
- One acid effluent lift station consisting of three pumps (one standby), each rated at 20.8 cubic metres per minute, for the transfer of acidic sewer effluent to the mix chamber of the aerated stabilization basin (ASB);
- One uncontaminated wastewater collection and transmission system for diversion of approximately 13.2 cubic metres per minute of uncontaminated wastewater to the storm sewer or aerated stabilization basin;
- One foul condensate steam stripping system consisting of a foul condensate storage tank, one preheater, one 14.6 metre high and 1.22 metre diameter stripping tower, one reflux condenser and a vapour superheater for removal of over 90% methanol and over 95% reduced sulphur compounds;
- One emergency spill pond with a volumetric capacity of 49,000 cubic metres;

To their Primary and Secondary Treatment System:

- Two reactor-type primary clarifiers operating in parallel, each having 45.7 metres in diameter and 4.27 metres in water depth with an upflow rate of 34.63 cubic metres per square metre per day, with effluent being discharged to the clarifier outlet structure;
- One acid sewer effluent neutralization system consisting of a scrubber slurry control valve, a 3785 litre mud mix tank, pH indicator, recorder and controller;
- One foam control tower;
- One mix chamber to blend acid effluent from the lift station and the alkaline effluent from the primary clarifiers;
- One concrete line settling basin with a volumetric capacity of approximately 47,300 cubic metres to provide additional solids removal from the effluent before the stabilization basin;
- One aerated stabilization basin consisting of three cells operating in series with approximate capacities of 499,600 cubic metres, 484,500 cubic metres and 151,400 cubic metres to provide a hydraulic retention time of eight days at a flow rate of 142,000 cubic metres per day;
- Thirty-six aerators in the aeration stabilization basin having a total power rating of 1857 kiloWatt;
- One extended aeration type packaged sanitary sewage treatment plant complete with chlorination chamber, having a design flow rate of 7 cubic metres per hour, with treated effluent being discharged to alkaline sewer system prior to transfer to the primary clarifiers;

To their Monitoring and Disposal System:

- One effluent monitoring and disposal system with effluent being discharged to the existing ditch leading to Moberly Bay and Lake Superior;

To their Sludge Handling System:

- One vacuum filter 3.66 metre in diameter and 5.5 metre face for handling approximately 40 tonnes per day of primary sludge;
- One sludge press to handle approximately 14.5 to 51.0 tonnes per day of primary sludge;

And finally to their Controls and Equipment System:

> All other controls, electrical equipment, instruments, piping, pumps, valves, appurtenances essential for the proper operation of the aforementioned sewage works;