The Development of a

TOXICITY EARLY WARNING SYSTEM

(TEW)

by:

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A thesis
submitted to Lakehead University
for the partial
fulfillment of the degree of
Masters of Science
in Biology

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2.0. The Use of the TEW system to Monitor the Toxicity of Pulp and Paper

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Abstract

Biomonitoring is a practice that has over time developed into a remarkably accurate form of detecting toxicity in the environment. The Toxicity Early Warning system (TEW) is a Real-Time Biomonitoring system developed to monitor the toxicity of industrial effluent to rainbow trout (*Oncorhynchus mykiss*) prior to the effluent entering into the environment. The development of a trout behavioural-response-to-toxicity library can eventually be used to monitor and prevent toxic industrial spills. In the TEW tests trout were exposed to three pulp and paper effluents (KRAFT, NEWS and KRAFT Clean Water Outfall). The TEW test is a 12hr test, consisting of an acclimation period and an effluent exposure period which runs off of an inexpensive, conductivity bridge circuit. Trout behavior was obtained via an oscillating signals produced by the conductivity bridge circuit. This signal was statistically analyzed using a single factor ANOVA, averages, and coefficient of variance. Results show that the normal ventilatory patterns of rainbow trout averaged between a range of 0.0138v – 0.7895v in ventilatory depth and 2-4 breaths per second. General activity was monitored as whole body movement (fin and body action). Trout on average were active and sporadic in their movement, averaging 2.39v during the acclimation periods. Exposure to KRAFT effluent resulted in severely reduced body movement at all concentrations, breathing patterns declined to an almost consistent 2.0v ventilatory depth, with a cyclical 2.0v – 3.5v ventilatory frequency. Exposure to NEWS effluent resulted in increased body movement, decreased ventilatory depth (0.0470v) and ventilatory rate (1.47 breaths per second). There were no significant behavioral results exhibited when trout were exposed to KCWO.

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General Introduction and Literature Review

The pulp and paper industry continuously utilizes large volumes of water, all of which must be monitored and treated for toxicity prior to its release from the mill into the natural environment. Modern techniques are normally adequate for this treatment, but isolated instances of toxicity may still occur. These events can result in the diminished quality of the ecosystem as well as negatively impact fisherman, farmers, industries, swimmers and other downstream water users.

Biomonitoring is described as the use of a living organism to monitor the quality of the surrounding environment. The establishment of a biomonitoring system in the pulp and paper industry, for the prevention of effluent spills, must be based on extensive research and experimentation if success is to be achieved. Typically, researchers developing biomonitoring systems initiate their experiments using a single species, over time, expanding to a battery of different species (Cairns *et al.* 1971). The habits, habitats and life requirements of any species chosen must be well established in order to elevate the possibility of unexplained behavioural responses. Each species chosen must exhibit sensitivity to a broad but very specific array of toxicants, therefore increasing the accuracy and sensitivity of the system (Cairns *et al.* 1971). As a result of this requirement, early scientists developing biomonitoring systems chose fish as their initial test species. The acute chemoreception ability of fish is far superior to that of terrestrial vertebrates, making them an ideal choice for biomonitoring (Kanwal and Doving 2003). In addition to the acute sensitivity most fish exhibit, there are many species known throughout the scientific community that show a specific sensitivity to water quality,

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residing in only clean, clear water systems (Baron 2004). The sensitivity of these species to pollutants makes them an ideal choice for toxicity research or use in biomonitoring.

Bowater, a mill in Thunder Bay, Ontario, Canada, in collaboration with Lakehead University has engaged in the development of an industrial, Real-Time Biomonitoring system, with the purpose of detecting and preventing the occurrence of toxic events. This system, referred to as the Toxicity Early Warning system (TEW), has been designed to continually monitor the toxicity of pulp mill effluent to aquatic organisms prior to the effluents release into the environment. The three pulp and paper effluents tested included KRAFT, NEWS and KRAFT clean water outfall (KCWO). The Kraft process, (sulfate process) as described by the Environmental Protection agency (1983), uses caustic sodium hydroxide and sodium sulfide to extract lignin from the wood fiber. The spent slurry, known as black liquor, is concentrated through evaporation and then burned to generate high pressure steam used to power other mechanical processes with in the mill. The remaining inorganic portion of the liquor is used to regenerate the sodium hydroxide and sodium sulfide needed during pulping. When softwood (conifer) wood chips are used in pulping a soap-like substance is collected from the liquor during evaporation, which is acidified to produce tall oil, a source of resin acids, fatty acids and other chemicals. The effluent produced from KRAFT processes, when untreated is highly toxic to both aquatic and terrestrial organisms. Although treatment processes under normal circumstances remove 100% of these toxicants.

The NEWS or newsprint processes as described by Natural Resources Canada (2005) utilizes elevated temperatures to soften the lignin locked within wood fibers. In this process wood chips are steamed by a process known as Thermomechanical pulping

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(TMP) just below the soften point of the lignin. The TMP treated wood chips are then passed through two rapidly spinning disks which allow for fiber separation to occur. This process is a high energy user but it is also a non-chemical process, producing effluents that are considered non-toxic prior to the required treatment processes that ensure effluents are safe when introduced into the natural water system.

KRAFT clean water outfall (KCWO) is a clean water source sampled from upstream of the mill. This water source flows through pipes located within and near KRAFT process machinery, but remains isolated from all KRAFT processes, simply acting as a cost effective cooling system. KCWO will re-enter the water system from which it was taken with no ramification on the natural environment.

The aquatic organism chosen to monitor the level of toxicity that these three effluents may present is rainbow trout (*Oncorhynchus mykiss*), an organisms whose life history and sensitivity to environmental toxicants have been well established in the scientific community since the early 1900's. This factor along with the provincially and federally required use of rainbow trout in the testing of industrial effluents via chronic LC50 tests, make it an ideal choice for the TEW system. The TEW system is designed to monitor the behavioral response(s) of the rainbow trout when exposed to a geometric series of the three effluents chosen (100%, 50%, 25%, 12.5%, 6.3%) creating a behavioral 'trout-response-to-toxicity-concentration' data library. Specific behavioral responses such as whole body movement, ventilatory frequency and depth, as well as trout activity level are correlated with a toxicity series in order to develop a biomonitoring system that accurately monitors effluent toxicity as it travels through the outflow pipes of the mill.

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Chemoreception in Fish: The Type of and Importance of Fish Sensitivity to Components within their Environments

The function of a sensory system in a living organism can be loosely defined as the activation of receptors cells as a result of external stimuli present in the organism's environment. Most organisms posses some form of sensory system, either rudimentary or complex, and each system has been adapted for the specific needs, and lifestyle of that particular organism. In the subphylum vertebrata the sensory system has five basic functions including; sight, hearing, taste, smell and touch. Fish sensory systems, in the Class Osteichthys (bony fishes) or Chondrichthyes (cartilaginous fishes) carry out all of these functions, but unlike terrestrial organisms, fish have special adaptations which accept large quantities of dissolved chemical stimuli from aqueous environments (Sorensen and Caprio 1998).

The efficiency of any sensory system of a resident fish is dependent on the habitat in which it is found. The vast array of fish species found in the oceans, rivers, streams, lakes and ponds provide a perfect example. The sense of smell in fish for example requires a continuous and steady flow of water through the dorsal external nares (Chiasson and Radke 1991). Fish such as those residing in perpetually moving water columns (rivers, streams and large lakes) will be exposed to higher turbidity and continuous water flow through their nares. These fish often have a very keen sense of smell and a more reduced sense of sight because they are exposed to a larger abundance of dissolved chemical stimulus (Chiasson and Radke 1991). Fish found in water bodies with minimal water movement such as ponds and small lakes will generally have an exceptional sense of sight and a more reduced sense of smell (Chiasson and Radke 1991).

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Chemoreception stimulates a physiological response in the organism that aids in its ability to make informed decisions (Moyle 1993). This sense has been divided and classed into two different sensory categories; the first being olfaction or simply stated a sense of smell and the second being gustation or sense of taste. They are shared by both terrestrial and aquatic organisms but because of the increased dependency aquatic organisms place on these functions, their chemoreceptive abilities usually far exceed that of those found in the terrestrial world. Olfactory and gustatory systems both require the use of receptor cells to receive and react to chemical stimuli, generating a chain reaction that passes biologically important information to the organism central nervous (CNS). The CNS discriminates between useful and redundant chemical stimuli found within the water column, filtering-out ineffectual 'background noise' and familiarizing the organism with its surrounding environment. This process will eventually result in an informed behavioral response from the organism, such as mating rituals, searching for food, or avoiding predators.

Olfaction and gustation both involve chemical stimuli and receptors, their general functions are quite similar. In olfaction the olfactory bulb acts as an interface, receiving chemical stimulus from the environment and passing information derived from that stimulus directly into the central nervous system (CNS) through the bipolar neurons of cranial nerve (Marui and Caprio 1992). Gustatory organs on the use specialized epithelial cells to transmitted information to the CNS by means of cranial nerve neurons (Marui and Caprio 1992).

Although the receiving of chemical stimuli in both olfaction and gustation are similar these two sensory systems are responsible for different behavioral responses. The

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gustatory system is responsible for short range responses such as fright, defense and territorial behavior (Marui and Caprio 1992). Olfaction on the other hand is responsible for long range responses such as locating, identifying and judging the distance between and from objects and organisms (Marui and Caprio 1992). Both olfaction and gustation are systems responsible for generating different types of responses, the responses produced are linked. They work in conjunction with each other providing aquatic organisms with an in-depth awareness of their surroundings.

Olfaction

Olfaction in fish plays an important role in many vital aspects of its life. Attracting mates for breeding purposes, avoiding potential predators, as well as searching out and catching prey are all a function of olfaction.

A report by Rehnberg and Schreck (1986) briefly summarized the results of Tomasso; Donaldson and Dye; and Schreck and Lorz, who each used various chemicals in their experiments to evoke behavioral response in the test species. These scientists used different concentrations of copper, ammonia, nitrate, endrine, kanamycin, phenol, and hydrogen ions. Each application of chemicals generated a recordable stress or avoidance responses from the fish being tested. An experiment performed by Brett and MacKinnon (1954) showed that when northern squawfish (*Ptychocheilus oregonensis*) and the large scale sucker (*Catostomus macrocheilus*) were exposed to L-serine, human skin rinse, whole body rinse, and cut skin, an avoidance response described as "fright" along with the production of rapid or erratic body movement. These studies used only a small variety of chemicals, but the fish displayed obvious and significant behavioral responses when exposed to the test chemicals. These tests produced very definitive

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results indicating avoidance (fright) of the stimuli applied. In a natural environment fish respond in a similar fashion as those tested *in situ.* A fish's chemoreceptive ability is one of the methods used to determine the safety an area is before it is entered.

Gustation

Taste or gustation is another form of chemoreception exhibited by fish. This sense in fish, unlike terrestrial vertebrates, is not confined to the buccal cavities. Gustatory sensory organs are distributed over the entire surface of the teleost organism, concentrated within the lips, mouth, gill rakers, pharynx, and barbels (Maslin 2000). The function of a gustatory sensory system is to receive and transmit chemical stimuli from taste buds to the central nervous system of the organism. A process, which allows the organism to determine and distinguish the source and locality of chemical stimuli as it moves throughout its environmental gradient (Kanwal 1992).

Weber in 1827 was one of the first scientists to study fish taste buds, later followed by Leydig in 1851, both observed that taste buds in fish are peripheral organs which constitute the structural basis of the gustatory system in all gnathostomes (Kasumyan and Doving 2003). One of the extraordinary features of the fish gustatory system is the quantity of taste buds present, numbers vary throughout different fish species but it has been estimated that fish have 100 times the number of taste buds than that of the average human, equaling approximately 680,000 taste buds (Kasumyan and Doving 2003).

Taste Classification

Fish exhibit different behaviors when exposed to different food types. This has allowed scientist such as Lindstedt (1971), and Mearns *et al.* (1987) to develop taste

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classification nomenclature for the different food stimulus found in an aquatic environment. Incitants, for example, are a food stimulus, which commonly evoke suction, grasping, snapping, biting, tearing or pinching responses in most fish as they attempt to capture the food source. The actual food capturing response is triggered by the extraoral taste system and will vary based on the physical characteristics of each fish species. Suppressants, also triggered by the extraoral taste system, are composed of food stimulus that deter a capture response from a fish, the rate of grasping, tearing, biting, excreta, is decreased. Stimulants are described as substances that increase the rate of ingestion by fish. This response is triggered by the oral taste system, promoting feeding, where as deterrents, also triggered by the oral taste system, evoke a food rejection response. In this situation the food is often captured by the fish but then rejected from the oral cavity, for a brief time period after the food has been rejected the fish will often exhibit a reduced interest in eating. Enhancers are not a feeding stimulant but appear to increase the rate of consumption by accentuating the flavor of the desired food. Detractors perform the opposite function, reducing the favorability of edible food; both of these are triggered by the oral taste system.

Typically scientists use the standard sweet, sour, bitter and salty taste sensations known to stimulate humane taste sensations in order to determine and compare the type of or arrangement of taste buds found in other organisms. In fish these taste sensations have been replicated using sucrose, acetic acid, quinine and sodium chloride, respectively, in order to determine what food types are preferred or avoided by whichever species of fish (Kasumyan and Doving 2003). The sweet tasting sucrose can be easily recognized by most fish species, evoking an indifference or positive taste response. For

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example fish such as the grass carp (*Ctenopharyn- godon idella*) are herbaceous and derive sucrose from aquatic grasses; in this case the sweet taste promotes a positive eating response (Kasumyan and Doving 2003). Carnivorous fish species such as atlantic navaga (*Eleginus navaga*), on the other hand typically display an indifference response to sucrose but show a strong attraction to more salty foods, replicated by sodium chloride (Kasumyan and Doving 2003). Table 1. provides a response gradient for 27 different types of fish, the positive numbers displaying a preference towards a specific taste, and a negative number indicating an avoidance response to a specific taste. Fish were fed different flavored pellets (sweet, salty, bitter, and sour). The number of pellets eaten or avoided for each type of flavor indicated the fish's preference.

Table 1. The Index of Palatability to Classical Taste Substances: 27 different fish species tested

Substances	Con.mm (%)	*Salmo trutta caspius*	*Salvelinus fontinalis*	*Salvelinus namaycush*	*Salvelinus alpinus erythrinus*	*Oncorhynch-us keta*	*Cyprinus carpio*	*Carassius carassius*	*Carassius auratus*	*Rutilus rutilus*
Citric acid	0.26 (5)	78.7***	95.2***	60.0***	53.4***	100***	53.9***	61.2***	31.0***	100**
Sucrose	0.29 (10)	10.2	90.6***	11.2	9.7	14.7	13.8	0	1.9	32.7*
		Leuciscus leuciscus	*Leuciscus cephalus*	*Phoxinus phoxinus*	*Tinca tinca*	*Rhodeus sericeus amarus*	*Ctenophar-ngodon idella*	*Puntius tetrazona*	*Brachydanio rerio*	*Pungitius pungitius*
Citric acid	0.26 (5)	17.1	9.8	10.3	56.4***	89.1***	46.0***	18.5	100	66.7***
Sodium chloride	1.73 (10)	24.4*	17.9	7.9	36.8***	6.3	5.1	11.0	100	10.4
Calcium chloride	0.9 (10)	9.2	44.0***	14.8	33.4***	7.4	20.9	15.7	0	11.0
Sucrose	0.29 (10)	28.6**	1.3	7.5	10.6	5.6	63.4***	10.0	100*	0.7
		Anarhichas lupus	*Heros severum*	*Poecilia sphenops*	*Poecilia reticulata*	*Xiphophorus maculatus*	*Acipenser baerii*	*Acipenser stellatus*	*Liopsetta glacialis*	*Eleginus navaga*
Citric acid	0.26 (5)	42.3*	22.0**	1.3	95.2***	77.2***	89.5**	89.5**	11.3*	28.9
Sodium chloride	1.73 (10)	35.3	73.5***	33.4***	100	87.1***	50.0**	47.5**	9.2*	100
Calcium chloride	0.9 (10)	31.1	21.5***	19.3	72.4	23.6	100***	83.7**	6.9	9.5
Sucrose	0.29 (10)	3.3	13.5	53.5***	83.7***	74.5***	12.5	7.1	2.0	20.3

The index of palatability (in percent) was calculated by the formula $I_{pal} = 100 \cdot (R\ C) \cdot (R + C)^{-1}$, where R is the number consumed of pellets containing a particular substance and C is the number of blank pellets consumed. See text for details.

(Kasumyan and Doving 2003)

This table shows that zebra danio (*Brachydanio rerio*) consumed 100% of citric acid, sodium chloride and sucrose, but completely avoided calcium chloride. Since this species is considered an omnivore it will consume both plant and animal that do not

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exhibit a bitter flavor. To species such as Baikal Sturgeon (*Acipenser baerii*) and Starry sturgeon (*Acipenser stellatus*) a bitter flavor appears to be more favorable than the sweet, salty and sour tasting chemicals (Kasumyan and Doving 2003). Both of these two sturgeons are commonly found consuming mollusks, crustaceans and worms which may indicate that these three food types trigger the bitter flavor taste buds of the sturgeon.

In summary the response of any teleost to a particular taste sensation is dependent on the eating habits of that species. Their gustatory sensory systems allow them to identify the desired food types. Herbaceous fish for example will be more attracted to plants due to the high sucrose levels found within vegetation. Whereas carnivores will be more attracted to the salty, acidic or bitter taste sensations produced by aquatic organisms in the water system.

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Rainbow Trout as Biological Indicators: The Life History and Living Requirements of the Rainbow Trout, and its Potential Use its use in a Real-Time Biomonitoring System

Rainbow trout (*Oncorhynchus mykiss*), among other species found within the family Salmonidae are well known for inhabiting clean and cold freshwater ecosystems. Their intolerance of pollution and habitat degradation from human encroachment can easily result in reduced health conditions, emigration to other regions or even death under extenuating circumstances. The utilization of this species as a biological indicator for hazardous waste has provided primary polluters, such as commercial and industrial enterprises, with a means of monitoring and controlling their impact on the environment.

Initially, during the North American industrial revolution, the negative effects of industrial discharge were unknown and steam, smoke and effluent were considered a sign of progress. It was not until the early 1960's that the negative impacts of industries to public health and the environment became a concern. It was at this time, in United States history, where policies such as the National Environment Policy of 1969 came into effect (Davis 1995). This act paved the way for the Clean Water Act (1972) which established a permit system for establishments discharging into American waters (Davis 1995). Later still the Safe Drinking Water Act (1974), Resource Conservation and Recovery Act (1976), and the Toxic Substance Control Act (1976), were presented as a means of establishing a water control program. These acts monitored the generation, transportation, treatment, storage, disposal and distribution of hazardous waste. Canada has emulated many of these acts and policies, enforced provincially by the Ministries of the Environment, and nationally by Environment Canada (Davis 1995).

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Today, both provincial and national laws require that effluent from commercial and industrial enterprises be tested for toxicity using a rainbow trout chronic toxicity test (Hardy 2002). These tests are labeled as Lethal Concentration Test (LC50), where the concentration of an effluent, that kills 50% of a sample population, determines the level of toxicity (Hardy 2002). This test is not used to describe the composition of the effluent, only the degree of toxicity present.

Rainbow Trout Habits, Habitats and Preferred Environment

The life span, living conditions and habitat requirements are of great importance when studying the usefulness of a species to civilization. Rainbow trout for example are currently used as indicator species in both laboratories as well as in river sample tests. They are used in provincially required effluent toxicity tests, the results of which are used to determine the environment impact of the industry in question. The results generated within these tests are reliable because living requirements and behavior patterns of rainbow trout have already been established. Knowledge of the morphology and physiology of rainbow trout are the foundation from which ecologically important decisions are made.

Morphological Traits

The physical characteristics of rainbow trout as described by Scott and Crossman (1973), and Baron (2004) are given below.

Rainbow trout's overall body shape is elongated, slightly compressed and approximately 12-18 inches in length with a head that contains a moderately sized eye. This species has a terminal mouth with a rounded snout that is large, and oblique, containing non-protractible premaxillaries. Rainbow trout have a dorsal adipose fin as

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well as a short, slightly square, soft rayed (10-12 principal rays) dorsal fin. The caudal fin is broad and short, moderately forked, and slightly square, composed of 8-12 principal rays. The pelvic fin located on the abdomen is small, short and rounded, containing between 11-17 rays. The scales covering rainbow trout's body are cycloid, and are usually small but may vary depending on the growing conditions, and habitat suitability. The lateral line is slightly curved and contains 100 to 150 scales. There are many minor variations noted within this species, this is a result of rainbow trout's adaptive behavior, and usually occurs within the head, mouth, color and body size of the fish.

Basic Spawning

Rainbow trout, are usually classified as spring spawners, and spawn from February to June, dependent on photoperiod and temperature (10.0^0C to 15.5^0C). This species almost exclusively spawns in streams, although some have successfully spawned in 'landlocked' lakes (Raleigh *et al.* 1984). The ideal spawning stream, described by Raleigh *et al.* (1984), consists of clear, cold, silt-free, highly oxygenated water with a gravel substrate that is ideal for the incubation of eggs and the building of a redd. An abundance of riffles with deep pools, including areas of deeper, slower moving water is most optimal. The stream should be well vegetated and have abundant in-stream cover, with highly vegetated banks.

Rainbow trout eggs require a 4-7 week incubation period, after which 800 to1000 alevins/redd are released (Scott and Crossman 1973). Maturity for most rainbow trout will occurs in males after 24-months, and for females after 36-months (Baron 2004). Their life expectancy ranges from approximately 3 to 4 years but individuals have been known to reach ages of 6 to 8 years.

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Rainbow Trout Diet

The diet of rainbow trout consists mainly of insects, zooplankton and small aquatic invertebrates; trout are however opportunistic feeders and will consume a large array of foods (McAfee 1966). Adult trout will feed mostly on smaller fish (including rainbow trout), whereas the juvenile and swim-up fry will feed mostly on the smaller sized menu of insects, and zooplankton (Hunt 1971).

The eating habits of the rainbow trout contributing to stream environments consist of a variety of different invertebrates. Oxygen rich pools and waterways both support an abundance of *Plecoptera* (stoneflies), *Trichoptera* (caddisflies), *Ephemeroptera* (mayflies) (Earle and Callaghan 1998). These invertebrates make up a large portion of the rainbow trout's diet so it is important that their population levels remain elevated. Lakes and Rivers that exhibit depleted levels of oxygen are not capable of supporting these aquatic insects, making food for the trout scarce.

Preferred Water Chemistry of Rainbow Trout

Water temperature, dissolved oxygen, dissolved carbon dioxide, water hardness, alkalinity, pH and conductivity all combined, describe the chemistry of a water system. United States Environmental Protection Series (1992) summarized the recommended water chemistry of rainbow trout, Table 2.

Table 2. Limits Based on Toxicity Tests Using Early Life Stages of Salmonid Fish

No.	Variable	Recommended limits
1	pH	6.5 - 8.5 (7.5 - 8.0 desirable)
2	Alkalinity	20 - 200 mg $CaCO_3L$
3	Dissolved carbon dioxide	0.03 - 15 mg/L
4	Dissolved oxygen	90 - 100% of saturation

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Rainbow Trout Response to Industrial Effluents

Since rainbow trout are known through previous studies to be an ecologically sensitive species and because they are currently utilized in chronic LC50 tests, which are required by provincial law, it makes sense that this species is chosen as a starting point for biomonitoring. Tests that have validated rainbow trout's sensitivity include work done by Valentincic *et al.* (1999) who discovered that when amino acid was placed in a tank with rainbow trout alevins, the acid would induce a snapping behavior from the trout. Amino acid is considered a potent olfactory and gustatory stimulus for adult rainbow trout, triggering swimming, turning and snapping responses (Valentincic *et al.* 1999). Amino acids are also known to influence the pH of a water system, another parameter that can be easily monitored through the application of pH probes. Rehnberg and Schreck (1986) performed other experiments in which it was discovered that trout exhibited avoidance or fright response when exposed to human skin water rinses, as well as 10^{-5} M_L-serine. Warner *et al.* (1966) discovered the same avoidance response when exposing the trout to DDT. In both cases the trout were placed in a Y-Trough, with the polluted substances flowing through one arm of the Y and clean water flowing through the opposite arm. The trout repeatedly chose the unpolluted arm of the Y indicating that when possible, a trout will avoid unsuitable water conditions. A feeding study performed by Brown *et al.* (1968) showed that damage could be caused to the peripheral organs of trout due to detergents. Brown showed that the taste buds were eroded off of the trout as a result of detergent exposure, resulting in the inability of the fish to ascertain the palatability of its food. The trout would take food into its mouth, but the food would not trigger an edibility response and the food would be spit back out.

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In another experiment trout were exposed to chemicals such as methanol, allyl acetate, allyl bromide, 2,4 Dichlorophenol, carbon tetrechloride, dichloromethane, 1,1,2,2-Tetrechloroethylene, Trichloroethylene (Kaiser *et al.* 1995). These chemicals, in varying concentrations were shown to produce behavioral responses such as increased and sporadic ventilatory rates and amplitude, frequent bouts of coughing, and increased swimming activity. Eventually chronic exposure resulted in more severe responses such as sideways swimming and eventually a reduction in ventilatory response leading to the death of the fish (Kaiser *et al.* 1995).

A review performed by Hutchins (1979) compared the reactions of rainbow trout to chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), perch (*Perca flavescens*), and many others to a 96h LC50 test in order to determine the sensitivity of each organisms. This review showed that rainbow trout when compared to other salmonids tested demonstrated equal or superior sensitivity to toxins. Which aids in the conformation that rainbow trout would be an excellent choice as an indicator species for industrial effluent, because it provides quantifiable behavioral responses to a varied range of chemicals and effluent concentrations.

A study performed by Leach and Thakore (1977) also exposed rainbow trout to effluent, in this case from a pulp mill. The results of their tests displayed swimming impairment and performance as well as biochemical changes. A report by O'Conner *et al.* (2000) exposed trout to oxygen activated sludge produced by a pulp mill. In this study the fish showed signs of stress in as little as one hour of effluent exposure. After prolonged exposure some of the trout responses included loss of equilibrium, erratic swimming, gasping at the water's surface, sinking to the bottom of the test container and paralysis.

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Once the trout were removed from the toxic environment they recovered and began to display normal behavior, whereas trout left within the effluent eventually died.

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Biomonitoring: The History, Development and Use of Real-Time Biomonitoring Systems for Monitoring the Quality of Aquatic Environments

Biomonitoring is referred to as the continuous assessment of potential toxicity by employing living organisms as sensors (Cairns *et al.* 1971). This is accomplished not by determining the type of chemicals found within the effluent but by observing how the effluent alters the behavior of the organism exposed to the effluent. The most important advantage of biomonitoring is the ability to visualize an organism's response to the totality of its environment. This ability, in essence, summarizes the effects of all toxic elements within the effluent, and when biomonitoring is coupled with standard regulatory testing, creates an advanced and efficient method of detecting toxicity (Cairns *et al.* 1977b).

Past Methods

There have been many varieties of early warning toxicity systems developed over the centuries. Canaries were one of the first, most highly recognized biomonitors. This species was used by miners as an environmental indicator for carbon monoxide in coal mines. When the levels of carbon monoxide reached unsafe levels the canary would slip into unconsciousness, signaling that the mine was no longer safe, resulting in the mines evacuation (BBC - News 1986). Within the last 35 yrs fish and other aquatic organisms have been used to monitor the affects of industrial pollution on water systems.

Henderson and Pickering (1963) were a few of the first documented scientists to monitor the behavioral response of fish as an indicator of toxicity. They placed fish into specific concentrations of known chemicals and used visual observations to determine the fishes' response to the chosen chemical prior to mortality. This method helped to provide

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an excellent indication of toxicity but exhibited a considerable time lag in providing an early warning of that toxicity.

As early as 1978 Adema (1978) and Leeuwangh (1978) began to re-establish the idea of biomonitoring for toxicity prediction, utilizing the movement of the water flea *Daphnia magna*. Effluent toxicity was detected from monitoring the dynamic movement of the *Daphnia* within the tank. The *Daphnia's* location, number of turns, velocity and direction of the travel over a pre-determined time period, all produced significant behavioral results that were recorded via cameras and electronics. These two scientists succeeded in measuring significant behavioral response in *Daphnia*, under a 2 hours exposure period to chemicals (Lindane for example) at concentrations less than or equal to 1ug/l.

Today *Daphnia* are frequently used for reference toxicology in North America. In fact *Daphnia* are currently used in the provincially and federally required toxicity tests for industrial and commercial effluents in Ontario (Maki 2004). Lethal concentration tests (LC50) monitor toxicity level by measuring the mortality rate of exposed populations. The sensitivity of this species in either a flow-through system or a standing water test has made them an ideal species for future studies.

Other studies employing aquatic organisms such as eukaryotic algae have also been investigated. Pandard, Vasseus and Rawson (1993) used these phyla to help develop two other potential pollution monitors. The first study monitored the reduction rate of a redox mediator by illuminating the biocatalyst, enzyme that initiates or modifies the rate of chemical reactions in a living body (Dictionary.com 2004). The second study successfully monitored the algae's production of oxygen. For both experiments the rate

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of reduction for a redox reaction, as well as the amount of oxygen algae are able to produce was previously known. Because of this base knowledge, the new data generated could be compared to existing data in order to identify patterns and significant responses to toxic events. The oxygen producing experiment generated consistently better results than the redox reaction experiment, but both tests effectively showed *Daphnia's* reaction to pollution.

In Germany Tahedl and Hader (1999) continued the quest for a sufficient biomonitoring system by observing the differential mobilization of motile unicellular flagellate (*Euglena gracilis*) as an endpoint. Orientation, velocity and mobility were used as the parameters and a computer system capable of analyzing and quantifying the results within minutes collecting and storing the data. This method of biomonitoring appeared to have many benefits. The organisms can both be grown and handled easily, the toxicity-monitoring unit is small and inexpensive, and the results are displayed within minutes. The only potential flaw with this system is that it is not a continuous monitoring, flow-through system. Samples of effluent must still be injected into the system in order to test for toxicity. While this system may work well, it is at present no better than the initial LC50 tests previously described.

It wasn't until 1971 when one of the more prominent biomonitoring systems in North America was established. W.A. Spoor along with some of his colleagues produced an early warning system that monitored the opercular rhythms and whole body movement of bluegill sunfish (*Lepomis macrochirus* Raf.) (Spoor *et al.*1971). They accomplished this by placing two stainless steel plates (electrodes) at either end of a fish tank. These electrodes were wired and attached to electronic filters and amplifiers in order to record

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an electrical signal produced by fish muscle contraction during ventilation and fish body movement.

Cairns, J. Jr., R. Sparks and W. Waller (1974) were a team of scientist that saw the promise in Spoor's research. They mimicked and altered his tank set-up in order to develop a more efficient tank, allowing for a larger array of toxicants to be monitored. By monitoring the frequency and intensity of ventilation and whole body movement they were able to create photoresistors employing light beams to monitor the location of the fish within the tank. When the fish swam through one of three light beams the counter was triggered. Compiling the results from the photoresistors and the electrodes generated large amounts of complex and useful data describing the sensitivity of fish to toxicants. Their experiments indicated that fish displayed less ambulatory movements during dark intervals (16/8hr light-dark photoperiod) than during of light intervals, fluctuation in body movement prior to fish mortality and increasing cough frequency (the number of times water is flushed backwards over the gills) in synchronization with increasing effluent concentration. Unfortunately the analysis for such data required the use of a highly trained professional. If the early warning system were operational within an industry it would be running continuously, both day and night; this would require the industry to hire several highly trained analysts. The employment of several new full-time staff members along with the purchase price as well as the cost of running the early warning system proved economically impractical for most industries. A second downfall to this system is the copious amount of data produced in contrast to the speed at which a single person could read through and interpret these data. Cairns *et al.* (1974) determined that fish exposure period, the speed of the polygraph recorder and the speed of a human

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operator, an early warning system would generate a response lag time from one to nine hours.

In further studies Cairns, Sparks and Waller were joined by Westlake and van der Schalie (1977) and Gruber *at el.* (1980) in attempting to reduce the effects of these two impediments and improve the reliability of the initial early warning system. Mini-computers were added to the system in order to store the data collected from the fish, and software was developed that could detect peaks, valleys and movement abnormalities in the fish's behavior. Both of these developments allowed for a more user-friendly system that could collect, store and analyze fish behavior, detecting some sub-lethal toxicants within a 30 minute to 1 hour time period (Gruber *et al.* 1980). After the development of a functional in-plant system the team moved towards an in-stream system. This system used coherent optical spatial filtering of diatoms. Spatial frequency filters then select diatoms of a specific structure from a mixture of diatoms. These act as an indicator of the stream's health. All possible diatoms are recorded and identified in a library file, a specific filter matching each type of diatom. When a diatom matches the filter it is tallied and viewed as a light on a computer screen. The density of lights or concentrations of a specific diatom within the water will indicate the health of that water system (Cairns *et al.* 1973a). These systems would be placed in the receiving waters and it was determined that by combining in-plant with in-stream biological monitoring systems, industries could increase their capability of managing the entire water system to better minimize the occurrence and magnitude of potential ecological disasters. Quality control would be extended from the manufacturing process to the waste treatment process, but also to the river itself (Cairns *et al.* 1974).

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Choosing a Species

If it is to be useful, a continuous biomonitoring system must rapidly detect toxins. Therefore, the species chosen must show obvious reactions to any and all aspects of the toxic effluent. If the chosen species only reacts to 50% of the toxic chemicals in the effluent then toxic waste may pass easily through the biomonitoring system unnoticed for a lengthy time period, before an alarm is initiated. The foresight of such an occurrence is one of the most important requirements for a biomonitoring system. Despite this requirement there are other factors not necessarily related to bio-sensitivity that come into play. The abundance of a species within the environment and its availability for research purposes can be detrimental. If a species is on the verge of extinction, but is sensitive to all industrial caused environmental changes, then the species would be an impractical choice for use in a biomonitoring system, even if the sensitivity of all other species pales in comparison.

The popularity of the test species employed both within science as well as in the eye of the public can also have a surprising affect on the success of the system. An organism well established in the scientific community is in most cases well researched, with most aspects of its morphology and physiology well known. This is important because it ensures confidence in measuring the response of an organism to applied toxicants in future tests. Chances are, an array of behavioral responses from the organism to specific chemicals have already been documented providing scientists with a history of reactions and concentration levels as a basis for comparing the responses generated within their own experiments. When the chosen organism is introduced into a new monitoring system its response has to some degree already been established. This allows

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for general comparisons between past tests in contrast to attempting an interpretation of the species responses based solely on the data obtained from a single experiment.

Some of the species chosen for past aquatic biomonitoring include diatoms, algae, daphnia, and blue gill sunfish, all of which performed adequately for the systems they inhabited. A fish species whether it be bluegills, or another fish species appear to be the most favored choice of both scientist and the corporations funding the research. Many biomonitoring systems have been developed using fish, not necessarily because they are reliable environmental indicators but because they are a highly visible, sought after organisms by anglers, and well recognized by both the scientific and general community, relative to other species.

Guidelines for Developing a Biomonitoring System

The following list describes biomonitoring systems which include description on organisms recommended and electronics used, creating a reliable, economical, and user friendly biomonitoring system for industrial application. This list is a compilation of suggestions from the various studies and experiments performed by Cairns *et al.* (1973a, 1973b, and 1977b), Westlake *et al.* (1977), Kingsbury and Rees (1978), and Sparks *et al.* (1978).

1. Previous studies show that the majority of the test species being used for biomonitoring react very quickly to toxic effluent anywhere from 5min up to several hours. Due to this short time lag effluent should not be tested at the outflow pipe, and not by grab samples. The effluent should be continuously monitored further up, along the pipeline to allow time for the indicator species to be affected by the effluent, react, and initiate a warning should a toxic event occur.

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2. To reduce the occurrence of false alarms all aspects of a test not directly generated from the effluent should be constant. Temperature, dissolved oxygen, total suspended solids, and pH will all effect the response as well as the health of the test organism. If the organism's health has deteriorated due to poor or inadequate living conditions they could have an increased or decreased susceptibility to effluent exposure; this will alter the levels at which the parameters are set. Therefore in order to reduce the occurrence of false alarms these parameters should be meticulously monitored and kept at a constant.
3. The parameters chosen for analysis should be easily quantifiable, their normal range of variation statistically chosen, allowing normal and abnormal responses to be easily distinguishable. It was also suggested that the test species should act as it own control, where the initial data used in setting the parameter ranges would be obtained. This was suggested due to the variation of behavior observed between each test organism within a species. The data would also only be collected after a two-week acclimation period, ensuring that the species response is only a response to the effluent and not from the stress of moving from one tank to another.
4. The test species would ideally be incapable of adapting to the effluent; a single organism could therefore be used in the biomonitoring system for several days, to several weeks, while still remaining sensitive to changes in toxicity. This would decrease the expense and time required for ordering and maintaining a large stock of the test organism. The organism should also be easy to obtain, and fairly inexpensive.

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5. Both control and test data should be easily digitized in order to increase the amount of and accuracy of the data collected as well as increasing the ease and speed in which that data is analyzed.
6. The biomonitoring system should be easy to operate and the data should be easy to interpret. This would increase the systems practicality, making it more appealing for industries that may not want to hire full-time, highly trained staff to run and interpret the tests.
7. Finally, the electrical system chosen to monitor behavior should be relatively maintenance free and reliable.

All of these suggestions are based on the advice of scientists that have developed their own variety of early warning systems. Each point developed from uncertainties, issues and concerns that occurred during their own test and trials. It is a list that will most likely continue to evolve as the development and use of early warning biomonitoring systems increase in popularity and complexity.

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Chapter 1
Design of a Rainbow Trout Sensory Monitoring System for Industrial Effluent Conductivity Sensing

Changes in the biochemical wellbeing of fish placed in a polluted environment are almost always accompanied by changes to their breathing, swimming and resting behavior as shown by Cairns *et al.* (1974), Spoor *et al.* (1971), and Henderson and Pickering (1963). Physiological activity in general is accompanied by changes in body movement and these changes can be used for assessing degrees of stress exhibited by unfavorable environments. Optics based image recording can clearly capture movements with time in an appropriate container. However, recording rates of thirty frames per second or more, for long imaging sessions, results in very large data files. Subsequent attempts to quantify changes in a specific behavior pose significant software challenges, expense, and time investment. While less complete in the recording of overall body conformation changes, electrical conductivity measurements of flow tanks containing fish, have become an accepted alternative to optical methods.

The individual efforts of scientist such as Cairns, Spoor, Henderson and Pickering for example, implemented the use of 10inch bluegill sunfish (*Lepomis macrochirus*) as indicators of toxicity. Through the application of invasive electrodes, sensitive to the minute electrical charges generated by muscle movement these scientists were able to establish the regular depth and frequency of bluegill ventilatory response to both toxic and non-toxic conditions (Westlake, G.F. and W.H. van der Schalie. 1977). The cough response, or reversal of water backwards over the gills and cough frequency as well as the whole body movement of the fish were recorded and along with ventilatory responses

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proved to be effective measures of aquatic toxicity (Westlake, G.F. and W.H. van der Schalie. 1977). At present the measurement from these biomonitoring systems are usually constructed from relatively simple and inexpensive analog electronics components and the output can be digitized for long term monitoring sessions. The waveforms generated can be analyzed visually, through the use of pattern recognition algorithms, or through power spectrum analysis techniques.

The Toxicity Early Warning system (TEW) described in this paper is a new biomonitoring system that utilizes fingerling rainbow trout (*Oncorhynchus mykiss*), a species well known for toxicity sensitivity (Valentincic *et al.* (1999), Rehnberg and Schreck (1986), Warner *et al.* (1966), Brown *et al.* (1968) and Kaiser *et al.* (1995)).

Experimental Considerations

The TEW system utilizes the conductivity of living organisms, which in general, are significantly higher than that of pH 7 water because of the high content of ions in the body tissue. Therefore, provided that the volume of the test species, namely rainbow trout, represent only 2%-10% of the overall tank conductivity volume, then changes in body conformation, in the parts per million level, such as ventilatory rates or frequencies can be detected. Once temporal baseline conductivity patterns for resting conditions of the trout can be obtained with adequate precision, and correlated with 'normal behavioral activity' deviations from these norms can be used as an indicator of stress induced by the external stimuli.

The process of monitoring the conductivity of minute body movement within a flow tank was complicated by several general factors, which were considered in the design of the sensors.

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(a) The sensitivity of the species to the electrical measurement process affects the allowable maximum probing voltage (current /electric field) and amplitude. In order to prevent stressing of the trout by the measurement process, electric fields in the tank, which drive the currents through the trout as well as the water, should be kept as small as possible.

(b) The concentration and chemical nature of the effluent used changes continuously over time.

(c) The design of flow-through tank geometry based on optimizing fish to water ratio via electrode sliders which move across the top of the tank to reduce the size of the fish enclosure.

(d) Choice of circuit type and design implementation. If DC currents are used, the chemical effects of the effluent may quickly lead to electrode polarization that further adds to long-term conductivity drift and/or electrode insulation. To avoid this, an AC measurement is required.

(e) Environmental electrical and mechanical noise factors that exist in the test environment. A high electrical signal to noise ratio is required if small changes in the physiological activity are to be observed with a high degree of reproducibility and precision. This factor also dictates that an AC technique be used and at a frequency beyond 1KHz so that the 1/f noise intrinsic to semiconductor electronic circuit components can be avoided.

Conditions (a) and (e) can be determined and optimized relatively easily, however variation in (b) in particular, can have a major impact on the overall choice of electrodes and the design considerations for the tank (c), and electronic measurement equipment (d). Effects of the effluent, on the detector design may increase the conductivity of the tank by a factor of two to more than ten. This is many times greater than that of the differential activity under study. Time dependent variations in the composition and molar concentration of the effluent will negate any static solution for the compensation of the effluent.

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Tank Design and Circuit Implementations

Acclimation Chamber and Trout Maintenance

The purpose of an acclimation chamber is to ensure the test species was disease free and to ensure acclimation of an organism to the environment to which it will be exposed during testing (Environment Canada 1992). This ensures that the organism responds to the stimulants within the test but not the properties of its surrounding environment. According to provincially required standard toxicity tests using rainbow trout and for the purpose of the TEW test, rainbow trout must be acclimated for at least two weeks prior to testing (Environment Canada 1992). The TEW acclimation chamber is shown in Figure 1., consisting of a glass tank containing both inflow and outflow tubing, and a maximum of 150 rainbow trout fingerlings (2-4g) at anytime.

The source for the dilution water used in the TEW system is the Kaministiquia River, upstream of the Bowater Inc., Thunder Bay, Ontario. The Kaministiquia River manifests zero to acceptable levels of chlorine and total suspended solids and the pH levels range between 6.5 to 8.5. Dissolved oxygen was added to the river water via standard aquatic air pumps and stones. An Orion benchtop 410A pH meter, Ag/AgCl Sureflow combination electrode and a stainless steel automatic temperature compensation probe, model # 917007 was used to monitored pH levels every 24hours. An Accument AP64 handheld DO meter ensured acceptable DO (90 to 100% saturation) levels were achieved. Both DO and pH are monitored daily and prior to testing. A Neslab Instruments Inc. Coil chiller maintained the temperature of the acclimation chamber at $15^0C^+/.1^0C$. The coil was made of stainless steel, and is approximately 20.32cm in length with a 5.08cm diameter. A thermostat with a copper probe was installed onto the chiller unit,

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providing an easy method for temperature regulation and adjustment. The use of copper presents copper toxicity issues with living organisms. Copper cannot come in to contact with the test species, or the environment of the test species (Environment Canada 1992). For this reason the copper probe was placed inside a 100 ml Nalgean container (filled with dilution water) suspended in the acclimation chamber via Masterflex tubing (#MFX9642017).

Acclimation water sampled from the Kaministiquia River flowed into the tank at a rate of 120 ml/min. Once 340 L has been reached a floating pump switch, attached to standard aquarium centrifugal pump is triggered, emptying the tank at a rate of 3.3 L/min. to a volume of 236 L. The Environmental Protection Agency (1992) recommends 1.0 L for every 10 grams of fish and the acclimation chamber greatly exceeded this volume.

The rainbow trout used in the TEW tests were obtained from the Aquatic Toxicology Research Center (ATRC) overflow supply, located at Lakehead University, Thunder Bay, Ontario. The ATRC purchase their trout from Rainbow Springs Trout Hatchery, in Thamesford, Ontario, and maintains the trout in accordance with the Lakehead University Center for Analytical Services (LUCAS): Standard Operation Procedures -SOP#AT001 (2003). The trout are certified free of disease or disease agents for live fish by the Government of Canada, Department of Fisheries and Oceans, SOP#AT002 (2003), They are composed of swim-up fry and fingerling life stages with a mean weight of 0.3 to 5.0 grams, and are fed granulated salmon fry feed (sinking) which contains 52/54% Protein, 14/17% Fat, 3/1% Fiber, 12/9% Ash and less than 10% moisture. The trout are acclimated to the ATRC testing environment at $15^{0}C^{+}/-2\ ^{0}C$ for two weeks prior to use in testing. A reference toxicant test was performed on the rainbow

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Figure 1.

The TEW Acclimation Chamber and the Chiller Unit used to Maintain the Acclimation Chamber at $15^0C^+/.2^0C$

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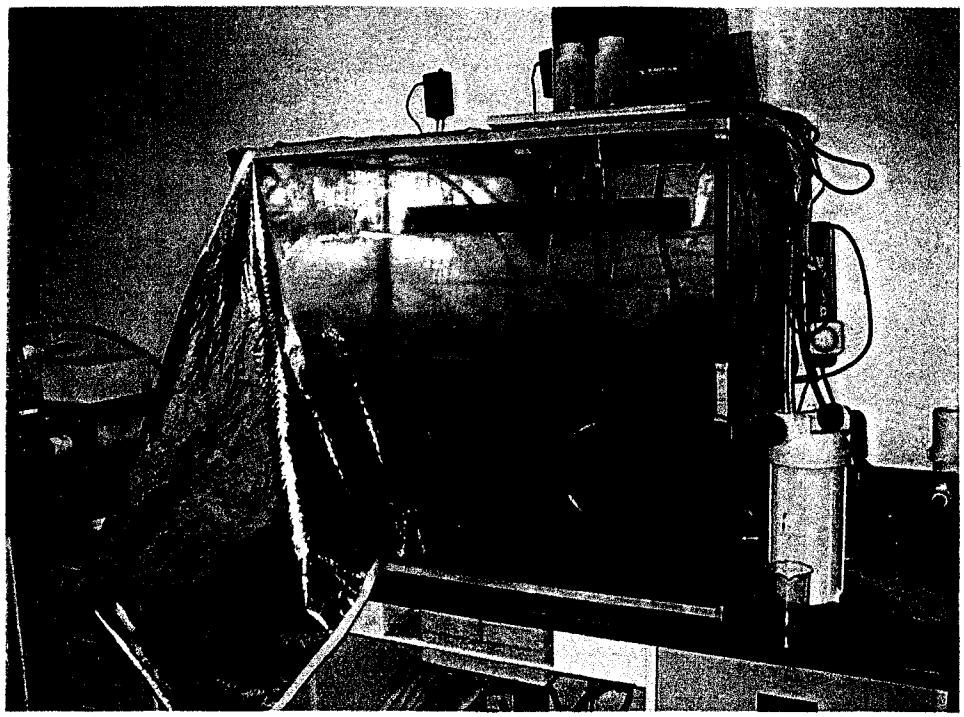

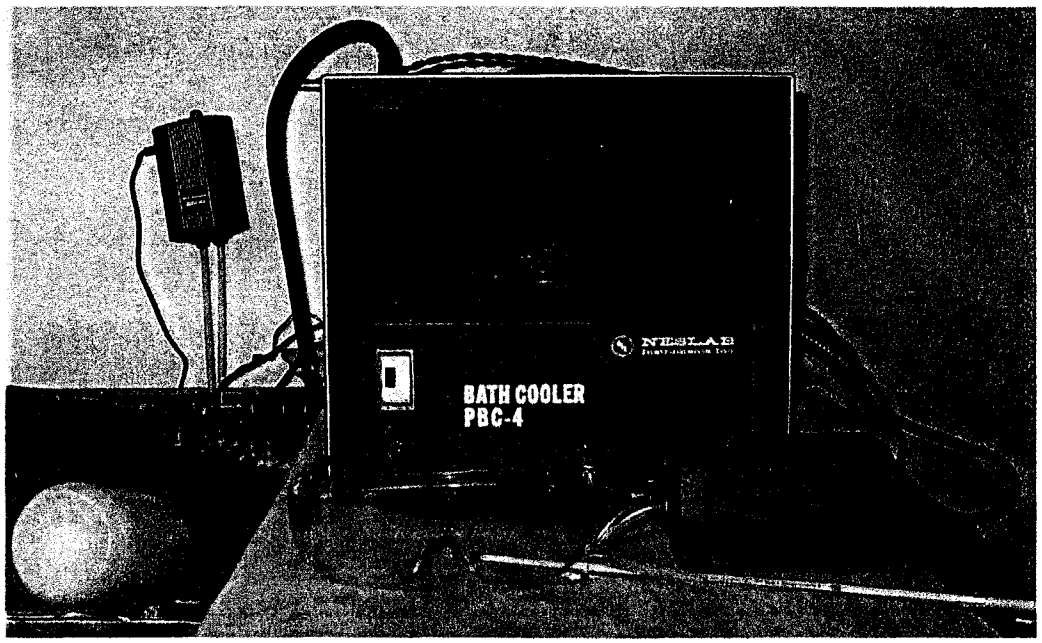

Figure1. TEW Acclimation Chamber and Chiller Unit: An aquatic light fixture provides >200 LUX at waters surface, 4 – 10cm long air stones maintain ~92 DO level, a NesLab Instruments bath cooler (chiller suspended on top of the acclimation chamber) maintains temperature at 15^0C+/-2^0C. Water continuously flows into the acclimation chamber at a rate of 0.120 l/min. and is pumped out of the tank once ~340 L has been reached, at a rate of 3.3 l/min. Chiller used for maintaining the temperature of the acclimation chamber at 15^0C+/-1^0C

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trout in accordance with SOP#AT003, and the results were validated using SOP#AT001, prior to their use in either LC50 or TEW testing.

Study Site, Sample Collection and Storage

The TEW study site was located at Bowater Inc. in Thunder Bay, Ontario. Three industrial effluents were sampled from this mill. For testing purposes grab samples of the effluents were taken twice a week. The collected effluent at each site was pumped into a 1500 L tote and placed in the box of a truck. Effluent was then transported into a Chiller room located above the acclimation chamber and TEW facility. This room contains ten 1000 L totes, all made from polypropylene, stacked in a staggered fashion to allow access to the center hole in the top of the tote. The chiller room was maintained at 4^0C and is located in the mezzanine level, located directly above TEW lab. Maintaining the effluents at this low temperature prevents the chemical composition of effluent to change over time. (Volatilization, pH, COD (MOE 2000)).

Effluent Storage, Preparation and Flow-Through

The effluent sample and storage methods were originally developed by Hardy (2000), and have been modified to fit the TEW system design and technique. A schematic of the TEW Laboratory set-up is shown in Figure 2.

Storage totes 1, 2, 6, 7 (effluent dilution) were connected to the TEW testing chambers within the TEW lab one floor below. Storage totes 5a and 5b were connected to the TEW acclimation chamber, while totes 4a and 4b, reserved for dilution river water storage, were both spliced and connected to testing chamber 1,2 (tote 4a) and 3,4 (tote 4b). These connections are shown in Figure 3.

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Figure 2.

System Schematic of TEW System

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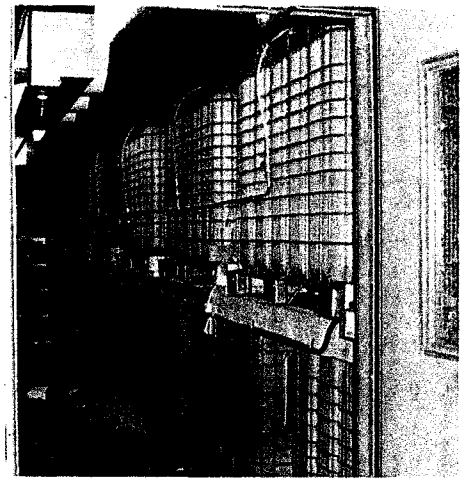

Chiller Room Above

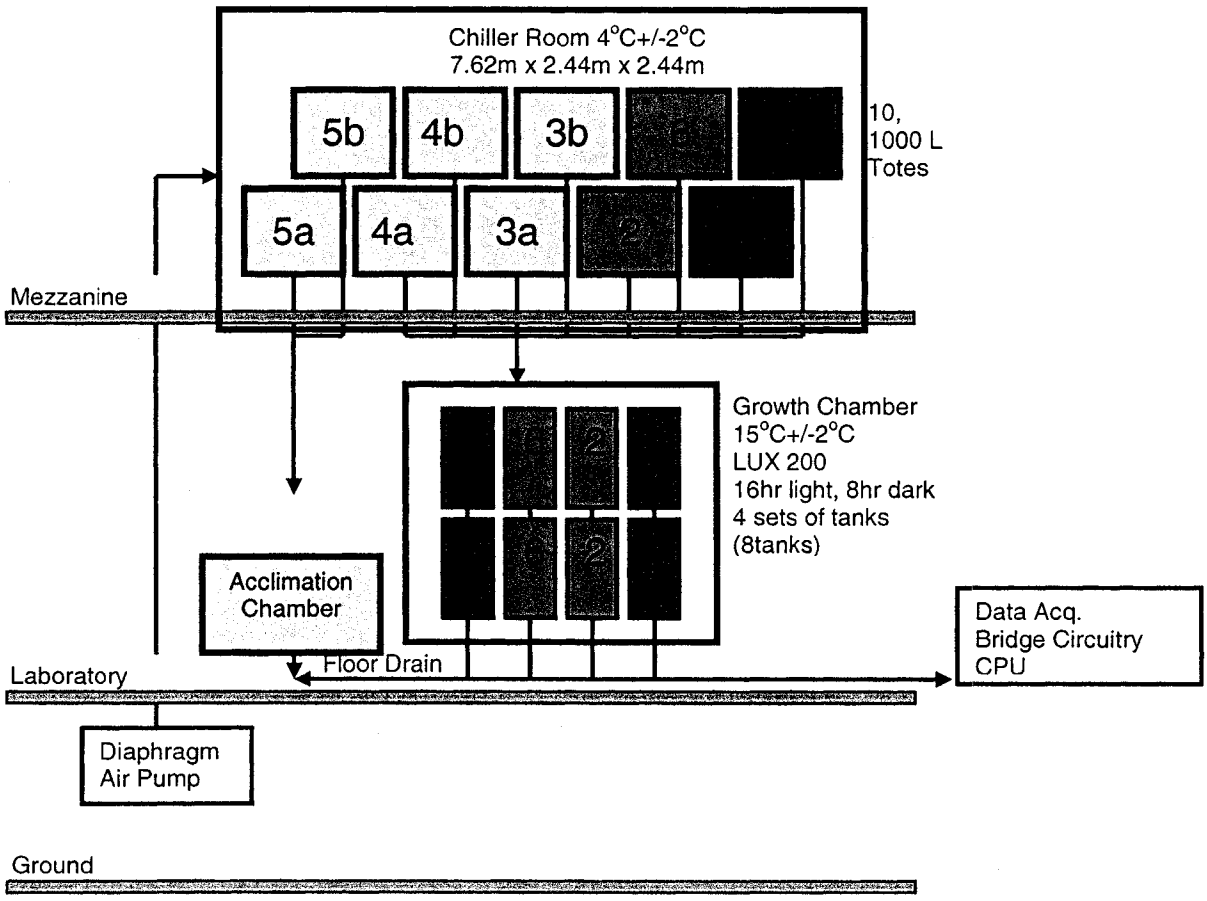

Figure 2. TEW Flow-Through System (sample pick-up to effluent testing stage): The interior of the Chiller room is displayed in the top left corner (contains ten 1000 L totes, and is maintained at a temperature of 4^{0}C). Effluent flows from the totes stored in the Chiller room to the laboratory and through the testing chambers containing the test species, rainbow trout.

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Figure 3.

System Schematic of TEW Flow-Through System

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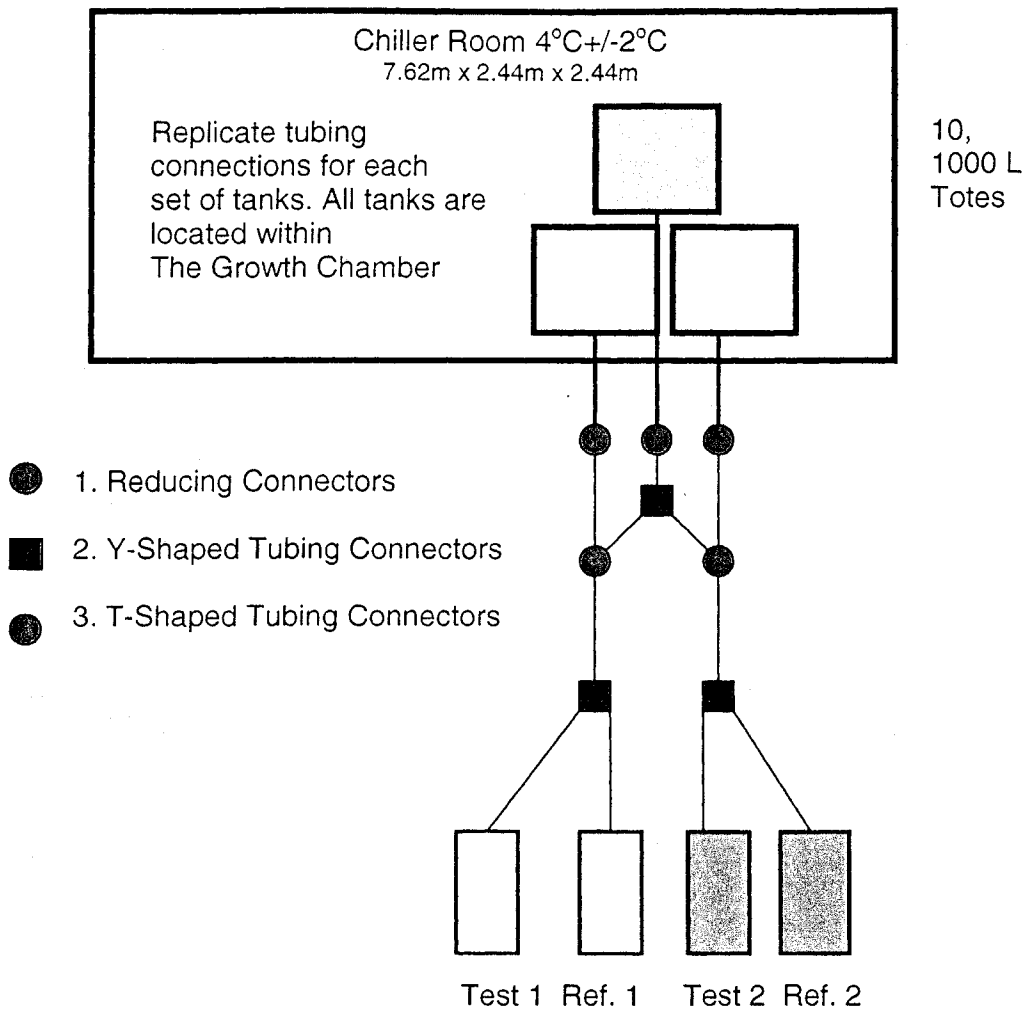

Figure 3. Tubing Connections and Configuration for TEW Flow-Through

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Hoffman Swinging Jaw Clamps #299-600.0 were used to control the inflow of both the dilution water and the effluent flow at a rate of 100 ml/min for each set of testing chambers. The 2cm HDPE tubing was spliced again prior to reaching the testing tank by more Y-shape tubing connectors in order to ensure that the inflow rate and type was identical between each test and reference chamber.

In order to prevent unnecessary stress to the rainbow trout, effluent must reach a temperature of 15^0C $^+/.1^0C$ prior to trout exposure as stated by the Environment Canada, Environmental Protection Series - Biological Test Methods: Using Early Life Stages of Salmonid Fish (1992). Due to the slow flow rate (100 ml/min) and the temperature of 13^0C maintained within the Growth Chamber, the desired effluent temperature was easily reached.

Growth Chamber, Data Acquisition, TEW Circuit Box

The growth chamber, housing the testing and references tanks, was a Conviron E7, environmental chamber (Figure 4a). It was modified to reduce compressor vibrations by relocating the compressor and motor to the base of the unit, temperature was maintained at 13^0C, and lighting was maintained on a 16/8hr light and dark cycle, 250 LUX. The TEW Circuit box houses the main data collection component of the TEW system (Figure 4b). The Dataq acquisition system, purchased from DATAQ Instruments (Figure 4c) records and displays trout activity on a PC.

The test tank and the reference tank (Figure 5), created by Surecraft Plastic, Thunder Bay, Ontario. Both tanks, which were stored in side the growth chamber, consist of two compartments, inflow and fish compartment, outflow occurs in the fish camber through a Plexiglas cylinder. The inflow compartment is 3.80cm x 5.08cm x 8.5725cm,

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contains 167ml of solution, the fish testing compartment is 20.32cm x 5.08cm x 8.5725cm and contains 891.19ml of solution. Inflow and outflow occurs at a rate of 100ml/min. The wall joining together the two compartments has two strips 0.635cm x 0.635cm removed to allow a gradual flow of solution from one compartment to the next. These strips have a spacer placed between them evenly covered by a 3.175cm x 0.635cm x 5.08cm plate. This forces the inflow-solution towards both the top and the bottom of the fish compartment.

Two electrodes were suspended on either end of both the testing tank and the reference tank, the electrode holders were designed to slide the electrodes freely over top of the tanks, while still allowing access to the testing area. Stainless steel washers, nuts and bolts were used to secure the electrode via coated copper wires, which were then connected to the conductivity bridge circuit and fed into the Dataq acquisition system.

Circuit Design and Test Chambers

The problem with the pulp and paper effluent is that it is not homogeneous due to this continuously changing matrix; therefore a Wheatstone bridge design was chosen (Figure 4b). Two identical tanks with flow directed equally to each, form two arms of a four-arm bridge (see Circuit Schematic, Figure 6). The two remaining bridge resistors are equal to within 0.5% and have a resistance of approximately the same value as the flow tanks without effluent. Differencing the signals from the two tanks, results in a signal that was due almost solely to the fish regardless of the effluent's nature or concentration.

Electrically grounding one side of the reference tank minimized input offset voltage to the preamp stage of the detector circuit. To achieve this, a 1:1 600Ω signal transformer was used to electrically isolate the single ended AC source output from the

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bridge circuit. The difference signal (now referred to as Ground) was fed via the 0.1μF capacitor into the OP277 pre-amplifying operational amplifier (op-amp). The gain of this stage was set to 1000x. Typically, the AC source operates at 1.2-1.8 KHz, so an amplified version of the bridge output AC signal must be rectified to produce a DC signal that is in proportion to the bridge imbalance. This was accomplished by the first OP07 op-amp. The second OP07 provides power amplification for this signal, which was smoothed to remove any residual AC by the RC integrator and final OP07 follower. An AC measurement voltage of 1 voltage peak-to-peak (equivalent to 0.35 volts DC) may be detected by the trout but appeared to have no influence over the trout's behavioral response to its environment.

The 600 Ω isolation transformer was chosen to simultaneously provide signal power to two identical bridge configurations. Since each one has an input impedance of about 6.8KΩ, there was negligible cross-talk between the two stages. Use of a single transformer and oscillator to power two stages reduced the overall cost and instrumentation footprint in a cramped environmental chamber lab setting.

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Figure 4.

Growth Chamber, TEW Bridge Circuitry and Data Acquisition System

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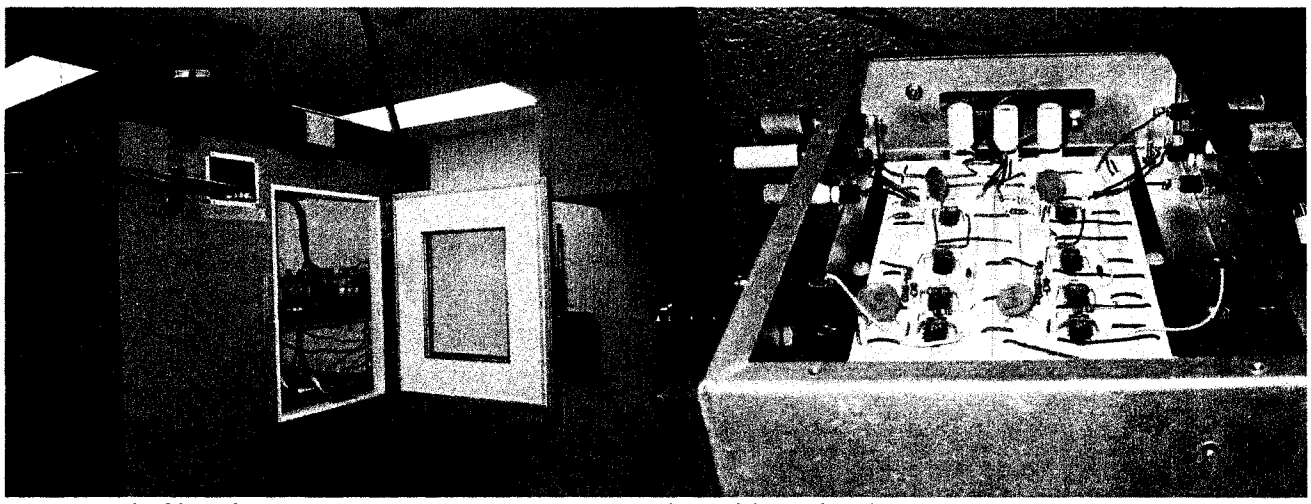

(a) Growth Chamber (b) Bridge Circuitry System

(c) DATAQ Instrumentation and Bridge Circuitry System

Figure 4. (a) Growth Chamber, (b) Conductivity bridge circuit, (c) Data acquisition system and Bridge Circuitry system.

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Figure 5.

TEW - Test Tank and Reference Tank Schematics

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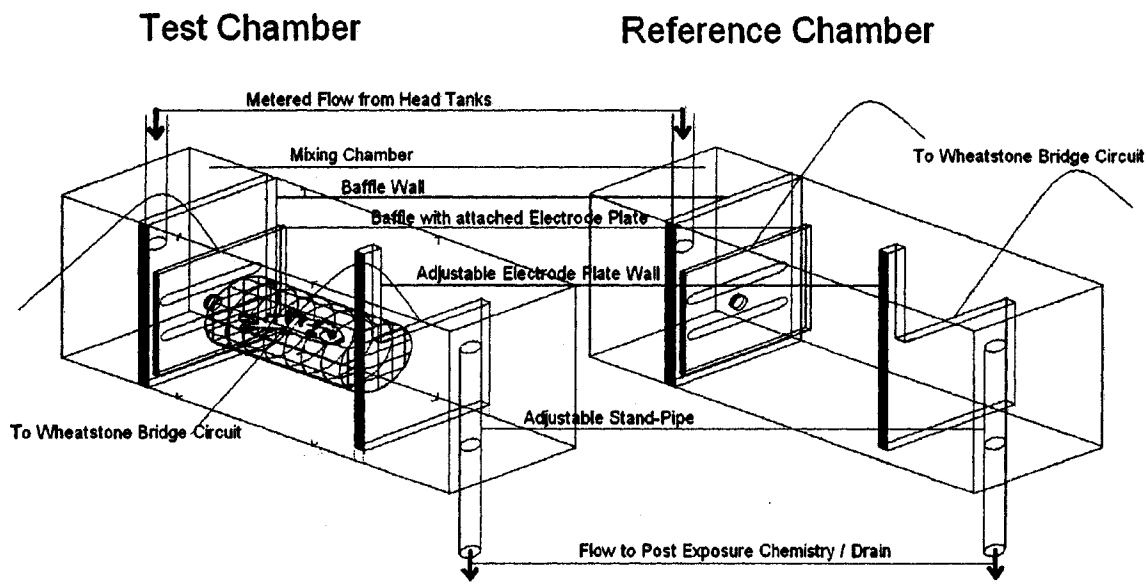
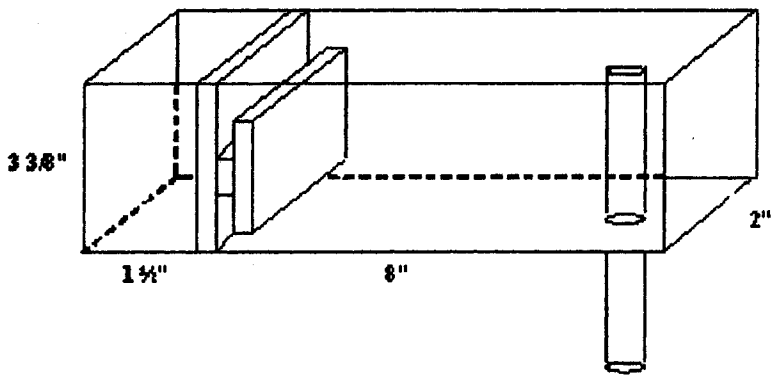
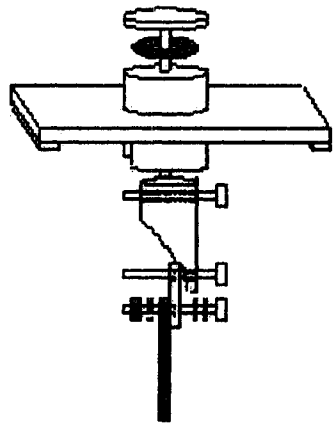


(a) Test Chamber and Reference Chamber of the TEW testing system



(b) Tank Design



(c) Electrode and Holder

Figure 5. TEW – Test Tank and Reference Tank Schematic: Rainbow Trout Effluent Testing Tank / Dimensions: (Left) Inflow and outflow of effluent and dilution water equals 100ml/min. (Right) Electrode and Electrode Holders: Electrodes are composed of #640 stainless steel plates (3.80cm x 3.80cm x 0.635cm). They are suspended over the tanks by electrode holders which are designed to slide smoothly overtop of the tank while remaining connected to the data acquisition system.

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Figure 6.

TEW – Toxicity Monitoring Conductive Bridge Circuitry System

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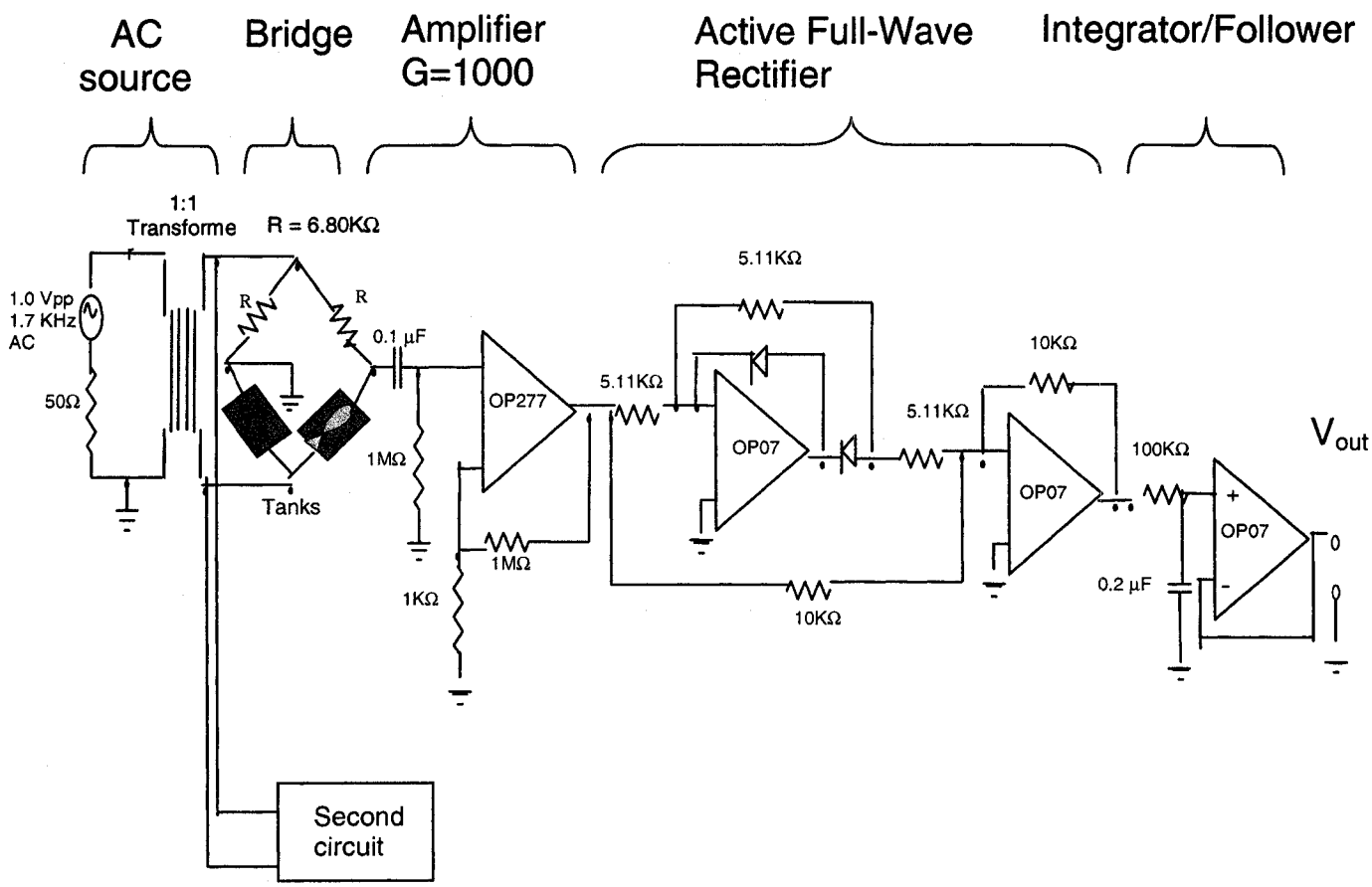

Figure 6. Toxicity Monitoring Conductivity Bridge Circuit:
Detailed layout of the AC source, bridge comparator, electronic amplifier, and active AC rectifier section. After the integrator/follower stage, a varying DC voltage that represents rainbow trout activity, is connected to the digitizer for computer input.

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Response Signal

The signal was acquired from the conductivity bridge circuitry system via a data acquisition system which displayed trout behavior on a PC via a DATAQ model banana jack input adapter and DI-720USB 32-channel data acquisition system. The data was collected for a 12hour-time period as a continuous waveform that provides instantaneous signs of fish stress through abnormal waveform actions such as peaks, valleys and erratic wave movement. The initial 6hrs acts as a test tank acclimation period where the fish was exposed to the dilution series (Kaministiquia River water); this period also acted as a baseline for each fish's normative behavior patterns. Since the behavior of each fish may differ, the individual control period provides a basis for comparison between behavior exhibited in both the dilution water and the effluent. At the 6-hr mark effluent was introduced into the testing chambers, producing an immediate behavioral response from the fish. Recorded data can be viewed during the test or after completion as a condensed file (Figure 7a). Regular breathing patterns appeared as steady peaks and valleys of approximately 0.1volts peak to peak (Figure 7b). Cough frequency was displayed as a double hitch in the normal breathing pattern and was caused by a reversal of water backwards over the fishes gills, two peaks were readily visible, the first approximating 0.05 volts peak to peak, followed by a 0.05 valley and a 0.1 volt peak (Figure 7d). Coughing is a normal occurrence in most fish its function either reflexive or simply a method of expelling waste and unwanted materials out of the gills. The increase of toxicants and particulate matter within the fish's environment has been noted to increase the trout's cough frequency. Whole body movement was measured by erratic waveforms exhibited by as small as 0.02 voltage change too as much as >2.0 volts (Figure 7c).

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Figure 7.

Comparison of Activity in Acclimation Chamber to Activity

During Effluent Exposure.

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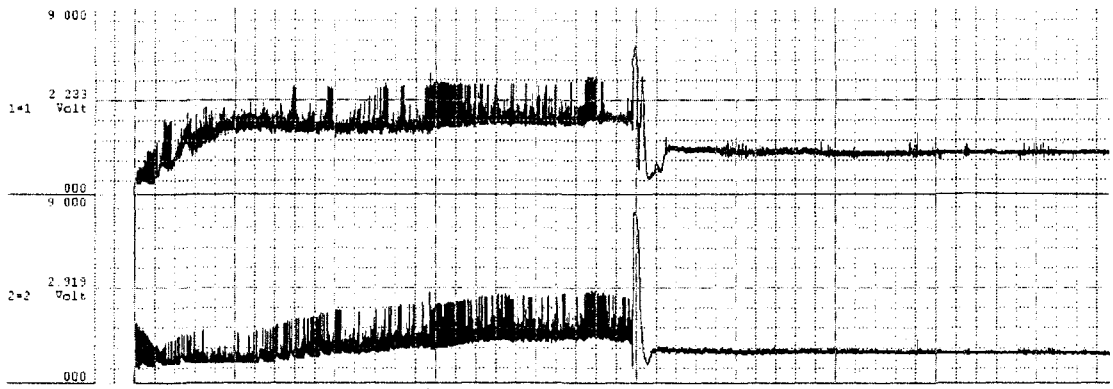
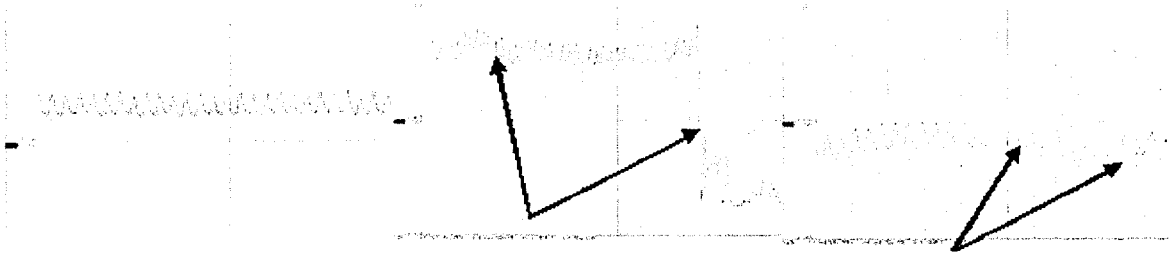


(a) Condensed 12hr test of 100% KRAFT effluent using two rainbow trout



(b) Breathing Pattern (c) Whole Body Movement (d) Cough Frequency

Figure 7. Acclimation Compared to KRAFT Exposure:

(a) 12hour toxicity test using two rainbow trout (6hr-dilution water exposure, 6hr 100% KRAFT effluent exposure). Trout acclimation and control period exhibit frequent and strong movement, trout effluent exposure period exhibits greatly reduce body movement, irregular ventilatory frequency and depth as well as increased cough frequency. (b) Breathing of rainbow trout exposed to dilution water – 0.5volts peak to peak, ~1.5 breaths/sec. (c) Whole body movement of rainbow trout – 2.45volts peak to peak, (d) Cough frequency of rainbow trout – Frequency undetermined.

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The response of the fish was dependant on the type of effluent used as well as the concentration of the effluent. Figure 7a. exhibits the response of two rainbow trout to the effluent sampled. Overall body movement was greatly reduced, an increase in cough frequency was observed and ventilatory depth and frequency both increased and became more erratic.

Once completed each test provides both the waveform patterns and a numerical recording of the physical parameters expressed by the trout. These data were retrieved as an excel file and any significant behavioral responses were flagged through the application of statistics, later to be used in the development of a response to toxicity correlation between rainbow trout and their surrounding environment. Through continuous testing of this system a library of toxicity responses can be developed. This toxicity detection system can eventually be setup in series with industrial outfall pipes, monitoring the effluent as it passes out of the industry thus creating an efficient and economically viable toxicity early warning system.

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Chapter 2
The Use of the TEW System to Monitor the Toxicity of Pulp and Paper

The Pulp and paper industry requires vast amounts of water, wood chips and chemicals in order to manufacture paper. Effluent, created as a side product of paper manufacturing, commonly contains such chemicals as chlorine (Cl_2), chlorine dioxide (ClO_2), ozone (O_3), oxygen (O_2), sodium hypochlorite (NaOCl), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) and over 500 other organic compounds (Hardy 2000). Due to the toxicity of these chemicals in the environment, industries such as pulp and paper are required by both provincial and federal laws to chemically, physically and biologically treat their effluent prior to its expulsion into the natural water systems. Physical chemical treatments are used to remove coarse fractions such as oil, fatty acids and suspended solids through a variety of methods (screens, coagulation, flocculation, flotation, centrifugation, fluidization, electrolysis, settling and precipitation). Biological treatments lower the organic load of solute organic compounds through aerobic and anaerobic treatments. Active sludge treatments use bacteria and microorganisms, which consume the organic matter within the sludge. Fine filters remove any remaining particulate matter from the now treated effluent, and disinfectants such as ozone, UV or chlorine dioxide (ClO_2) are used to make the water safe for redistribution back into the environment (Lenntech 2005).

Government also requires that effluent toxicity is monitored via laboratory testing, regular sampling and testing of grab samples (Davis 1995). These tests include bioassays such as the chronic rainbow trout Lethal Concentration test (LC50) test, where

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the concentration of an effluent, which kills 50% of a sample population, determines the level of effluent toxicity (Hardy 2002). An LC50 fails to describe the composition of the effluent rather it determines the degree of toxicity present within the effluent tested.

Under normal circumstances effluent treatment processes remove 100% of toxins from what would otherwise be extremely toxic effluent. The expulsion of properly treated effluent into natural waterways results in minimal negative environmental ramifications. Despite all of the precautions in place on the treatment of pulp and paper effluents, mechanical and structural errors do occur. This results in the occasional occurrence of a toxic event. The occurrence of toxic spills has prompted the development of toxicity warning system, usually consisting of a biological monitoring component, for example the Dynamic *Daphnia* Test which monitors the movement of *Daphnia magna* as they swim through chemical solutions. This results in a response-to-toxicity data base describing *Daphnia* movement at different levels of toxicity (Leeuwangh 1978). Other toxicity warning systems promote the use of sunfish, algae, and/or mussels.

The preliminary test describing the development and function of the Toxicity Early Warning system (TEW) used in this study are described in Chapter 1. Detailed testing methods and procedures can be found in the Lakehead University Toxicity Early Warning System (TEW) - Rainbow Trout Standard Operating Procedure SOP#TEW001, given in Appendix V.

The objective outlined in this study was to relate the TEW systems sensitivity to the pulp and paper industry by developing a rainbow trout (*Oncorhynchus mykiss*) trout response-to-toxicity library to three different pulp and paper mill effluents.

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Methods

Study Site, Sampling Requirements and Handling

The study site was located at Bowater Inc., a local pulp and paper mill based in Thunder Bay, Ontario. Three effluents were sampled from this site for use in the development of the TEW tests, these included KRAFT Clean Water Outfall (KCWO), KRAFT and NEWS effluents. The untreated KRAFT effluent was sampled from the KRAFT cooling towers. The untreated NEWS effluent was sampled from the NEWS lifting station, located near the NEWS distribution chambers. The treated KCWO was sampled from the KCWO sample building located along the north shore of the Kaministiquia River. Once samples were collected they were transported to and stored in a chiller room which maintained the samples temperature at 4^0C. All effluents were diluted using the geometric concentration series (100%, 50%, 25%, 12.5%, 6.3%) with Kaministiquia River water sampled upstream of the mill. Testing will usually commence 24-hrs after sampling, but in accordance with EPA (1992) must be tested within 3 days and no later than 5 days from the sampling date. Kaministiquia River water was employed for the acclimation chamber, the 6-hr acclimation period of testing and effluent concentration dilutions.

The temperature, pH and the DO of the test tank were monitored daily and prior to testing. Testing conditions must remain at a temperature of $15^{+}/.1^0$C, pH between 6.5 – 8.0, and the dissolved oxygen level must remain over 90% saturation and below 100% saturation. The testing photoperiod was kept on a $16^{+}/.1$-hr light, $8^{+}/.$ 1-hr dark cycle, with a light intensity of 200 LUX as required by EPS 1/RM/9&13 (1990). Upon test

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completion trout were euthanized through submersion in Tricaine methane sulfonate (TMS), the fork length and weight of each trout was measured and recorded.

Lethal Concentration tests (LC50) were performed on two samples of KRAFT, and one sample of NEWS.

Test Apparatus

The TEW system was described in Chapter 1. Essentially the system consists of 4 sets of test and reference tanks, each individually connected via electrodes to the Conductivity Bridge circuitry system and then into a PC for visual display, recording, and analysis capabilities. Four fingerling rainbow trout (0.3-5.0 grams) were randomly selected for each test, with an additional test replicate. This resulted in the use of 8 trout for each effluent concentration, 40 trout for each effluent sample, and 120 trout total. Each test was composed of a 6-hr, test tank acclimation period and a 6-hr effluent exposure period

All results for both the test tank acclimation period and the effluent exposure periods were obtained by randomly sampling fifty, ten second data sets within both exposure period for each effluent concentration tested. Behavioral responses (body movement, breathing depth, breathing frequency and overall activity level) were statistically analyzed through calculating averages, coefficient of variance and ANOVA's.

Results

Normal Trout Behavior

A "Normal" rainbow trout behavioral response was established by monitoring the ventilatory rates, frequency, and whole body movement of the trout for a 6-hr time period

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prior to every test performed is shown in Table 1. The results show that trout normally breathe at a depth ranging from 0.0138v - 0.7895v, with a breathing rate of 2-4 breaths per second. When viewed electronically trout ventilation appear as a two abrupt spikes in the data (~0.15v in height) followed by a dramatic decline of approximately 0.2volts (Figure 1a). Trout body movement during the acclimation period of the test appears as erratic movement ranging from 1v - 4v, as shown in Figure 1b.

Due to signal interference caused by excessive trout body movement, cough behavior (movement of water backwards over the gills to remove foreign/unwanted matter) could not be distinguished from the regular breathing pattern output and was not a useful measurement of effluent toxicity with the current TEW approach.

Trout Behavior During Effluent Exposure Period

The TEW tests suggested that changes in ventilatory depth provided the first indication of toxicity, followed by a decrease in ventilatory rate and then whole body movement. The ventilatory patterns displayed in Figure 2 (a-d) were derived from four different juvenile rainbow trout. These patterns are a representative of the ventilatory behavior most commonly displayed by the trout. Ventilatory depth varied the least and provided an easy identification of potential toxicity. The ventilatory frequency was much more variable. There were four distinct patterns, but pattern (b) and (d) were the most common. Out of 120 fish tested 55 fish exhibited patterns similar to (b), and 34 fish exhibited patterns similar to (d). Pattern (a) was displayed by 12 trout, and pattern (c) was displayed by 19 trout. All four patterns have one key characteristic determining trout health, namely the pattern is regular, and does not drag. When the trout are healthy breathing is a consistent and regular process;

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Table 1. Whole Body Movement, Ventilatory Frequency and Ventilatory Depth of Rainbow Trout During the 6hr Test Tank Acclimation Period, using Kaministiquia Dilution Water

Ventilatory Length Breaths / Sec.			Ventilatory Depth (Volts)			Body Movement (Volts)		
KRAFT	NEWS	KCWO	KRAFT	NEWS	KCWO	KRAFT	NEWS	KCWO
2.94	2.38	2.00	0.0066	0.0138	0.0983	3.58	2.87	1.43
3.85	3.23	3.70	0.0197	0.0526	0.2154	2.66	1.22	2.05
2.94	1.82	1.79	0.0164	0.0164	0.2030	1.79	3.97	3.26
2.94	2.94	2.44	0.2632	0.0428	0.0337	1.13	2.44	2.18
1.79	2.38	2.13	0.0658	0.1711	0.1670	2.87	2.81	3.19
1.79	2.38	2.22	0.0329	0.2627	0.0253	3.02	1.64	1.19
3.33	1.92	2.00	0.0263	0.1402	0.0649	3.78	1.75	1.96
2.00	2.86	2.08	0.0197	0.1101	0.2130	3.45	4.00	2.97
1.43	3.13	2.00	0.0263	0.0267	0.1951	2.06	1.52	1.09
3.13	2.13	3.45	0.0164	0.2405	0.1996	3.58	1.75	2.11
2.17	2.56	3.45	0.0230	0.0714	0.1163	1.18	1.85	1.63
1.43	2.44	1.89	0.0263	0.0851	0.0612	2.38	1.83	3.01
2.27	2.17	3.57	0.7895	0.0534	0.1286	2.02	1.13	3.49
2.08	2.04	2.22	0.3947	0.1539	0.0946	3.03	1.12	3.12
2.38	2.00	2.56	0.3290	0.1967	0.2296	2.39	3.82	1.62
2.17	1.79	2.13	0.3618	0.1388	0.0817	3.75	1.58	3.82
2.94	1.96	2.17	0.3290	0.0982	0.1838	2.19	1.66	2.96
2.00	2.33	3.23	0.5921	0.2529	0.0612	1.06	2.20	1.47
2.08	2.04	3.57	0.0197	0.0632	0.0744	2.99	1.46	3.29
2.38	3.23	2.56	0.0192	0.2560	0.1885	3.15	1.76	3.35
Combined Average = 2.45 Breaths / Second			**Combined Average = 0.14volts**			**Combined Average = 2.39volts**		

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Figure 1.

Ventilatory Frequency and Depth, and Whole Body Movement of Rainbow Trout Recorded During the 6-hr Acclimation Period Prior to Effluent Exposure

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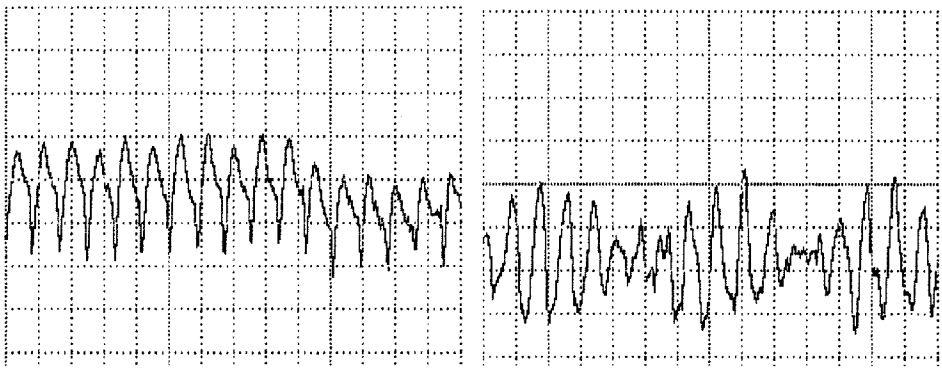

Figure 1a. Ventilatory rates and depths of Rainbow Trout

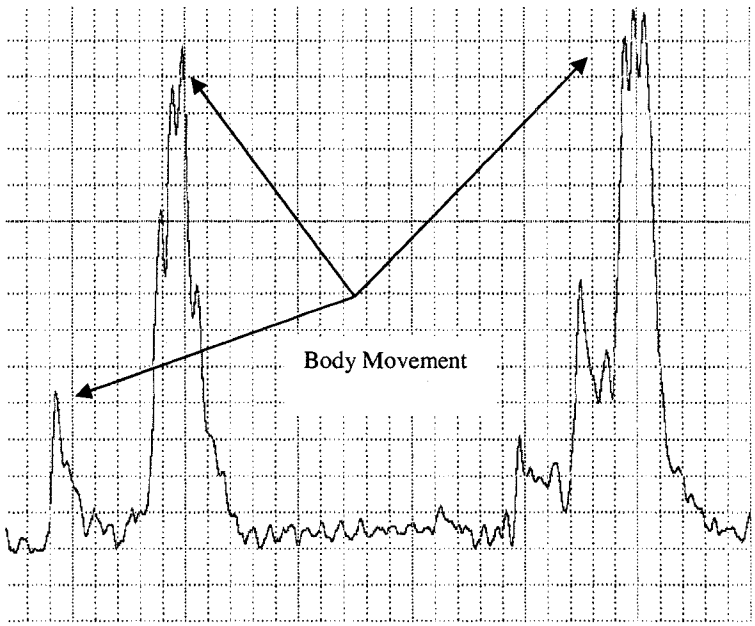

Figure 1b. Rainbow trout whole body movement

Figure 1. Ventilatory Frequency and Depth, and Whole Body Movement of Rainbow Trout Recorded During the 6-hr Acclimation Period Prior to Effluent Exposure: (a) Ventilatory rates and depths of rainbow trout recorded during the six hour test tank acclimation period of the TEW tests. These are the control results exhibiting trout exposure to Kaministiquia River water. (b) Rainbow trout whole body movement: The three peaks represent the body movement of a rainbow trout (*Oncorhynchus mykiss*) during the acclimation period of TEW testing.

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inhalation breathing is shown as a rapid increase of voltage, followed almost immediately by an exhalation, the spacing between the two remaining almost equal.

Figure 2 (e-l). displays the ventilatory patterns exhibited by trout exposed to 100% KRAFT effluent (the same scale was used). Stressed trout behavior patterns were displayed as either erratic ventilation (g) and (h), shallow (e) and (i) or labored (f) and (j). Labored breathing patterns can be identified by a sharp change in voltage followed by a slower change. Pattern (f) for example shows a quick inhalation followed by a very labored exhalation whereas pattern (j) displays the opposite. The ventilatory depth appears as greatly reduced for most of the trout.

Normal and abnormal trout activity or whole body movement is displayed in Figure 2 (k, l). Whole body movement provided a very quick indication of potential toxicity when acclimation behavior patterns could be compared to that of the effluent exposure patterns.

Trout were very active during the acclimation period [Left: (k), Left: (l)] but their activity levels were greatly reduced when exposed to KRAFT and the higher concentrations of NEWS effluents [Right: (k), Right: (l)]. In the presence of 100% KRAFT trout would reduce movement from whole body to only fin and gills [Right: (l)].

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Figure 2.

Comparison of trout ventilatory library (a-d) to Abnormal trout ventilatory library (e-j). Ventilatory patterns were derived from rainbow trout exposure to 100% KRAFT effluent, scale (0-0.5votls). Whole body movement (k-l) during rainbow trout exposure to 50% KRAFT effluent, compared to trout exposure to 100% KRAFT effluent.

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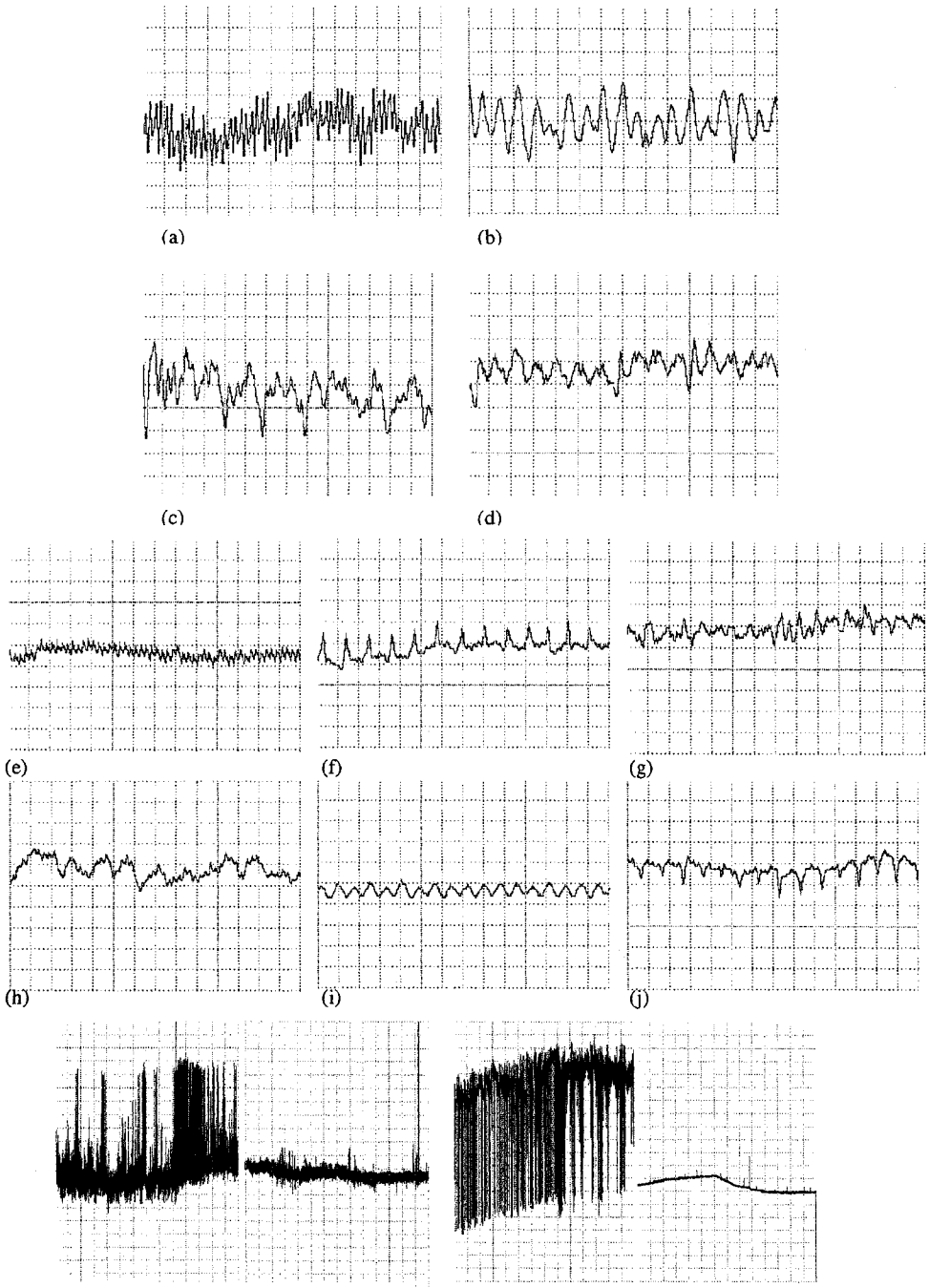

(k) Scale: 0-6.0volts – Left: 5hour acclimation period, Right: 5hour effluent exposure period (50% KRAFT)

(l) Scale: 0-7.5volts – Left: 5hour acclimation period, Right:5hour effluent exposure period (100% KRAFT)

Figure 2. Comparison of Normal trout ventilatory library (a-d) to Abnormal trout ventilatory library (e-j) Ventilatory patterns, all derived from rainbow trout exposure to 100%KRAFT effluent, scale (0-0.5volts). Whole body movement (k, l) displaying acclimation vs. effluent exposure period.

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KRAFT Clean Water Outfall (KCWO)

The KCWO data points were collected and the averages can be viewed in Table 2. The acclimation period exhibited an average body movement of 2.46v, breathing depth of 0.1318v and 2.56 breaths per second. The effluent exposure period exhibited an average body movement of 2.52v, breathing depth of 0.1278v and 2.62 breaths per second. An Analysis of Variation (ANOVA) were performed for each of the parameters listed (Table 2). The Null Hypothesis used in ANOVA calculations assumes the theory that there are no significant differences found within the data, the data being the behavior displayed in the acclimation period compared to the behavior displayed in the effluent exposure period. When the null hypothesis is rejected a significant difference between the data has been found. The ANOVA showed that the Null Hypothesis was accepted, and there was no significant difference found between the 6-hr acclimation period and the 6-hr KCWO effluent exposure period.

KRAFT and NEWS

The KRAFT average body movement, breathing depth, and breaths per second for both the acclimation period and the effluent exposure period can be viewed in Table 3a. The KRAFT acclimation period exhibited an average body movement ranging from 2.28v to 2.69v, breathing depth ranged from 1.2449v to 1.8469v and breaths per second ranged from 2.47 to 2.77. The effluent exposure period of 100% KRAFT exhibited an average body movement of 0.87v, which increased to 1.33v in the 50% dilution series, and decreased to 0.97v in the 25% dilution series. Both of the 12.5% and 6.3% concentrations showed an increase in body movement, averaging 2.03v, 3.62v respectively. The average breathing depth for 100% concentration was 0.0240v, which

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Table 2. KCWO –Rainbow Trout Ventilation

KCWO - 100%

Body Movement and Ventilatory Rate and Frequency

	Acclimation Period			Effluent Exposure Period		
	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second
	1.43	0.0983	2.00	1.83	0.1445	2.94
	2.05	0.2154	3.70	3.29	0.0337	2.08
	3.26	0.2030	1.79	2.19	0.1903	2.38
	2.18	0.0337	2.44	1.44	0.1434	4.76
	3.19	0.1670	2.13	2.88	0.0470	2.13
	1.19	0.0253	2.22	1.98	0.1667	2.86
	1.96	0.0649	2.00	3.23	0.0523	2.33
	2.97	0.2130	2.08	1.96	0.1850	2.00
	1.09	0.1951	2.00	1.02	0.0548	2.27
	2.11	0.1996	3.45	3.06	0.1148	3.03
	1.63	0.1163	3.45	3.81	0.1996	2.27
	3.01	0.0612	1.89	1.93	0.1045	3.23
	3.49	0.1286	3.57	1.54	0.1058	2.22
	3.12	0.0946	2.22	3.10	0.1392	2.22
	1.62	0.2296	2.56	1.69	0.1036	2.04
	3.82	0.0817	2.13	3.35	0.1548	2.08
	2.96	0.1838	2.17	1.45	0.1618	3.03
	1.47	0.0612	3.23	3.45	0.0772	2.33
	3.29	0.0744	3.57	3.29	0.1810	3.85
	3.35	0.1885	2.56	3.97	0.1964	2.27
Ave.	**2.46**	**0.1318**	**2.56**	**2.52**	**0.1278**	**2.62**

* Indicates significant trout behavioral response at $P<0.05$ when acclimation period is compared to effluent exposure period.

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increased to 0.0343v at 50% concentration, decreased to 0.0172v and then displayed a gradual incline of 0.0242v and 0.0313v at 25%, 12.5%, and 6.3% concentrations. The average ventilatory rate at 100% concentration was 1.76v, which increased to 1.99v, and 4.67v and then decreased to 1.85v and 1.92v in their respective, declining, effluent concentrations. The results completed for both KRAFT and NEWS tests can be viewed in Appendix II, and III, respectively.

The NEWS average body movement, breathing depth, and breaths per second for both the acclimation period and the effluent exposure period are contained in Table 3b. The NEWS acclimation period exhibited an average body movement ranging from 2.42v to 2.95v, breathing depth ranged from 0.1541v to 0.1386v and breaths per second ranged from 2.33v to 2.63v. The effluent exposure period of 100% NEWS exhibited an average body movement of 5.75v, which gradually decreased through each successive effluent concentration from 4.74v, 3.32v, 3.17v, to 3.09v at 6.3%. A breathing depth of 0.0714v gradually decreased to 0.0442v in 12.5% effluent concentration and then increased slightly to 0.0470v in the 6.3% dilution series. At 100% concentration the breaths per second was 2.16, decreasing to 1.45v at 50%, increasing slightly to 1.67v and 1.72v in 25% and 12.5% concentration, then dropping to 1.47v in the 6.3% dilution series.

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Table 3a. KRAFT Ventilatory Depth, Frequency and Whole Body Movement

	KRAFT					
	Acclimation Period			Effluent Exposure Period		
Concentration	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second
*100%	2.53	1.2613	2.74	0.87	0.024	1.76
*50%	2.54	1.2449	2.47	1.33	0.0343	1.99
*25%	2.59	1.6468	2.67	0.97	0.0172	4.67
*12.50%	2.28	1.8469	2.77	2.03	0.0242	1.85
*6.30%	2.69	1.3644	2.71	3.62	0.0313	1.92

* Indicates significant trout behavioral response at $P<0.05$ when acclimation period is compared to effluent exposure period.

Table 3b. NEWS Ventilatory Depth, Frequency and Whole Body Movement

	NEWS					
	Acclimation Period			Effluent Exposure Period		
Concentration	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second
*100%	2.42	0.1386	2.33	5.75	0.0714	2.16
*50%	2.65	0.1541	2.71	4.74	0.0607	1.45
25%	2.49	0.1449	2.56	3.32	0.0493	1.67
12.50%	2.95	0.1514	2.6	3.17	0.0442	1.72
6.30%	2.65	0.1504	2.63	3.09	0.047	1.47

* Indicates significant trout behavioral response at $P<0.05$ when acclimation period is compared to effluent exposure period.

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Average Activity Level of Rainbow Trout

The average activity level (all movement: ventilatory and whole body movement combined) for each of the three effluents and subsequent concentrations were calculated by repeatedly averaging 20min of data for the duration of a full 12-hr test (Figure 3). Results for the analysis of variances (ANOVAs) for each effluent concentration, comparing the river water exposure period (acclimation period) to the effluent exposure period (KCWO, KRAFT, and NEWS) are shown by Figure 3. Figure 3a shows that trout did not display statistically significant responses from the acclimation period to the KCWO effluent exposure period at 100% concentration. When trout were exposed to KRAFT, their activity levels drastically reduced in all effluent concentrations (6.3% - 100%), resulting in statistically significant activity levels for each dilution (Figure 3b). When trout were exposed to NEWS effluent they displayed an increase of average activity levels in all effluent concentrations but only 100%, and 50% dilutions were found to be statistically significant (Figure 3c).

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Figure 3

Average Activity Level of Rainbow Trout – Comparison of Acclimation Period to Effluent Exposure Period (KCWO, KRAFT and NEWS).

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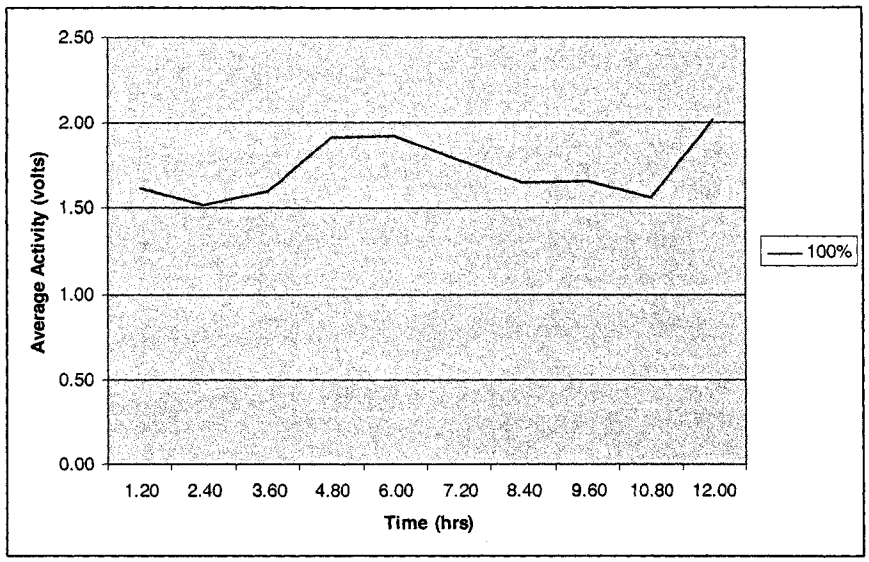

Figure 3a. Average Activity Level of Rainbow Trout Exposed to KCWO

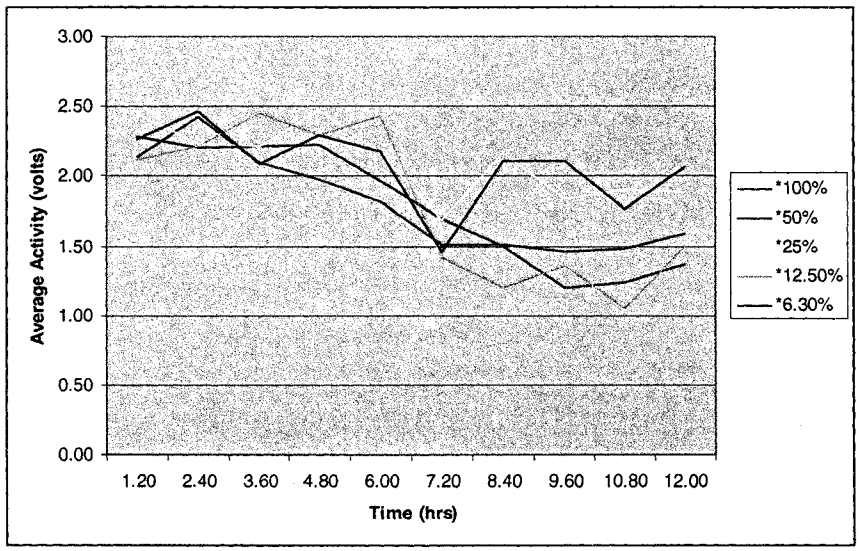

Figure 3b. Average Activity Level of Rainbow Trout Exposed to KRAFT

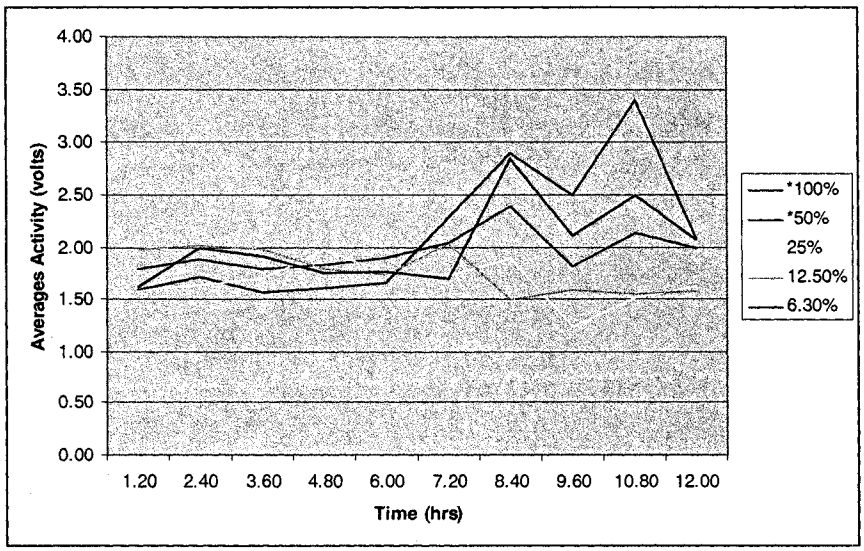

Figure 3c. Average Activity Level of Rainbow Trout Exposed to NEWS

Figure 3. Average Activity Level of Rainbow Trout - Comparison of Acclimation Period to Effluent Exposure Period (KCWO, KRAFT and NEWS). Time (hrs): 0-6 is acclimation (river water exposure) period, 6-12 is effluent exposure period, * Indicates statistical significance at $P<0.05$.

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Lethal Concentration (LC50)

Lethal Concentration tests (LC50) were performed on two samples of KRAFT, and one sample of NEWS to determine if the toxicity levels within the effluent were comparable to the behavioral responses displayed by the trout. The results of the LC50 tests were calculated using the Spearman-Karber test method and are displayed in Appendix IV. A summary of the LC50 results are displayed in Table 4.

Table 4. Lethal Concentration 50% (LC50), Summary of Mortality

	KRAFT (1)	KRAFT(2)	NEWS
100%	9	10	2
50%	0	10	0
25%	0	5	0
13%	0	1	0
6%	0	0	0
Control	0	0	0
Spear-K	73.49%	23.33%	N/A

The first KRAFT LC50 resulted in partial mortality within 100% effluent concentration. Three trout died on day two of testing, one trout died on day three of testing and five died on day 4 of testing, totaling nine dead trout out of the ten required within that concentration. The LC50, calculated using the Spearman-Karber method was 73.49%. The second KRAFT LC50 test resulted in five dead trout on day two in 25% effluent concentration, nine in 50% and nine in 100%. Mortality continued through out the testing period in these three buckets resulting in complete mortality in 100% and 50% concentrations, 50% mortality in 25% concentrations and 10% mortality in 12.5% concentration. No fish died in either the control or at the 6.3% effluent concentration. The LC50 was 23.33%, which can be described as fairly toxic. The NEWS LC50 results were not calculated because morality was below the 50% required to perform a Spearman-

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Karber calculation. Only two fish died, at 100% concentration on day two. There was no LC50 test performed on the KCWO due to insignificant behavioral response exhibited by the trout.

Discussion

The Toxicity Early Warning system, (TEW) was established as a potential method for early toxicity detection in industrial effluent. By using rainbow trout as an indicator species, this system has begun the initial steps in developing a trout response-to-toxicity library, which will be used as a behavior reference when future effluents are monitored. The TEW systems design, although unique, was modeled from the initial work performed by scientist such as Spoor *et al.* (1971), Cairns and Spark (1971), and Gruber *et al.* (1989). They focused on the behavioral response of bluegill sunfish to specific chemicals stimuli.

Pulp and Paper Effluent Composition

One of the difficulties in using biomonitoring systems for industrial effluents is that these effluents, especially in pulp and paper mills, are not always homogeneous. Effluents may contain high levels of residual material, or even moderately sized, semi-solid particulates. In many biomonitoring systems effluent must be filtered prior to monitoring, otherwise clogging will occur. Unfortunately for these systems any particulate material removed from the effluent will decrease its accuracy. The filtered material may possess high levels of toxicants that due to filtering have been removed from the effluent. The TEW system has been designed to allow for heterogeneous effluents to flow through the trout test tanks with out clogging. The TEW Wheatstone Bridge Circuitry system has also been developed to respond only to the behaviour of the

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organism within the tank, therefore the signal displayed on the computer is based entirely on trout behaviour and not changing effluent heterogeneity.

The Development of a Trout-Toxicity-Library

When the trout were exposed to either KRAFT or NEWS effluents their ventilatory depth and frequency generally decreased by at least 25% for all effluent concentrations. This decrease may be due to a variety of physical and chemical parameters, not all of which may negatively impact the trout in a natural environment. Future experimentation and tissue sampling would be required to determine the actual effect of effluent on the trout.

Based on visual observations made during TEW testing (Figure 2), KRAFT effluents often appeared to act as a sedative, their whole body movement slowing and ventilatory rates slowing. In the natural environment this coma-like state may eventually lead to the death of the organisms either through starvation or predation from more virile organisms. Another potential theory for trout sedation may be that the epithelial layers in the trout's gills (parts of the gill structure responsible for the transfer of oxygen from water into the fish) are able to absorb a higher percentage of oxygen per breath from the effluent vs. that of the regular river water. This would consequently result in slower breathing patterns, but does not explain the reduced activity levels. A third theory may be that the trout are in the late stages of physical shock. Mammals that are in the shock initially increasing their ventilatory frequency but greatly decrease their ventilatory depth, they gasp for air (EPA 2005). Eventually, when the mammal is on the verge of death the breathing frequency dramatically declines, resulting in similar patterns

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displayed by the trout. In this situation the trout would be considered to be on the verge of death, and would only recover if it were removed entirely from the effluent.

A more accurate indication of trout health can be established by comparing TEW ventilatory observations to the results from the LC50 tests. Two KRAFT LC50 tests were performed, followed by one NEWS LC50 test.

The two KRAFT LC50 tests that were run showed that KRAFT toxicity ranged from 23% to 79%. Although the level of toxicity was not the same, they still proved that KRAFT effluent when untreated is toxic to rainbow trout. The TEW system did not show much mortality during the test run on KRAFT effluents but at all dilution levels there were statistically significant behavioral responses noted.

For the primary NEWS effluent exposure period, it was observed that trout ventilatory responses decreased for all NEWS concentration levels although statistically significant response were only noted at 100% and 50% effluent concentrations. The LC50 test for NEWS could not calculate a mortality level because 50% of the trout population did not die (LC50 can only be calculated at $\leq$ 50% mortality). There was mortality in the LC50 test, but not enough to calculate toxicity. When comparing these results with the TEW results, trout displayed very minor behavioural alterations when exposed to lower NEWS effluent dilutions but did display significant response to the higher effluent concentrations. Based on the LC50 and TEW tests run on both primary KRAFT and primary NEWS it can be concluded that KRAFT is toxic at all concentrations tested and NEWS is only moderately toxic at high concentrations. These comparisons are the initial steps of developing a TEW trout response-to-toxicity library.

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Future research will identify specific TEW trout responses to additional pulp and paper effluent matrices, such as black, white and green liquor. This will eventually form a response: addition library for pulp and paper effluents.

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Appendices

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APPENDIX I - Proposed TEW Alterations

The development of a new biomonitoring system requires much mechanical experimentation. The accuracy of the TEW system will increase over time. Many simple mechanical altercations could ensure that effluent is properly cooled and stored, that the length of a test is applicable to effluents other than pulp and paper and that the tank design itself ensures continuous, even effluent flow. The four main areas needing reassessment are; the Acclimation Chamber, the Testing Tanks, the Growth Chamber and the Chiller Room.

Chiller Room / Effluent Storage

The type of Chiller room required depends on the function of the proposed TEW laboratory. A mobile lab, the future goal of the TEW system, will require a much smaller Chiller room than a stationary lab. Ideally effluent will only be stored in the case of a toxic event because the TEW system will be tapped directly into the Industrial outflow pipes, receiving a constant flow of fresh effluent. If a Storage/Chiller room is required, then it should be located in close proximity to the TEW testing area (within the TEW laboratory). Tubing should be changed to stainless steel pipes to avoid effluent clogs and to ensure flow rate remains constant.

Acclimation Chamber

The Acclimation chamber consists of a large tank with continuous inflow and intermittent outflow, triggered by an aquatic pump switch. This chamber ensures the health of the species and allows the species to adapt to the conditions of its new environment. The TEW system utilizes one 550 L tank for trout acclimation; the tank size was excessive for the number of trout tested per effluent dilution. Cleaning was difficult

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due to the dimensions of the tank. It is recommended that two to three tanks, 20-50L tanks be used instead. This would allow for one or two sets of fish to be acclimated while another set can be used for testing. This system will ensure that fish are always accessible to the operator.

A continuous flow through system should be installed with flow meters and water flow control. Water outflow should be setup using an overflow standpipe system. This inflow-outflow system will allow for the continuous mixing which will help to maintain an equalized temperature throughout the water column.

Growth Chamber

The Growth Chamber was a Conviron product that regulates the temperature, humidity, and lighting conditions, the compressor and motor were removed from the bottom of the chamber and placed on the floor underneath the chamber to reduce the occurrence of vibrations. To increase maneuverability within the growth chamber operators could install a model that rest on the floor, allowing for overhead access, or a water bath cooling method could be employed as alternative.

Test Tanks

There were many unforeseen problems with the test tanks which accumulated during the duration of the TEW tests. The water flow in the TEW system is slow but must be continuous; alterations in the flow rate alter the conductivity of the water column and will therefore affect the output signal produced. Water flow was regulated using tube clamps. This made it difficult to ensure that flow rates within the tanks were equal. It is highly recommended that a flow meter be attached to the inflow tubes and that the flow is regulated using a speed adjustable pump. Masterflex tubing was used for both tank inflow

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and outflow. The tubing was suspended from the ceiling and traveled from the Chiller room, through the growth chamber and into the testing tanks. The extensive travel distance, the inconsistent tube lengths and the slow flow rate resulted in inconsistent timing for effluent inflow into the test tanks. Therefore, the effluent from the chiller room should be transported using stainless steel piping to the growth chamber, ending with a flowmeter and adjustable dials for flow rate. If tubing is to be used then it should be placed on level surfaces. The downward bowing of the tubes caused by gravity and the slow flow rates results in the buildup of sediments in the bowed section, resulting in clogged tubes.

The new proposed tank design is displayed in Figure 8. The proposed test tank should be placed in either a cool water bath or a growth chamber to ensure temperature requirements are met. A mixing and aeration chamber will ensure that dissolved oxygen levels are reached and that suspended particulate matter will not settle to the bottom of the tank. The inflow chamber will reduce effluent turbidity that would otherwise alter the volume and conductivity maintained within the testing chamber. The weir system currently employed by the TEW tank will remain the same, but the pieces will be removable to allow for proper cleaning. The effluent will flow out into a collection trough that will drain using a single pipe into an effluent collection container or into the waste disposal system. The current standing pipe will be replaced by a waterspout located on the middle, upper side of the tank's far/end wall. This will reduce any effluent back flow into the testing chamber, due to the slow flow rate, or during the occurrence of tubing clogging.

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Proposed Testing Tank for Future TEW Design

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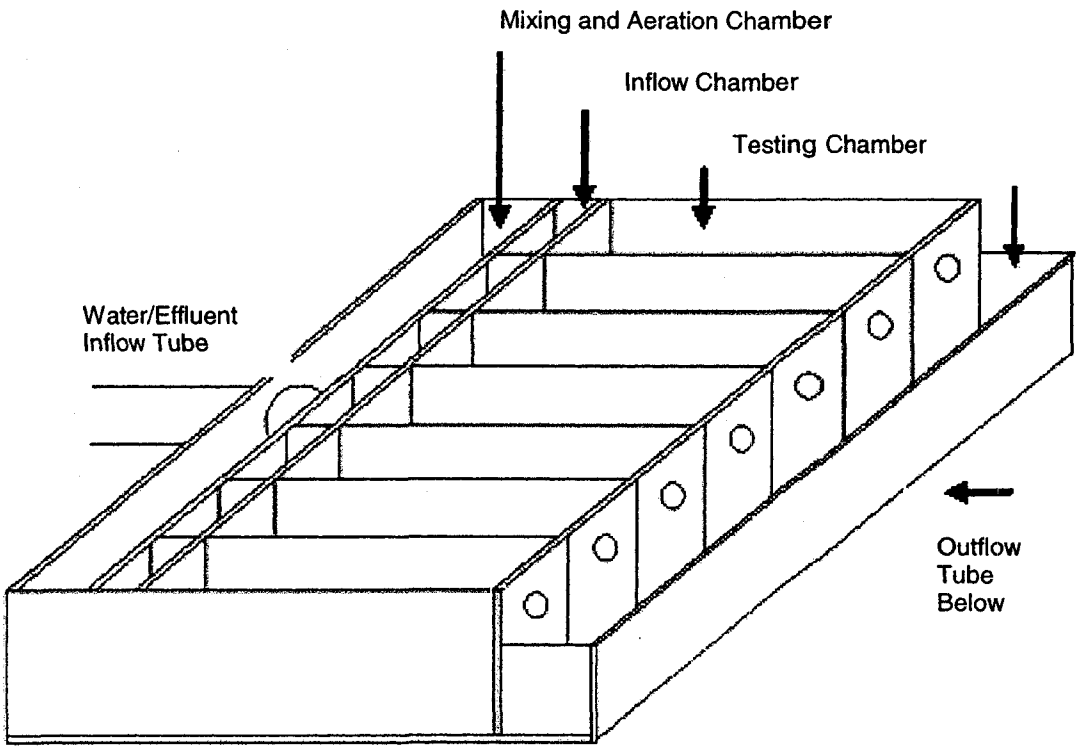

Proposed Testing Tanks for Future TEW Design.

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The testing tanks will be comprised of eight individual test tanks (or any other suitable denomination) placed on a fitted tray; each can be removed for easy cleaning. There will be at least two sets of 16 tanks available, allowing for the testing of up to a total of 16 fish (one fish for every two tanks). This ensures that a minimum of eight fish will be monitoring effluent, with a set of eight backup fish in the event of mechanical failure, a toxic event, or unintentional injury to a fish set. This will also allow for a set of fish to be acclimated to the testing conditions, ensuring that tests are in fact continuous, with no gap occurring during fish change over.

Further alterations to the TEW system would include the development of an independent, mobile system that can travel from one industrial site to another. Through future testing this can easily be accomplished, ensuring that the TEW system will evolve into an efficient, accurate and economical early warning toxicity identification system.

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APPENDIX II - KRAFT

KRAFT - 100%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
3.28	2.7489	3.68	0.44	0.0156	1.66
2.97	0.0994	1.65	0.66	0.0156	1.66
2.29	2.0747	3.24	0.95	0.0125	1.76
2.64	1.0909	1.60	1.12	0.0156	1.76
2.66	0.7112	2.57	0.25	0.0156	1.66
3.37	2.5925	2.16	1.02	0.0156	1.66
3.56	1.1324	2.86	0.79	0.0312	1.76
1.37	0.3875	3.12	1.03	0.0469	1.76
2.27	1.6872	2.67	0.92	0.0313	1.88
1.49	1.1186	1.87	0.89	0.0313	1.66
3.18	1.6237	3.78	1.04	0.0156	1.76
2.13	1.4327	3.36	0.88	0.0156	1.88
3.52	1.0960	3.33	1.26	0.0156	2.00
3.14	1.3088	3.26	1.15	0.0156	1.76
1.98	1.1655	2.82	0.45	0.0313	1.76
2.17	0.0958	2.85	1.19	0.0313	1.76
3.74	0.3930	1.84	1.23	0.0313	1.76
1.39	1.2267	2.95	1.00	0.0313	1.76
1.66	1.8112	3.19	0.70	0.0313	1.76
1.82	1.4292	2.09	0.67	0.0313	1.76
***2.53**	***1.2613**	***2.74**	***0.87**	***0.0240**	***1.76**

*Averages

KRAFT - 50%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
1.43	1.1851	1.62	1.33	0.0156	1.76
1.73	1.0628	3.62	0.63	0.0156	1.88
3.60	1.0199	3.49	1.63	0.0156	2.14
3.07	1.1303	2.60	1.31	0.0156	2.14
2.10	1.8381	2.19	0.72	0.0469	2.14
3.27	2.0106	2.55	2.27	0.0469	1.76
2.77	1.8431	3.03	1.02	0.0625	1.76
3.34	1.6124	2.56	1.67	0.0625	1.66
2.36	0.1039	1.98	1.95	0.0625	1.57
3.28	0.3400	1.77	1.52	0.0938	1.66
3.59	1.1015	2.69	1.07	0.0313	2.50
2.44	2.4628	2.95	0.72	0.0469	2.50
1.21	2.1177	2.73	2.18	0.0156	2.50
3.57	0.5130	1.82	1.36	0.0156	2.14
1.15	2.8349	2.06	0.78	0.0156	2.14
2.10	0.9261	2.59	0.56	0.0156	2.00
1.84	0.2574	1.68	1.87	0.0156	2.14
3.64	0.3088	1.72	1.74	0.0156	2.14
2.20	0.8321	3.21	1.66	0.0156	2.14
2.14	1.3980	2.51	0.66	0.0605	1.08
***2.54**	***1.2449**	***2.47**	***1.33**	***0.0343**	***1.99**

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APPENDIX II - KRAFT

KRAFT - 25%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
3.58	1.5127	3.29	0.73	0.0156	4.28
3.80	1.3941	3.10	0.73	0.0156	4.28
1.12	2.8152	2.67	1.00	0.0156	5.00
2.95	1.3284	1.77	0.36	0.0156	3.75
3.66	1.9789	2.82	0.78	0.0156	6.00
1.31	1.5159	1.99	0.78	0.0156	4.28
3.47	1.0456	3.29	1.00	0.0156	6.00
1.24	2.4815	2.03	1.00	0.0156	6.00
2.18	1.2566	2.40	0.47	0.0156	4.28
3.74	0.2623	1.62	0.68	0.0156	5.00
1.61	1.6478	3.89	0.78	0.0156	3.75
1.68	2.7785	3.43	0.57	0.0156	6.00
1.76	0.9217	3.26	0.78	0.0156	4.28
2.54	2.3816	1.84	1.00	0.0156	5.00
3.40	1.8143	3.13	1.00	0.0156	6.00
3.53	2.2608	2.80	1.00	0.0156	3.75
1.92	0.9084	2.00	1.00	0.0156	3.75
2.86	2.8700	2.60	1.00	0.0156	4.28
2.32	1.6499	3.02	1.00	0.0156	6.00
3.20	0.1112	2.54	3.78	0.0474	1.75
***2.59**	***1.6468**	***2.67**	***0.97**	***0.0172**	***4.67**

*Averages

KRAFT - 12.5%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
1.31	0.1891	1.64	2.14	0.03125	1.88
2.06	1.5264	2.28	2.14	0.03125	1.76
3.84	2.8560	2.65	3.35	0.03125	1.76
2.67	2.1696	2.15	2.14	0.03125	1.66
1.62	1.6334	3.39	2.14	0.03125	1.88
1.95	2.2820	2.91	1.00	0.03125	1.88
1.43	0.4205	2.39	1.56	0.0156	1.76
2.63	2.1505	3.69	2.14	0.0156	1.88
3.15	1.6130	3.84	2.14	0.0156	1.88
2.41	2.6008	2.02	2.14	0.0156	1.88
2.10	2.5076	2.86	2.14	0.03125	2.00
2.53	2.0713	1.66	2.14	0.03125	2.00
2.09	0.2797	2.84	1.00	0.03125	1.88
3.81	2.4462	3.74	2.14	0.0156	1.76
1.90	2.3882	2.55	1.00	0.0156	1.88
1.13	1.8618	2.52	1.00	0.0156	1.88
1.36	2.1701	3.27	3.35	0.0156	2.00
2.10	2.8009	2.54	2.14	0.0156	1.88
3.47	0.2940	3.31	1.00	0.0156	2.00
1.97	2.6768	3.19	3.78	0.0474	1.64
***2.28**	***1.8469**	***2.77**	***2.03**	***0.0242**	***1.85**

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APPENDIX II - KRAFT

KRAFT - 6.3%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
2.68	0.5915	1.99	2.14	0.03125	1.88
1.65	2.1986	3.40	2.14	0.03125	1.88
1.60	2.8122	3.16	3.35	0.03125	1.88
2.34	1.2403	3.53	2.14	0.03125	1.88
3.34	1.9478	3.20	3.35	0.03125	2.00
1.89	0.3142	3.29	2.14	0.03125	2.00
3.15	1.1990	1.96	5.87	0.03125	1.88
3.24	2.8582	3.14	5.87	0.03125	2.00
3.83	1.6616	3.28	2.14	0.03125	2.00
2.85	0.3741	3.22	7.18	0.03125	1.88
2.80	1.0726	1.76	2.14	0.03125	1.88
1.50	0.1802	2.45	3.35	0.03125	2.14
2.89	0.1333	1.92	3.35	0.03125	1.76
3.39	2.6708	3.30	3.35	0.03125	1.88
2.45	2.2716	2.08	4.59	0.03125	2.00
3.54	0.3497	2.47	2.14	0.03125	2.00
1.44	1.7562	3.82	3.35	0.03125	1.88
3.57	0.8442	2.44	5.87	0.03125	1.88
3.90	1.0854	2.11	4.59	0.03125	1.88
1.75	1.7259	1.69	3.35	0.0313	1.88
***2.69**	***1.3644**	***2.71**	***3.62**	***0.0313**	***1.92**

*Averages

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APPENDIX III - NEWS

NEWS - 100% Body Movement and Ventilatory Rate and Frequency						NEWS - 50% Body Movement and Ventilatory Rate and Frequency					
Acclimation Period			Effluent Exposure Period			Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second	Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
1.82	0.1846	1.74	3.35	0.0474	1.12	1.33	0.2259	3.44	2.14	0.0605	1.22
2.20	0.1117	2.42	4.59	0.0345	1.33	2.62	0.1525	2.60	2.08	0.0474	1.33
3.42	0.1135	1.73	2.74	0.0221	1.03	3.78	0.1257	2.89	10.53	0.0740	1.08
2.95	0.1170	2.32	8.50	0.0103	4.59	2.67	0.0778	1.61	4.59	0.0605	1.28
1.38	0.1634	2.16	7.18	0.0173	1.28	1.43	0.0909	1.64	6.52	0.0081	1.75
3.51	0.2520	3.04	8.50	0.0221	1.40	3.82	0.0952	2.69	10.53	0.0081	1.22
2.49	0.2217	2.19	7.18	0.0345	1.56	2.91	0.2066	3.36	3.35	0.1128	1.28
2.24	0.1162	2.80	5.87	0.0126	1.64	3.69	0.1897	2.52	2.14	0.0474	1.75
1.79	0.0966	2.13	8.50	0.3400	0.79	2.40	0.2406	3.44	4.59	0.0740	1.56
3.19	0.1367	1.61	2.74	0.0103	3.35	1.53	0.1878	3.25	5.87	0.0605	1.33
2.28	0.0734	2.59	2.14	0.0103	4.59	2.09	0.1457	3.27	3.35	0.0474	1.03
2.31	0.2383	2.45	2.08	0.0081	7.18	3.25	0.0785	1.62	3.35	0.0605	1.03
1.93	0.2254	2.30	6.52	0.0474	1.48	3.57	0.1845	2.95	5.87	0.0877	2.50
1.16	0.0892	2.80	2.74	0.3400	1.75	3.48	0.1700	3.40	2.74	0.0877	1.86
2.65	0.1173	1.89	9.85	0.0474	1.22	2.03	0.1581	2.46	5.87	0.0877	1.75
3.35	0.0808	2.78	15.39	0.0345	1.33	1.51	0.1628	1.88	3.35	0.0740	1.33
1.68	0.1325	2.58	3.35	0.0173	1.64	2.59	0.1484	2.66	3.35	0.0474	1.08
3.17	0.1015	1.64	5.87	0.0103	3.35	2.63	0.1670	3.16	6.26	0.0474	1.75
1.43	0.0902	2.28	4.59	0.0221	1.28	3.58	0.0783	3.41	4.91	0.0605	1.86
3.50	0.1102	3.24	3.35	0.3400	1.17	2.13	0.1951	1.99	3.35	0.0605	1.08
***2.42**	***0.1386**	***2.33**	***5.75**	***0.0714**	***2.16**	***2.65**	***0.1541**	***2.71**	***4.74**	***0.0607**	***1.45**

*Averages

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APPENDIX III - NEWS

NEWS - 25%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
1.93	0.0864	1.77	4.91	0.0474	1.64
3.25	0.1921	1.98	1.00	0.0474	1.64
1.64	0.1315	2.99	2.14	0.0474	1.64
2.05	0.1181	2.86	5.87	0.0474	1.64
2.14	0.1809	2.65	4.59	0.0474	1.64
2.02	0.2469	2.73	3.35	0.0474	1.64
2.88	0.1430	1.74	5.87	0.0474	1.64
2.53	0.1095	2.21	2.14	0.0474	1.64
3.16	0.2450	2.53	2.14	0.0474	1.64
1.87	0.1082	3.28	1.56	0.0474	1.64
1.39	0.1204	3.28	2.14	0.0474	1.75
2.17	0.0905	2.20	7.18	0.0474	1.56
1.95	0.1755	3.44	3.35	0.0474	1.64
3.34	0.1517	2.05	2.44	0.0474	1.64
3.42	0.0894	3.11	3.35	0.0474	1.75
3.51	0.0794	2.25	2.14	0.0605	1.64
3.17	0.1813	3.45	3.35	0.0605	1.75
1.85	0.1801	1.81	3.35	0.0474	1.75
3.56	0.1053	3.16	2.14	0.0605	1.75
1.93	0.1636	1.63	3.35	0.0474	1.75
***2.49**	***0.1449**	***2.56**	***3.32**	***0.0493**	***1.67**

*Averages

NEWS - 12.5%

Body Movement and Ventilatory Rate and Frequency

Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
2.42	0.1649	1.91	5.87	0.0474	1.28
3.57	0.1426	2.85	5.87	0.0474	1.28
3.29	0.1844	2.63	4.59	0.0474	1.56
3.45	0.2323	2.90	2.14	0.0474	1.64
3.78	0.0653	2.33	2.14	0.0345	1.75
3.70	0.2267	2.37	3.35	0.0345	1.56
1.66	0.0822	3.35	2.74	0.0605	1.28
3.46	0.2491	2.91	3.35	0.0474	1.75
3.18	0.1468	2.93	2.14	0.0474	2.00
3.58	0.1348	2.65	3.35	0.0345	2.14
2.57	0.0698	2.26	2.44	0.0345	1.75
3.12	0.2046	2.93	2.74	0.0345	1.86
3.42	0.1139	2.35	3.35	0.0345	2.00
1.76	0.0842	2.78	2.44	0.0345	2.74
2.86	0.2291	1.68	2.14	0.0474	1.75
3.98	0.2237	2.05	2.44	0.0474	1.56
2.01	0.0601	2.39	2.14	0.0474	1.64
1.87	0.2262	3.31	4.59	0.0605	1.64
3.30	0.0762	2.06	2.14	0.0474	1.64
2.06	0.1118	3.42	3.35	0.0474	1.64
***2.95**	***0.1514**	***2.60**	***3.17**	***0.0442**	***1.72**

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APPENDIX III - NEWS

NEWS - 6.3%					
Body Movement and Ventilatory Rate and Frequency					
Acclimation Period			Effluent Exposure Period		
Body Movement (Volts)	Breathing Depth (Volts)	Breaths per second	Body Movement	Breathing Depth (Volts)	Breaths per second
2.75	0.0925	2.43	2.14	0.0740	1.40
2.61	0.1741	2.63	1.85	0.0474	1.22
1.15	0.0613	2.07	3.35	0.0474	1.40
3.26	0.1260	2.07	7.18	0.0221	1.64
3.65	0.2013	3.27	2.14	0.0474	1.56
3.83	0.0737	3.06	2.14	0.0740	1.28
3.10	0.1289	3.25	2.14	0.0474	1.75
1.32	0.2134	1.82	5.87	0.0474	1.64
2.86	0.2130	3.02	1.85	0.0474	1.33
1.36	0.2399	3.37	2.25	0.0474	1.17
3.78	0.2216	3.08	2.14	0.0605	1.28
1.60	0.0957	3.12	3.35	0.0221	1.56
2.52	0.1205	1.67	4.59	0.0103	1.56
1.91	0.1765	2.05	2.14	0.0474	1.64
3.41	0.0619	2.88	2.14	0.0474	1.47
3.81	0.2316	2.16	3.35	0.0474	1.40
1.24	0.1851	2.90	1.85	0.0605	1.75
3.70	0.1299	3.24	3.35	0.0474	1.86
3.23	0.1082	2.25	4.59	0.0474	1.28
1.86	0.1529	2.22	3.35	0.0474	1.28
***2.65**	***0.1504**	***2.63**	***3.09**	***0.0470**	***1.47**

*Averages

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APPENDIX IV – EFFLUENT BIOASSAY

EFFLUENT BIOASSAY: RAINBOW TROUT: EPS 1/RM/13, 1990/1996.

DATE SAMPLED: 07-20-05 TIME: 14:00 SAMPLE METHOD: GRAB
COMPANY: BOWATER (INGRAM) LOCATION: BOWATER T-BAY
SAMPLE CODE: SAMPLER: INGRAM SOURCE: PRIMARY KRAFT
ARRIVED: 07-20-05 14:00PM TESTED: 07-22-05 14:00 L-RASTE: OVER

INITIAL CHEMIOCAL PARAMETERS OF TEST SOLUTIONS

SOLUTION	pH	DO	TEMP	COND	pH	DO	TEMP
CONTROL	7.64	7.8	16.2	0.12	8.15	9.4	15.4
-1	7.43	7.2	16.5	0.24	8.19	9.8	15.3
-2	7.34	8.1	16.3	0.36	8.17	9.1	15.4
-3	7.36	9.0	15.6	0.65	8.13	10.0	15.4
-4	7.32	8.7	15.8	1.08	8.19	10.0	15.5
-5	7.58	7.5	16.7	2.70	8.30	9.6	15.2

MORTALITY								
CONTROL	---	---	---	---	---	---	---	MG/L
-1	---	---	---	---	---	---	---	BATCH #: 06230502
-2	---	---	---	---	---	1	1	%PRE-TEST MORTALITY: 1.09
-3	---	---	---	---	5	5	5	
-4	---	---	---	---	9	9	10	ANALYST: Mike,Mary
-5	---	---	---	---	9	10	10	COMMENTS: GOOD Aeration off pat of July 3, 05

LOADING RATE

	LENGTH (mm)	WEIGHT (g)
1	34.02	0.433
2	34.23	0.491
3	39.25	0.692
4	31.09	0.367
5	35.58	0.684
6	35.69	0.631
7	37.48	0.455
8	35.55	0.348
9	31.05	0.449
10	36.17	0.535
x	35.02	0.508
(std)+/-	2.56	0.124

0.2g/L (fish density)

Concentrations: 100, 50, 25, 12.5, 6.25%
Program: Effluent LC50 version 1.0 Spearman/stephen
Test: Spearman – Kamben test
LC50: 23.33% (Fairly toxic)

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APPENDIX IV – EFFLUENT BIOASSAY

EFFLUENT BIOASSAY: RAINBOW TROUT: EPS 1/RM/13, 1990/1996.

DATE SAMPLED: 06-21-05 TIME: 10:00 SAMPLE METHOD: GRAB
COMPANY: BOWATER (INGRAM) LOCATION: BOWATER T-BAY
SAMPLE CODE: SAMPLER: INGRAM SOURCE: PRIMARY KRAFT
ARRIVED: 06-29-05 11:00PM TESTED: 06-30-05 13:00 L-RASTE: OVER

INITIAL CHEMIOCAL PARAMETERS OF TEST SOLUTIONS

SOLUTION	pH	DO	TEMP	COND	pH	DO	TEMP
CONTROL	7.62	9.3	16.1	0.12	7.62	9.5	14.5
-1	7.44	7.6	16.8	0.24	7.72	9.8	14.3
-2	7.44	9.2	16.0	0.36	7.76	9.7	13.7
-3	7.33	8.9	16.2	0.65	7.82	9.4	13.6
-4	7.24	8.7	16.5	1.08	7.89	9.4	13.8
-5	7.07	7.0	17.0	2.70	7.89	9.0	13.6

MORTALITY								
CONTROL	---	---	---	---	---	---	---	---MG/L
-1	---	---	---	---	---	---	---	---BATCH #: 06020504
-2	---	---	---	---	---	---	---	---%PRE-TEST MORTALITY: 0.00
-3	---	---	---	---	---	---	---	
-4	---	---	---	---	---	---	---	---ANALYST: Mike,Mary
-5	---	---	---	---	3	4	9	---COMMENTS: GOOD Aeration off pat of July 3, 05

LOADING RATE

	LENGTH (mm)	WEIGHT (g)
1	34.96	0.509
2	32.95	0.395
3	36.47	0.561
4	42.34	0.908
5	30.89	0.378
6	40.36	0.737
7	31.00	0.340
8	35.06	0.458
9	34.60	0.557
10	33.30	0.463
x	34.19	0.531
(std)+/-	3.72	0.175

0.2g/L (fish density)

Concentrations: 100, 50, 25, 12.5, 6.25%
Program: Effluent LC50 version 1.0 Spearman/stephen
Test: Spearman – Kamben test
LC50: 73.49% (Not extremely toxic)

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APPENDIX IV– EFFLUENT BIOASSAY

EFFLUENT BIOASSAY: RAINBOW TROUT: EPS 1/RM/13, 1990/1996.

DATE SAMPLED: 06-21-05 TIME: 10:00 SAMPLE METHOD: GRAB
COMPANY: BOWATER (INGRAM) LOCATION: BOWATER T-BAY
SAMPLE CODE: SAMPLER: INGRAM SOURCE: NEWS SHOWER WATER
ARRIVED: 06-21-05 2:00PM TESTED: 06-23-05 10:00 L-RASTE: OVER

INITIAL CHEMIOCAL PARAMETERS OF TEST SOLUTIONS

SOLUTION	pH	DO	TEMP	COND	pH	DO	TEMP
CONTROL	7.34	9.5	15.6	114	7.72	19.3	13.2
-1	7.37	9.6	15.4	115	7.69	10.2	13.2
-2	7.37	9.6	14.7	115	7.6	9.9	14.1
-3	7.34	9.5	14.7	116	7.62	10.2	13.6
-4	7.33	9.4	14.8	119	7.51	10.7	14.0
-5	7.29	9.0	16.2	124	7.42	9.9	14.1

MORTALITY								
CONTROL	---	---	---	---	---	---	---	---MG/L
-1	---	---	---	---	---	---	---	---BATCH #: 06020504
-2	---	---	---	---	---	---	---	---%PRE-TEST MORTALITY: 0.07
-3	---	---	---	---	---	---	---	
-4	---	---	---	---	---	---	---	---ANALYST: Mike,Mary
-5	---	---	---	---	2	2	2	---COMMENTS: GOOD

LOADING RATE

	LENGTH (mm)	WEIGHT (g)
1	29.19	0.294
2	33.85	0.413
3	35.04	0.454
4	35.11	0.471
5	27.48	0.272
6	34.74	0.517
7	32.42	0.393
8	30.37	0.302
9	30.67	0.361
10	33.40	0.396
x	32.23	0.387
+/-	2.67	0.081S

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Appendix IV

Lakehead University Toxicity Early Warning System (TEW)

Rainbow Trout Standard Operating Procedure SOP#TEW001

Prepared by: Mary Kate Ingram

Revision No.: 3
Revision Date: July 6, 2004
Original Date: May 1, 2004
File Name: SOP#TEW001

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INTRODUCTION AND SCOPE

Single Concentration of Effluent

The end point is reported at 12 hours. A 6-hr control period using river water will be used for each test run, followed by a 6-hr effluent exposure period.

Principle of the method

Test organisms, namely rainbow trout are exposed to a single concentration of effluent, a set of tests exposing the organism to a geometric dilution series, in which each successive concentration is fifty per cent of the previous one, each test running for 12 hours. Observed behavioral responses and potentially mortality during the test and at the end of the test period are used to estimate the relative toxicity of the effluent.

Safety

Wear gloves and safety goggles when handling effluent. Avoid skin contact and use in a well-ventilated area. Wash thoroughly after handling. Consult Material Safety Data Sheet (MSDS) for information on chemical prior to use.

TEST ORGANISM REQUIREMENTS

The organism tested is the *Oncorhynchus mykiss* commonly known as the rainbow trout. Fingerling life stage will be used for toxicity testing. The mean weight of test fish must be between .3 and 5.0 grams. See Rainbow Trout History/Acclimation Standard Procedure SOP#AT002. Fish are acclimatized at 15+/- 2^{0C} for two weeks immediately prior to use in testing. The batch of organisms to be used for testing must have passed the Reference Trout Test, SOP#AT003. The result of the test must be within the accepted Control Limit, see Method Validation Section of SOP#AT001, for the batch to be accepted for testing.

QUALITY ASSURANE/QUALITY CONTROL

Test conditions contained in this document comply with EPS 1/RM/9 and 1/RM/13 requirements and checklists. Test conditions are quality control checks which must be within acceptable range to ensure a reference toxicant test is preformed monthly to ensure acclimation organisms in tests demonstrate good survival, health and growth. Calculation for series dilution or preparing reagent must be checked by another technician.

METHOD OF VALIDATION

All testing techniques and procedures have been developed from the Environmental Protection Series, Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon). Report EPS 1/RM/28, Dec. 1992.

SAMPLING REQUIRMENTS AND HANDLING

Sample Required

One thousand liters of sample is required for 100% concentration testing, for successive concentration series the geometric dilution series will be used.

Sample Collection

Samples are collected by grab method for regulatory testing. Samples will be collected at the kraft, news effluent outfalls as well as the kraft clean water outfall. A truck along with a one thousand-liter tote will be supplied from Bowater in order to obtain the samples at each sample station. Once collected the sample will be pumped from ground level up in to the chiller room located within Bowater. Testing should commence as soon as possible after sample arrival, but

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within 3 days and no later than 5 days from sampling date. If the sample is tested at >5 days after sampling, bench sheet and report must indicate that sample is stale-sated (i.e. exceeds the allowable storage requirements under EPS 1/RM/13, 2nd edition, December 2000. The sampling stations, sample date, time, source and samplers name will be recorded for each sample used. For the purpose of Bowater testing sample will occur twice a week on every Monday and Thursday, or every Tuesday and Friday, depending on availability of the Lakehead University Truck.

Storage

After logging in the samples are placed in the chiller room and maintained at 4^{0C} until testing. Testing should commence within 24 hours and must be started no later that 5 days after date of sampling. Temperature in the chiller room may range from 1 to 8^{0C} but must not exceed these limits. While samples are stored in the chiller, monitor and record the temperature daily in the Chiller room temperature log posted on the wall next to the door in the chiller room. If the temperature of the chiller room in outside the range of 1^{0C} to 8^{0C}, the samples are run as usual, the results are flagged and the client is notified.

EQUIPMENT, REAGENTS AND SUPPLIES

Accument AP64 Series Handheld Dissolved Oxygen Meter

Manufacturer	- Fisher Scientific
Model No.	- AP64, Part No. 7713
Accessories	- DO Probe (12ft cable) Catalog No. 13-620-F12
	- DO Probe (25 ft. cable) Catalog No. 13-620-F25
	- Membrane Kit Catalog No. 13-637-DOM
	- Calibrate Quiver Catalog No. 13-637-QVR

Note: *Dissolved oxygen probe instruction can be viewed in Accument AP64 Series Handheld Dissolved Oxygen Meter Pamphlet*

Benchtop pH/ISE Meter

Manufacturer	- Orion
Model	- 410A
Serial No.	- 45632
Accessories	- Automatic Temperature Compensation Probe
	(Epoxy body), Catalog No. 917005
	(Glass body), Catalog No. 917006
	- Orion ROSS Sure-Flow pH Electrode, Catalog No. 8165BN
	(Epoxy Body)
	Ag/AgCl SURE-FLOW Electrode, Catalog No. 9165BN

Note: *Benchtop pH/ISE Meter Instruction Manual Temp. Probe and Ag/AgCl probe instruction can be found in the Orion Automatic Temperature Compensation probe instruction manual and the Orion Ag/AgCl Sure-Flow Electrode instruction manual (In pamphlet form, kept inside Orion Benchtop pH/ ISE meter instruction manual)*

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EZ OS-5020/5020C Analog Oscilloscope

Manufacture	- EZ Digital Co., LTD.
Model	- OS-5020
Accessories	- Oscilloscope Probe Kit, Model. HP-2040
Purchase Info.	- http://www.ezdgt.com

Note: *Analog Oscilloscope instructions can be viewed in the OS-5020 Analog Oscilloscope Operation Manual*

EZ FG-8002 Function Generator

Manufacture	- EZ Digital Co., LTD>
Model	- FG-8002
Purchase Info.	- http://www.ezdgt.com

Note: *Function Generator instructions can be viewed in the FG-8002 Function Generator Operation Manual*

Auto-Range Dual Display Digital Multimeter

Manufacture	- METEX,
Model	- M-3860D
Serial No.	- DJ168523

Note: *Multimeter instructions can be viewed in the Dual Display Digital Multimeter Owner's Manual*

Conductivity Meter

Manufacture	-Yellow Springs Instrument Co., Inc.
Model	- 35
Serial No.	- 96A45116

Note: *Use conductivity meter in conjunction with Multimeter, there is no standard instruction manual for this instrument as of yet.*

DATAQ hardware

Manufacture	-DATAQ Instruments
Model	-DI-720 Series Acquisition system
Purchase Info.	-http://www.dataq.com/products/hardware/di720.htm
Accessories	-BNC cables with banana plug adapters, 32 input Banana Board

Note: *DATAQ hardware instructions can be viewed in the DATAQ hardware instruction manual*

DATAQ software

Manufacture	-DATAQ Instruments
Model	-Windaq Pro data acquisition software, CODAS, Windaq/XL

Note: *DATAQ software instructions can be viewed in the DATAQ software instruction manual*

REAGENTS

PH Reference Standard Solution; pH=4.00, 7.00, and 10.01 are purchased from Corning, catalogue #478551, 478552, and 478553 respectively. These solutions are logged in the Reagent Prep logbook when received. The reception date is written on the bottle and the expiry date circled. These solutions must be kept on the chemical shelf in the laboratory and are not to be used past the expiry date.

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Conductivity NIST Reference Standard Solution; Conductivity standard solution is purchased from Cole Parmer, catalogue #473623. This solution is logged in the Reagent Prep logbook when received. The reception date is written on the bottle and the expiry date circled. This solution must be kept on the chemical shelf in the laboratory and is not to be used past the expiry date.

Dissolved Oxygen Electrolyte Solution; electrolyte solution is purchased from VWR Symphony, catalogue #14002-830. This solution is logged in the Reagent Prep logbook when received. . The reception date is written on the bottle and the expiry date circled. This solution must be kept on the chemical shelf in the laboratory and is not to be used past the expiry date.

TMS tricaine methanesulfonate; This anesthesia is purchased from Syndel Laboratories Ltd. Vancover, B.C. Canada, V6P 6R5. DIN 02168510, Product code 18323. The reception date is written on the bottle and the expiry date circled. This solution must be kept on the chemical shelf in the laboratory and is not to be used past the expiry date.

Control/Dilution Water

Kaministiquia River Water; Receiving water collected upstream from the source of contamination. LUCAS will test a sample of the collected river water in order to ensure that it can reliably support good survival, health and growth of the test species. Monitoring and assessment of variables such as pH, alkalinity, hardness, total organic carbon, conductivity, total dissolved gasses, chemical oxygen demand ammonia nitrogen, nitrite, and metals will be preformed before testing commences. The water will be sampled from the center of the river, 2 to 4 ft from the water surface in order to minimizes the particulate and vegetative matter collected. The control/dilution water will have an oxygen content of 90-100% air saturation before use.

SUPPLIES

Use only nontoxic materials such as stainless steel, porcelain, fiberglass-reinforced polyester, acrylic, polyethylene, polypropylene, or glass when measuring or handling test samples, dilution water, test organism.
Natural rubber, copper, brass, galvanized metal, and lead must not come into contact with tanks, test vessels, dilution water, test solutions and test organisms.

TEST PROCEDURE

All toxicity tests initiated must be reported and recorded

1.0 Equipment Calibration

1.1 Calibrate the following daily when measurements are required;

1.1.1. Conductivity meter; See Conductivity Meter SOP#TEW021 (PENDING)
1.1.2. Dissolved Oxygen Meter: See Dissolved Oxygen Meter SOPTEW022 (PENDING)
1.1.3. pH electrode: See pH Meter SOP#TEW023 (PENDING)

1.0 Bench Sheet Information

1.1 Fill in the information on the Industrial Effluent Bioassays: Trout Sheet.

DATE SAMPLED	=	date sample was collected
TIME	=	time of sample collection
SAMPLE METHOD	=	grab, batch or composite
COMPANY	=	name of company
LOCATION	=	city or town
SAMPLE CODE	=	assigned a 4-digit code to sample at time of Testing samples are coded consecutively starting with 0001 January 1 of each year.

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SAMPLER	=	name of person collecting sample
SOURCE	=	source of sample within operation
ARRIVED:DATE&TIME	=	date sample arrived in Chiller room
TESTED:DATE &TIME	=	date of sample testing
INITIAL TEMPERATURE	=	temperature (0C) of sample upon arrival
WEIGHT/LENGTH	=	average weight and mean fork length of fish determined at the end of the test
ACCLIMATION	=	the number of days the test rainbow trout were acclimated prior to testing
%PRE-TEST MORTALITY	=	% mortality of fish in stock tank for 7 days immediately preceding the test
BATCH NUMBER	=	batch number of fish used for test
ANALYST	=	initials of analyst who performs test
CHEMICAL PARAMETER MEASURMENTS	=	temperature, dissolved oxygen, pH, conductivity, mortality
COMMENTS	=	any observation/comments of sample

NOTE: Recall that time lapsed between date sampled and date tested must be equal to or less than five days, and preferably within 3 days

2.0 Sample Preparation
2.1 Pump sample from storage tote on truck into totes in chiller room
2.2 Turn on stainless steel agitators to ensure proper mixing of effluent
2.3 Pump effluent into head tanks in TEW lab
2.4 Check temperature of effluent in chiller room once daily
2.5 Check temperature of effluent during test twice daily
2.6 Check DO, conductivity, pH twice daily and record in appropriate Log sheet

TEST SET-UP

1.0 Calibration
1.1 Place clean test tanks into growth chamber
1.2 Place inflow tubing into inflow chamber
1.3 Attach outflow tubing to outflow standing pipe
1.4 Place electrodes in testing chamber of test tanks
1.5 Calibrate Function Generator (FG) and Oscilloscope
- Turn on Power board, oscilloscope, and function generator
- Plug FG into oscilloscope, set oscilloscope VOLTS/DIV dials to 1 volt.
- Set FG amplitude (DC current in fish tank) to 1.8 by turning the amplitude dial
- Place FG voltage dial to 1.2
- Re-set oscilloscope VOLTS/DIV dials to 0.1 volts
- Place oscilloscope channels to GND and zero DC current by turning the POSTION dial

1.6 Fill 25L bucket with water from the Acclimation Chamber
1.7 Using a fish net and the White Acclimation Tank Divider obtain four test fish and place into 25L bucket

1.8 Capture one fish from bucket and place into containment sleeve, tightly secure using yellow fishing wire while the fish and the sleeve are in the water, place contained fish directly into the test chamber of the test tanks
1.9 Repeat step 1.8 for the remaining three fish

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1.10 Once trout is in the test chamber zero the DC current on the oscilloscope by sliding the electrode holders back and forth over the top of the tank. When zero (or closest possible proximity) is reached the signal displayed on the oscilloscope will be positioned nearest to the bottom of the screen.

Note: $V_P/(2)^{1/2} = V_P/(1.414)^2$, $0.7V_P = 0.7V$ if $V_P=1V$
$V_{RMS} = V_{DC}$ for $V_{RMS} = V_{DC} = 1$

$V_{PP} = 1 * (2)^{1/2} * 2$
$= 1.414 * 2$
$= 2.8V$

V_{PP} = Distance from the top of a sin wave to the bottom (-1 to +1)
V_P = Distance from the top of a sin wave to the center line of that wave (0 to +1 or –1)

1.11 Once FG and Oscilloscope are calibrated turn on computer and calibrate Dataq software
- Open WinDaq Pro Data Acq (DI-7x0 USBO
- Plug banana plugs (attached to electrodes within the fish tank) to the Banana board. There is a notch located on one side of the banana plug and not on the other side. The notched side is placed in the black outlet and the smooth side is placed in the red outlet.
- Using the Dataq software click on EDIT, CHANNELS and then click a check mark on each channel in use, click OKAY.
- EDIT, SAMPLE RATE, set sample rate to 250, OKAY
- EDIT, CHANNEL SETTINGS, set channel settings to (Gain of 1, -FS Volt of –10.000, +FS Volt of 10.000), Acquisition Method is average, OKAY
- VIEW, FORMAT SCREEN, at least four sets of tanks will be in use, click on 4 waveforms to view four separate signals, OKAY.
- SCALING, LIMTS, set Limits to 9 (Top Limit), 0.000 (Bottom Limit) for each channel, the channel highlighted will be the channel that changes. To select a channel Click on the numbers on the left hand side of the screen i.e. 1=1 is channel 1, from banana board signal 1.

1.12 Create a folder on your hard drive that all of your data will be stored in, i.e. FISH TESTS

2 Calibrate Growth Chamber

The growth chamber must be programmed twice one for day light hours and the other for night time hours. Click HOLD, PROGRAM. Type in the beginning time of the daylight hours (7am) then ENTER, the temperature (15^{0}C) then ENTER, humidity (0) then ENTER, Light (3 0) then ENTER, AUX (0 0 0) then ENTER. Hit RUN, HOLD, PROGRAM to create the nighttime hours requirements. Type in the begging time of the nighttime hours (11pm) then hit ENTER, the temperature, humidity and AUX will remain the same as the daylight hours. Type in (0 0) for Lighting conditions. When finished click ENTER, RUN. The growth chamber will now follow the two programs exhibiting a 16/8 hour photoperiod.

2.1	Date / Time of Test	(7am-light, 11pm-dark)
2.2	Lighting	(30 – 3 referring to the bulbs, 0 referring to the fluorescence)
2.3	Temperature	(15^{0}C)
2.4	Temperature Alarm	(12^{0}C min. – 17^{0}C max.)

3 ICP Analysis for Determination of Sample Hardness Based on Calcium and Magnesium Concentrations

3.1 Take a 250 ml plastic polyvinyl chloride bottle and lid and label bottle with the sample code assigned the sample

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3.2 Filter sample with Fisherbrand P5 (porosity-medium, flow rate=slow) filter paper before taking sample to UC-0001 for ICP analysis.

3.3 Fill 15 ml cleaned and labeled plastic tube with at least 12 ml of filtered sample and close lid. Store in fridge until analysis. Sample may be stored for up to one week. Analysis must be done within one week. Bring sample tubes to UC0001 for ICP analysis of calcium and magnesium

4.0 River Water and Effluent Concentrations

4.1 At least one day prior to testing, preferable two, a schedule driver, truck and 1000L tote will aid in sample collection (arranged by Jackie Lurant (807)473-2867, and Alicjia Augustyns (807)475-2469). First collect Dilution water to fill Acclimation Chamber totes (5a,b) and Test totes 1,2,3,6. For river water test

4.2 For KRAFT, NEWS and KCWO tests river water will be collected for the acclimation chamber and enough to fill totes 1,2,3,6 half way up. 2000L of Effluent (either KRAFT, or NEWS or KCWO) will be placed in tote 4 and 7. Only sample one of the three stations for each series of test a series of tests include two 12hr test at 100%, two 12hr test at 50%, two 12hr test at 25%, two 12hr test at 12.5, and two 12hr tests at 6.3% effluent concentration.

4.3 Effluent will be pumped from ground level in the truck to the second floor of the BOWATER pulp mill, NEWS print section, located in the chiller room. Make sure that the correct valves are open of closed during pumping into chiller room.

5.0 Effluent Concentrations

5.1 Suggested concentrations by the EPS1/RM/28 manual include:

1. 100, 32, 10, 3.2, 1.0 (ICP or NOEC/LOEC Test)
2. 100, 46, 22, 10, 4.6, 2.2, 1.0 (ICP or NOEC/LOEC Test)
3. 100, 50, 25, 12.5, 6.3 (used when there is uncertainty over toxicity)

For initial testing the third concentration series will be used

5.2 Test will commence at the highest concentration level and work down to 6.3%

5.3 The test totes (1,2,3,6) will be filled up with the following ratio or dilution water to effluent depending at what concentration is desired, all four totes (1,2,3,6) will be filled with the same effluent concentration. Effluent and dilution water will be agitated with a stainless steel agitator to prevent settling of effluent.

Concentrations	= Effluent: Dilution Water
100%	= 1000 L : 000 L
50%	= 500 L : 500 L
25%	= 250 L : 750 L
12.5%	= 125 L : 875 L
6.3%	= 63 L : 937 L

5.4 A full test is complete when all 5 dilutions have been tested

5.5 Once the set-up is done, proceed with testing (TESTING-X: 1.0 to 1.9)

TESTING -BASELINE TROUT TEST

1.0 Initiating Baseline Trout Respiration Test

1.1. Measure and Record the following Chemical Parameters of Test Solutions:

1.1.1.1 Conductivity

1.1.1.2 Temperature of the test solution and Acclimation Water

1.1.1.3 Temperature must be 15+/-1^{0}C in the growth chamber and 12+/-1^{0}C in the Head tanks both before and during testing.

1.1.1.4 Dissolved Oxygen, if the dissolved oxygen is between 90-100% proceed with test.

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1.1.1.5 Observed sample and solution colour, turbidity, foaming and precipitation, etc., (as described in EPS 1/RM/9) during solution preparation and during tests.

1.1.1.6 Stirrers located underneath each Head tank are turned on until stirrer is circling freely.

1.2 Using a clean Beaker place dilution water from acclimation chamber into testing chamber

1.3 Obtain Chemical parameters of effluent in Head tanks and Acclimation water in testing chambers.

1.4 Placing Trout in Test Solution: One fish required for each set of tanks, therefore one fish for every two tanks.

1.4.1 Fish loading density is </= 0.5 g/L

1.4.2 A large fish net is used to capture fish from the holding tank, which are then placed into their individual mesh constraint and are immediately placed into the testing chamber (TEST SETUP 1.6-1.9). One fish for each set of tanks, four fish in total.

1.4.3 Trout in acclimation chambers are not to be fed 16 hours prior to testing.

1.4.4 Trout in test chamber are not to be fed during testing

Note: fish should be transferred quickly as to minimize stress. Any fish dropped or injured during transfer should be discarded.

1.5 Turn on Masterflexs pumps, flow rate set at 2, from totes containing only dilution river water.

1.6 Zero the DC current displayed on Oscilloscope as described in TEST SET-UP 1.7

1.7 Insure that the signal viewed on Oscilloscope is the same as that viewed on the Dataq monitoring program

1.8 Click FILE, RECORD on Dataq software. Locate and select previously created data storage folder, see TEST SET-UP 1.9 (i.e. Fish Tests), label file with effluent type, date, and concentration (i.e. KRAFT_041231_25). Set TEST RECORDING TIME to 12:00:00hrs, OKAY

1.9 Leave Fish undisturbed for length of test

1.10Monitor the temperature, pH and the DO of the head tanks twice daily

2.0 Test Conditions

2.1 Temperature within the Growth Chamber is kept at 15+/-1^{0C}.

2.2 Photoperiod must be 16+/-1 hr light 8+/- 1hr dark as required by EPS 1/RM/9&13.

2.3 Light intensity should be within the required range: 100 to 500 LUX at surface of water as required by EPS/1/RM/9&13.

3.0 Mortality

3.1 Test is invalid if fish dies during Baseline Trout Respiration Test, during acclimation to test chamber conditions, or in control dilution

3.2 Death is defined when the trout lacks evidence of opercular or other activity, and does not respond to gentle prodding.

4.0 Test End

4.1 On the Dataq software click FILE, STOP, CLOSE to stop and save recording

4.2 Remove trout from fish chambers and pour out of mesh constraint and immobilizes using a solution of tricaine methanesulfonate. Measure and record weight (g) and length (mm) on End of Test bench sheet, located in Trout Testing Binder.

4.2.1 Length of Trout

4.2.2 Measure fork length of fish using ruler found on shelf under Head tanks

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4.2.3 Place one end of ruler (location 0.00mm) at the tip of the trout's nose, line ruler parallel along trout body, take measurement at fork of trout tail (i.e. the end of the trout body not the end of the trout's tail). Record value onto bench sheet. Repeat steps 4.2.3 for all fish

4.2.4 Weight of Trout

4.2.5 Place a weighing boat on the balance pan and wait for the numbers to stabilizes. Press TARE. The display should read 0.000. If the display does not read 0.000, press TARE again.

4.2.6 Lightly blot trout with paper towel. Using tweezers, transfer one trout to the weigh boat and allow numbers to stabilizes. Record value on bench sheet.

4.2.7 Using tweezers, remove trout and discard. Repeat steps 4.2.5 to4.2.7 for other trout used in test.

4.3 After all data has been collected and recorded, dead fish are discarded in the garbage. Used test solutions are poured down the drain in the lab. Testing Tanks, and other equipment exposed to either trout or testing solution during testing are washed according to SOPTEW00C

CONDUCTIVITY TROUT TEST

1.0 Initiating Conductivity Trout Test

1.1 Follow procedures described in Initiating Baseline Trout Respiration Test; 1.0 to 1.7

1.2 Click FILE, RECORD on Dataq software. Locate and select previously created data storage folder, see TEST SET-UP 1.9 (i.e. Fish Tests), label file with effluent type, date, and concentration (i.e. CODUCT_041231_25). Set TEST RECORDING TIME to 1:00:00hrs, OKAY

TROUT EFFLUENT TOXICITY TEST

1.0 Initiating Toxicity Test

1.1 Follow procedures described in Initiating Baseline Trout Respiration Test; 1.0 to 1.10

1.2 After a 6hr marked time period add effluent to tote 1,2,3,6 creating desired concentration. i.e. 50% effluent and 50% river water. The 6hr river water exposure is preformed in order to give the trout time to acclimate to their new surroundings (recommended by the EPA). This is also done because the response of one trout to toxicity may be very different to that of another fish. The response before and after effluent exposure for each fish will be the determining factor for response to toxicity.

1.3 Since time is required to mix effluent concentrations they must be preformed in sequence and the time of mixing recorded for comparison later with acquired fish response data.

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