ASSESSING THE TOXICITY OF AQUATIC SEDIMENTS USING JAPANESE MEDAKA (*ORYZIAS LATIPES*) EMBRYOLARVAL BIOASSAYS

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ABSTRACT

Soil erosion from agricultural fields is a major environmental concern on Prince Edward Island (PEI). The Wilmot River watershed is one of the most intensively farmed regions of PEI with approximately 77% of its land designated to agriculture. The main objectives of this study were to test techniques that would provide more effective and reliable analysis of aquatic sediments. These techniques were used in two initial experiments; the first to assess different sediment storage methods and the second to assess the toxicity of three different carrier solvents.

The first experiment assessed different storage methods of field collected sediment. Japanese medaka (*Oryzias latipes*) embryos were exposed to Wilmot River sediments that had been refrigerated, frozen, and freeze-dried. Hatching success, time to hatch, length at hatch, and developmental abnormalities were used as endpoints. Medaka exposed to freeze-dried sediments had a drastic reduction in hatching with only 2 of 72 individuals surviving to hatch. There were no significant differences found between medaka exposed to refrigerated or frozen stored sediments with 75% and 69.4% of embryos hatching, respectively. These results indicate that short term freezing does not increase the toxicity of sediments and that freeze-drying is not an appropriate method of storage for sediments to be used in bioassays.

In the second experiment, Japanese medaka embryos were used to assess the toxicity of three different carrier solvents, ethanol, methanol and acetone. Heart rate, hatching success, time to hatch, length at hatch, and developmental abnormalities were used as endpoints. This was conducted in order to select the most appropriate carrier

solvent for sediment extracts to be used when comparing the toxicity of Wilmot River sediments from different sites or time points. Methanol proved to be the least toxic to medaka. With increasing concentrations, individuals exposed to ethanol and acetone exhibited delayed heart rate, reduced hatching success, and delayed time to hatch.

Finally the sediment toxicity of an upstream site (S1) and a downstream site (S2) in a river heavily impacted by agriculture (the Wilmot River) was compared. The rate of developmental abnormalities from the downstream site was also compared with results from a previous study done on at the same site on the Wilmot River in 2005. Medaka exposed to downstream sediments showed decreased hatching success, increased time to hatch, and decreased length at hatching when compared to upstream spring sediments. When compared to results from a similar study done in 2005 there were fewer developmental abnormalities. When exposed to sediment extracts from the 2005 study as well as the present study, medaka exhibited no signs of exposure to toxicants. Pesticide analysis showed trace amounts of several pesticides (e.g. linuron and imidacloprid), with concentrations varying between the years. Analysis of metals (copper, cadmium, and zinc) showed that levels of all three remained constant between the two time points. The techniques assessed here represent a substantial contribution to our knowledge of aquatic sediment analysis.

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CHAPTER 1:

OBJECTIVES AND LITERATURE REVIEW

OBJECTIVES

Intensive farming can place high levels of stress on stream ecosystems. Chemical agents from adjacent land can enter surrounding streams and result in acute and sublethal toxicity. Sediments within these streams are expected to accumulate sublethal concentrations of contaminants that can negatively impact fishes over long time periods. Sublethal concentrations of contaminants are associated with reduced growth, decreased mobility, changes in hatching times, reduced fecundity rates, and increased number of developmental abnormalities. Varying concentrations of a wide variety of pesticides have been detected in river sediments of Prince Edward Island (PEI) (Mutch *et al.* 2002).

Standardized bioassays are becoming increasingly important in testing the toxicity and bioavailability of chemical compounds in sediments. Acute toxicity generally occurs when organisms are subjected to a single large dose of a contaminant and often results in death, while sublethal effects occur after long term exposure to lower doses. While acute toxicity tests are useful to assess tolerances of different species to harmful chemicals, more sensitive bioassays should be performed in order to determine chronic effects as these may be important in influencing the dynamics of natural populations. Test organisms most commonly used in sublethal bioassays include crustaceans (amphipods, waterfleas, grass shrimp, mysids, copepods), mollusks (clams), bacteria (Microtox®), and to a much lesser extent fishes (Japanese medaka (*Oryzias latipes*), various salmonids, fathead minnows (*Pimephales promelas*), and zebrafish (*Danio rerio*)).

The first objective of the present study was to assess the effects of different storage methods on the toxicity of field collected sediments. Although it is generally advised that sediments be used as soon as possible after collection, it is often necessary to be able to store sediment for longer periods of time. During this study, Japanese medaka embryolarval bioassays were used to assess the toxicity of fresh (refrigerated), frozen, and freeze-dried sediments. Results from this experiment were used to determine the proper storage method for sediments to be used in subsequent experiments.

The second objective was to test the toxicity of different carrier solvents commonly used in sediment extracts, using the medaka bioassays. Organic solvents assist in the solubilization and dispersion of lipophilic pollutants into the test media prior to exposure of the test organism. Common solvents include ethanol (Hallare *et al.* 2006, Okumura *et al.* 2001), dimethyl sulphoxide (Hallare *et al.* 2006, Hallare *et al.* 2004, El Jay 1996), acetone (Hallare *et al.* 2006, Okumura *et al.* 2001), methanol (Hallare *et al.* 2006, Okumura *et al.* 2001), dimethylformamide (El Jay 1996), and triethylene glycol (Hutchinson *et al.* 2006). From this list three of the most common solvents, ethanol, methanol, and acetone were chosen for further study. Results from this experiment were used to select the proper solvent for a sediment extract carrier used in the third part of this study.

The third objective was to compare the toxicity of sediment and sediment extracts from the Wilmot River, an intensely farmed watershed, from two different sites on the river (upstream vs. downstream). The rate of developmental abnormalities from was also compared with those from a previous study at the downstream site (Jardine 2005).

GENERAL INTRODUCTION

Sediment is an integral part of the aquatic environment, providing habitat, food, and breeding sites for many organisms (Geffard *et al.* 2004a). However, sediments have a large capacity for storing contaminants and represent a major sink for pollutants in the aquatic environment (Viganò *et al.* 1995) that may be introduced through runoff or via groundwater (Greig *et al.* 2005). Thus, human activities that exacerbate soil erosion in catchments may lead to sediment habitat degradation in receiving watercourses (Evans *et al.* 2006). Contaminated sediment can be lethal or sublethal to sediment dwelling organisms (U.S. EPA 2001) and is likely to negatively impact fishes. Studies involving fishes are increasingly important as they represent higher trophic level organisms in stream ecosystems.

Sedimentation

Early studies in aquatic toxicology focused on the quality of water, that had traditionally been viewed as the medium for contaminants. It is currently recognized that an almost uncontaminated water column may lie above sediments which have become contaminated following long periods of chemical deposition (Baudo *et al.* 1999). Many toxic substances entering aquatic ecosystems accumulate in the sediment, that constitutes a large reservoir of potentially bioavailable contaminants. Therefore, protecting sediment quality is an important part of restoring and maintaining the biological integrity of surface waters as well as protecting aquatic life, wildlife, and human health.

Soil erosion and the subsequent introduction of silt or sediment into watercourses

represent the most important threats to stream water and aquatic habitat quality on Prince Edward Island (PEI) (PEI Department of Fisheries and Environment 2000). Soil erosion and associated sedimentation are natural processes caused by wind, water, and ice. However several anthropogenic activities such as deforestation, overgrazing, changes in land use, and non-sustainable farming practices accelerate these processes (Zapata *et al.* 2002). Although all land is subject to geomorphological processes, particular attention must be given to the erosion of sediment, especially of finer particles (Harrod and Theurer 2002). Silt can kill aquatic plants and animals and negatively impact organisms by blocking sunlight necessary for plant growth, suffocating fish eggs, killing aquatic invertebrates that serve as food for fish, covering gravel spawning areas, and smothering shellfish beds (Waters 1995). Greig *et al.* (2005) reviewed studies which showed that an increase in silt within salmon redds has a deleterious effect upon embryo survival. Successful incubation requires that the oxygen concentration within the redd is sufficient to support the oxygen gradient required to drive oxygen diffusion across the egg membrane at varying water temperatures and stages of embryonic development. Fine sediment intrusion into the incubation zone will decrease the passage of oxygenated water by blocking interstitial pore spaces and reducing the interstitial flow velocities.

On PEI, high siltation rates are commonly associated with agriculture but may also result from road construction, use of unpaved roads, improper watercourse alteration, and building construction. Poor agricultural practices such as planting to the water's edge, removing hedgerows, ploughing directly up and down slopes, and leaving fields bare over winter (Waters 1995) account for much of the soil erosion on PEI.

Agriculture on Prince Edward Island

The soils of PEI are shallow, acidic, low in organic matter, and highly erodible (Jacobs and Associates Ltd. 1997). This is largely because the soils are high in silt and fine sand, and low in clay. Most Island soils also have low subsoil permeability. They are considered moderate to well drained and seldom remain saturated for long periods of time during the summer (Jacobs and Associates Ltd. 1997).

According to the Prince Edward Island Department of Agriculture, Fisheries, and Aquaculture (2005) the total land area of Prince Edward Island is 566,560 hectares with approximately 261,427 hectares cleared for farm use; potatoes represent PEI's single largest agricultural commodity (Fig. 1-1). PEI commonly experiences heavy rainfall events during the crop season and repeated freeze-thaw cycles during winter, both of which increase risk of soil erosion. Soils are particularly at risk to water erosion during the crop growing season from high intensity summer storms, especially on fields where potatoes have been planted up and down a slope with little residue or crop canopy cover (Jacobs and Associates Ltd 1997). PEI also has moderately windy conditions, that increase the risk of wind erosion, especially on fields that are left bare over the winter or those which have little shelter from hedgerows or woodlands (DeHaan 2002). Annual soil erosion rates of 25 tonnes/ha are common on agricultural land in row crop production on PEI (Smith *et al.* 2002). In an attempt to limit the amount of sediment entering rivers and streams provincial legislation was introduced in 2001 that prohibits planting an

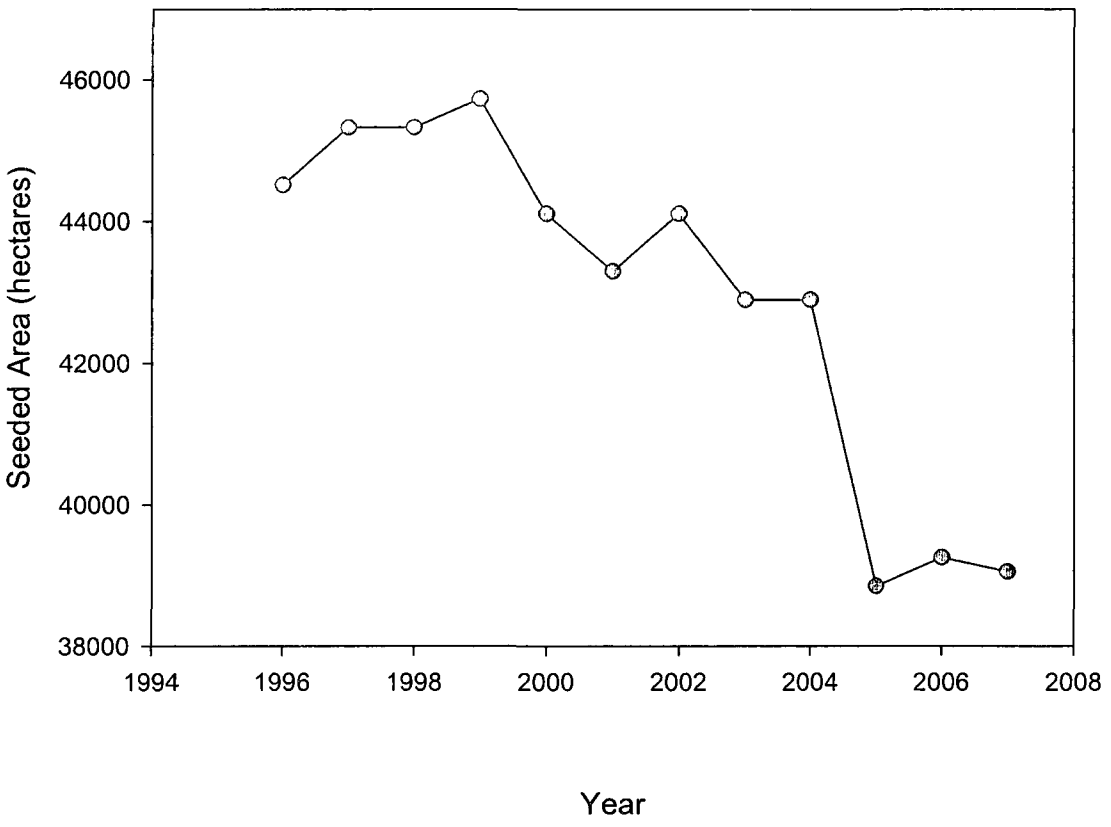


Figure 1-1. Hectares of potatoes planted in Prince Edward Island, Canada from 1996-2007 (Agriculture, Fisheries, and Aquaculture 2005). This number accounts for approximately one third of the total land used for potatoes due to mandatory three year crop rotation.

agricultural crop within 10 m - 90 m of a watercourse or designated wetland depending on the slope of the field and other factors such as type of agricultural activity.

The majority of pesticide use on PEI results from potato production as current growing methods are heavily dependent on pesticides, with up to 15 pesticide applications per growing season (Mutch *et al.* 2002). The main pesticides used on PEI can be found in Table 1-1. According to Rachael Cheverie (Integrated Pest Management Specialist, PEI Department of Agriculture, Fisheries, and Aquaculture, pers. comm. July 14, 2006) pesticide use begins in late May with the application of pre-emergent herbicides such as linuron and metribuzin to kill unwanted growth during the seed stage before germination. Seed piece treatments are commonly used on PEI and involve applying insecticides or fungicides in powder form to crop seeds before planting begins. The most commonly used insecticide to treat seeds is Admire® (active ingredient Imidacloprid) that is systemic and provides the developing crop with protection from insects such as Colorado potato beatles, corn borers, and aphids for much of the growing season. Seeds may also be treated with Ridomil Gold® (active ingredient Mefenozam™) or Quadris® (active ingredient Azoxystrobin) to protect against soil born fungal diseases. Once crops reach 10-15 cm in height, preventative fungicides such as Bravo® (active ingredient Chlorothalonil), are applied to crops from July to mid-September. Although all pesticides have some potential to damage non-target organisms, insecticides are generally considered most hazardous to humans and non-target wildlife (Büyüksönmez *et al.* 1999). The majority of insecticides currently in use are organophosphate, carbamate, and synthetic pyrethroid, and to a lesser extent organochlorine compounds (widely used in the

Table 1-1. List of pesticides used in Prince Edward Island along with the hazard, volume used, persistence, and pesticide class (Environment Canada 2004).

Active Ingredient	Hazard Rank	Volume Rank	Half-life in soil	Pesticide class
Carbofuran	1	19	30-120 days	Carbamate
Endosulfan	2	17	730 days	Organochlorine
Azinphos-methyl	3	18	5-21 days	Organophosphorus
Chlorothalonil	4	2	90 days	Oraganochlorine
Methamidophos	5	11	4-21 days	Organophosphorus
Chloropicrin	6	27	Unknown	Organochlorine
Phosmet	7	24	4-20 days	Organophosphorus
Carbaryl	8	29	7-28 days	Carbamate
1,3 Dichloropropene	9	15	Non-persistent	Organochlorine
Dimethoate	10	10	2-16 days	Organophosphorus
Diquat dibromide	11	7	Highly persistent	Bipyridyl
Mancozeb	12	1	6-15 days	Dithiocarbamate
Metiram	13	4	Low persistence	Carbamate
Paraquat	15	20	1000 days	Pyridine
Linuron	16	14	30-150 days	Substituted-urea herbicide
Thiram	17	31	1-60 days	Dithiocarbamate
Propiconazole	18	30	40-70 days	Triazole
Imidacloprid	19	13	730 days	Chloronicotinyl

Continued

Table 1-1, continued.

Active Ingredient	Hazard Rank	Volume Rank	Half-life in soil	Pesticide class
Atrazine	20	26	60-100 days	Triazine herbicide
Metribuzin	21	8	17-60 days	Triazine herbicide
2,4-D	22	25	7 days	Aryloxyalkanoic acid
Metobromuron	23	9	30 days	Substituted-urea herbicide
Thiophanate-methyl	25	12	21-28 days	Carbamate
Fluazifop-p-butyl	26	21	7 days	Aryloxyphenoxypropionic herbicide
MCPA	27	3	14-42 days	Phenoxyacetic herbicide
Metalaxyl-M	28	6	7-170 days	Acylamino acid
Rimsulfuron	29	16	5-10 days	Urea herbicide
Glyphosphate	30	5	60 days	Aliphatic acid
Hexazinone	31	22	30-365 days	Triazine herbicide

past). Because they are less persistent than many of the other chemicals, organophosphate compounds have become one of the most widely used classes of insecticides in the world (Fulton and Key 2001). However, they generally have a greater acute toxicity compared to organochlorine insecticides. Organophosphate and carbamate insecticides work by inhibiting acetylcholinesterase, an important enzyme in transmission of nerve impulses from presynaptic to postsynaptic nerve cells (Büyüksönmez *et al.* 1999). As a group, carbamate insecticides are considered to be non-persistent and to have low chronic toxicity (Büyüksönmez *et al.* 1999). Pyrethroids are synthetic organic insecticides related to pyrethrum, a natural plant-derived insecticide, altered to be stable in sunlight (Büyüksönmez *et al.* 1999). Pyrethroid insecticides also interfere with nervous system functions such as transmission of nerve impulses. They are effective at relatively low application rates and provide residual effectiveness.

Due to the number of compounds and chemical categories, herbicides are normally classified by their effect, such as contact or systemic, or their time of application, such as preemergence or postemergence (Gevao and Jones 2002). Half-life is an estimate of the number of days required for a pesticide to degrade to half its original concentration, and is a general indicator of pesticide persistence. Pesticides with a half-life in soil of less than 30 days are considered non-persistent while pesticides with a half-life over 100 days are considered persistent (Büyüksönmez *et al.* 1999). In general, herbicides are less toxic to non-target animals than insecticides and are not as persistent, i.e. they have a shorter half-life. There are exceptions such as Paraquat, which is moderately toxic and has a half-life of 500 days (Büyüksönmez *et al.* 1999).

Fungicides are used to control both parasitic fungi and fungal-like protists that cause destruction or deterioration in the quality of agricultural and forest products. Functionally, fungicides are divided into three groups: 1) protectants that are used to form a surface deposit on foliage to prevent germination, 2) eradicants that control established fungal infection, and 3) wood preservers (Büyüksönmez *et al.* 1999). On PEI, fungicides are the most commonly applied pesticides and are used to treat both early and late blight. The environmental persistence of fungicides in water ranges from a half-life of 1-2 days for Mancozeb to a half-life of over 4 weeks for Metalaxyl (Environment Canada 2004).

Fates of Pesticides and Fertilizers

All pesticides are subject to decomposition. This may occur through many processes, such as microbial decomposition, hydrolysis, oxidation-reduction reactions, and photolysis (Gevao and Jones 2002). During decomposition, pesticide compounds progressively break down to metabolites, ions, and elements. It is possible that a pesticide may degrade partially to a secondary compound which has similar toxicity or is more toxic then the parent chemical (Büyüksönmez *et al.* 1999). Physicochemical properties of pesticides such as molecular weight, melting/boiling point, solubility, and partition coefficient between water and sediment can ultimately affect the movement of pesticides in the environment.

Excessive use of nitrogen fertilizer for crop production can also cause deterioration of water quality (Udawatta *et al.* 2006). As a major plant nutrient, nitrogen (in the form of nitrate) is often applied in large amounts to agricultural land to achieve optimal yields. Any excess nitrate is susceptible to loss from fields via leaching into ground water (Hatch *et al.* 2002).

Phosphorus is an essential plant nutrient and is necessary for optimal crop yields (National Research Council Staff 1993) yet it is also one of the least abundant nutrients relative to its demand in the environment (Leinweber *et al.* 2002). In natural systems phosphorus is tightly cycled through the plant-soil continuum, but in agricultural systems soil phosphorus is taken up by the crop and must be replaced if a deficiency is to be avoided (Leinweber *et al.* 2002). Phosphorus fertilizers (in the form of phosphate) have traditionally been applied to agricultural land with no concern over losses into water because inorganic phosphorus is strongly fixed in the soil and largely prevented from leaching. However, even small amounts of phosphate lost from the soil into the water can have severe impacts on water quality in receiving catchments.

Both nitrogen and phosphorus limit the growth of phytoplankton, macroalgae, and vascular plants in freshwater and marine ecosystems. Phosphorus is the primary limiting nutrient in most fresh water systems while nitrogen is considered the primary limiting nutrient in marine ecosystems (Rabalais 2002). During the winter months these limiting nutrients can be reversed. For example, while nitrogen is the primary limiting nutrient in most marine ecosystems, phosphorus can be limiting (or co-limiting) in the winter (Pinckney *et al.* 2001). Inputs of these nutrients into aquatic ecosystems can lead to algal

blooms which can result in oxygen depletion, fish kills, and other water quality problems (National Research Council Staff 1993).

Effects of Pesticides on Fishes

Aquatic systems that run through agricultural areas have a high risk of being contaminated by a variety of chemicals through runoff and groundwater leaching (Todd and Van Leeuwen 2002). Although sublethal effects of chemicals in the aquatic environment are major issues in ecological risk assessment, most laboratory testing and evaluations have focused on lethal endpoints. Sublethal effect tests are normally measured in terms of the effects of a chemical on the growth and reproduction of organisms (Chaisuksant *et al.* 1998). Although early life stages of fishes are generally considered to be the most sensitive to waterborne toxicants, juvenile and adult fishes are often used to evaluate the acute toxicity of pesticides (Phillips *et al.* 2002). Acute toxicity is used to describe the adverse affects of a substance which results either from a single exposure or multiple exposures in a short period of time (usually less than 24 h). Lethal and sublethal effects on larvae from exposure to toxicants have obvious implications for recruitment into adult populations. The following are a few of many examples of studies conducted at each of three commonly used life stages.

Immersion exposures with eggs of frequently tested species have proven quite useful in studies of field collected samples. *In situ* exposures and evaluations of moderately hydrophobic components provide a realistic expression of exposure that most closely simulate passive exposure observed in nature (Helmstetter and Alden 1995).

Many studies have found that embryonic exposure to pesticides results in adverse effects to developing embryos. For example, González-Doncel *et al.* (2003) found that eggs exposed to permethrin at 100 μg/L and 300 μg/L took significantly longer to hatch compared to control groups. Trembling, hyperactivity, and spastic body contractions were observed in the embryos at higher levels of exposure. Newly hatched larvae were unable to inflate swim bladders or respond to stimuli, had uncoordinated movement, and displayed evidence of spinal curvature. Fent and Meier (1994) exposed European minnow (*Phoxinus phoxinus*) eggs to triphenyltin and observed delayed development, decreased hatching success, spinal deformity, delayed yolk sac absorption, and pericardial edema. Exposure of zebrafish eggs to the carbaryl insecticide Sevin® resulted in delayed development and smaller larvae at hatching (Todd and Van Leeuwen 2002). Spinal alterations, pericardial edema, heart abnormalities, deformed tails, and tail lesions were observed in Japanese medaka embryos exposed to sublethal concentrations of the fungicides Acrobat MZ® (active ingredient dimorph) and Tattoo C® (active ingredients propamocarb HCl and chlorothalonil) (Teather *et al.* 2001). Another study exposing Japanese medaka eggs to pentachlorophenol resulted in swollen abdomens, underdevelopment, pericardial edema, lethargy, and delayed or incomplete hatching (Helmstetter and Alden 1995).

Many other studies have exposed larval and adult fish to varying concentrations of pesticides. Van Leeuwen *et al.* (1985) found the period of yolk sac resorption to be the most sensitive stage of embryolarval exposures. The accumulation of toxicants followed by metabolism of triglycerides and proteins during yolk sac resorption increases the

concentration and possibly the bioavailability of contaminants, and therefore their toxicity. Exposure to carbofuran and molinate reduced the growth rate, swimming ability, and swimming speed in larval fathead minnows (Heath *et al.* 1997). Increased respiration rates and decreased heart rates were observed in juvenile rainbow trout (*Oncorhynchus mykiss*) after being exposed to 350 ppb and 140 ppb DDT. These signs are characteristic of fish suffering from hypoxia, which indicates that oxygen uptake may be affected following exposure to DDT (Lunn *et al.* 1976).

Three specific chemicals have been associated with fish kills on PEI to date. Chlorothalonil (Mutch 2001b), endosulfan (Mutch 2001c), and azinphos-methyl (Mutch 2001a, Mutch 2001c) have all been found in streams and surrounding standing water following these events. In a laboratory study by Davies and White (1985) adult rainbow trout, common jollytail (*Galaxias truttaceus*), and spotted galaxias (*Galaxias auratus*) were subjected to varying concentrations of the fungicide Chlorothalonil. Increasing concentrations resulted in a decrease in blood haematocrit and inhibition of key respiratory enzymes. Chlorothalonil has been listed as highly hazardous in a document listing the relative risk ranking of pesticides to fishes (Mutch 1999). Similarly, endosulfan has been listed as extremely hazardous. Exposure to endosulfan has been shown to produce excessive mucous and gill lesions, suggesting that endosulfan irritates the gills and impedes respiratory diffusion across the gills (Nowak 1992). Azinphos-methyl was also listed as extremely hazardous. Adult bluegill sunfish (*Lepomis macrochirus*) exposed to azinphos-methyl were observed coughing, rolling, exhibiting darting movements, and showing an overall lightening or darkening of body colouration.

Teather *el al.* (2001) found reduced reproductive performance in adult males that had been exposed to Chlorothalonil.

Sediment Collection and Storage

When collecting and storing sediment, a major concern is to maintain the integrity of the samples until their chemical characteristics and/or toxicity are evaluated. This is best achieved by minimizing changes to the physical, chemical, and biological properties of the samples that might result from transportation, storage, and preparation for analysis (Environment Canada 1994). Manipulation of sediments in the laboratory is often required to achieve certain desired characteristics for toxicity testing and chemical analysis. As all manipulation procedures may alter some qualities of field samples, it is critical to evaluate the extent to which this occurs (U.S. EPA 2001). To ensure minimum disturbance of the upper layer of sediment, sampling at least the upper 6-8 cm is recommended, with 10-15 cm being optimal (U.S. EPA 2001, Environment Canada 1994). Sampling of the surface layer provides information on the properties of interest of the most recently deposited material (U.S. EPA 2001).

In general, sieving is not recommended because it can substantially change the physicochemical characteristics of the sediment sample (U.S. EPA 2001, Environment Canada 1994). However, in some cases sieving might be necessary to remove indigenous and predatory organisms which can interfere with subsequent toxicity testing and results (Ferretti *et al.* 2002, Lewis *et al.* 2001, Besser *et al.* 1998, Nebeker *et al.* 1984). Different sized screens have been suggested in different studies; mesh sizes such as, 850 μm

(Ingersoll *et al.* 2005), 0.5 mm (Ferretti *et al.* 2002, Besser *et al.* 1998, Nipper *et al.* 1998, Ozretich and Schults 1998), 1 mm (Lewis *et al.* 2001), and 2 mm (Geffard *et al.* 2004a, Geffard *et al.* 2003, Daniel *et al.* 2002, Moore *et al.* 1995) have been used. The smaller the particle size, the greater the surface area exposed, and therefore there is an increase in bioavailability of toxicants. Sieving through a 2 mm sieve is acceptable to discriminate between sediment and other materials (ASTM 2000).

It is often necessary to homogenize subsamples, a process in which sediment is mixed to obtain consistent physicochemical properties throughout the sample to be analyzed. Homogenization can be done either in the field or in the laboratory (U.S. EPA 2001) and should be performed as quickly and efficiently as possible as prolonged homogenization can alter the particle size distribution in a sample and cause oxidation of the sediments (Ditsworth *et al.* 1990, Stemmer *et al.* 1990). Oxidation of the sample can alter the bioavailability of contaminants, particularly metals (Ankley *et al.* 1996).

Sediment Exposure

Burton (1991) stated that the most important issue in sediment toxicity testing is the use of the appropriate test phase. Test phase systems can be categorized as extractable, elutriate (water taken from the top of sediment that has been re-suspended and let settle out), and interstitial (pore) water phases, whole sediment, and *in situ*. No phase can meet all study objectives and each has strengths and weaknesses; therefore each would have to be evaluated for suitability in any study (Burton 1991). These issues are summarized in Table 1-2.

Several studies have compared test phases as treatments (Geffard *et al.* 2004b, Geffard *et*

al. 2003, Geffard *et al.* 2002, Hoke *et al.* 1995, Sasson-Brickson and Burton 1991), while others have used only one (Viganò *et al.* 1995, Geffard *et al.* 2004a, Mondon *et al.* 2001). Geffard *et al.* (2002) compared the toxicity of whole sediment and elutriates to the embryos of the oyster (*Crassostrea gigas*) and found whole sediment to be toxic 90% of the time, while elutriates were toxic 30% of the time. Sasson-Brickson and Burton (1991) compared whole sediment, elutriate, interstitial water, and *in situ* test phases and found that elutriates were less toxic than interstitial water or whole sediment, while all three were more toxic than the *in situ* experiment.

Bioavailability and Routes of Uptake

Bioavailability of contaminants in sediment is complex and includes many relationships between the concentration of contaminants in the environment, the amount of contaminant that an organism is able to take up, and the duration of exposure (Sijm *et al.* 2000). Bioavailability of contaminants in sediment depends on physical properties of the sediment such as grain size, chemical properties of the contaminant such as solubility, and biological properties such as the variety of organisms that inhabit the sediment. One of the best methods for assessing the bioavailability of sediment associated contaminants is to observe its accumulation in organisms (Borgmann 2000, Connell *et al.* 1999, McCarty and Mackay 1993). This approach measures toxicant concentration at target sites (within the organism) rather than in the surrounding environment.

Fish in agricultural areas are especially susceptible to contaminant exposure caused by pesticides moving from agricultural fields in runoff water and sediment. Fish

Table 1-2. Sediment phases used in toxicity tests.

Phase	Strengths	Weaknesses	Routine Uses
Extractable phase	- used with all sediment types - sequentially extract different degrees of bioavailable fractions - determine dose-response	- ecosystem realism: bioavailability unknown	- rapid screen
Elutriate phase	- used with all sediment types - methods relatively standardized - determine dose-response	- ecosystem realism: never occurs in equilibrium *in situ*	- rapid screen - dredging evaluations
Interstitial water	- direct route of uptake for some species - semi-direct exposure phase for some species - methods of exposure relatively standardized - determine dose-response	- cannot be collected from some sediments - limited volumes can be collected - constituents altered by all methods - exposure phase altered chemically and physically when isolated from whole sediment	- rapid screen - initial survey
Whole sediment	- used with all sediment - relative realism is high - determine dose-response - holistic versus reductionist approach	- some physical, chemical, and/or microbial alteration from field collection - few standards methods - indigenous biota may be present in sample	- rapid screen - chronic surveys - initial survey

Continued

Table 1-2, continued. Sediment phases used in toxicity tests.

In situ	- real measure integrating all key components - resuspension/suspended solids effects assessed	- few methods and endpoints - not as rapid as some assay systems - predation by indigenous biota	- resuspension effects - intensive system monitoring

may be exposed to contaminants via ambient water and food. As a result, the most important sites of absorption in fish are the gills and the gastrointestinal tract (Franklin *et al.* 2005). Absorption through the skin occurs to a lesser extent. Since gills are continuously in contact with water and have such a large surface area, they also have the ability to continuously transfer pollutants from water and suspended sediment particles via the gill surface (Yang *et al.* 2007). The primary route of uptake differs depending on the type of contaminant. For example, Borgå *et al.* (2004) found that dietary uptake is the main route for organochlorine contaminants, while Jonsson *et al.* (2004) stated that the most significant route of uptake of polycyclic aromatic hydrocarbons (PAH's) is across the gills. According to Newman and Unger (2002), uptake by a cell can be achieved via lipid, aqueous, and endocytic routes. The lipid route involves the passage of lipophilic contaminants through the lipid bilayer of the cell membrane. The aqueous route employs membrane transport proteins that form channels or act as carrier proteins in the membrane and also those that transfer hydrophilic contaminants into cells. For example, waterborne cadmium enters the gill epithelium through the same pathway as calcium (Ca^{2+}) channels and blocks active Ca^{2+} uptake (Franklin *et al.* 2005). This disturbs the Ca^{2+} balance, leading to hypocalcemia and cell death. Endocytic uptake occurs when cells take in material from outside the cell by engulfing it with the cell membrane.

The overall objective of the present study was first to assess the methodology used to determine sediment toxicity and then employ these methods to assess potentially contaminated sediments from a stream in Prince Edward Island. First sediment storage methods were assessed, comparing fresh sediments to those frozen or freeze-dried using

Japanese medaka embryolarval bioassays. There are conflicting opinions in the literature on the different storage methods and no other studies have determined the effects of sediment storage methods on a vertebrate model. Depending on what is to be done with the sediment extracts, the toxicity of the carrier solvent can be very important. Next the toxicity of three common carrier solvents was examined using similar medaka bioassays. Finally, results of embryolarval bioassays with whole sediment and sediment extracts from two sediment collection periods were compared to assess sediment quality in the Wilmot River in the years following massive fish loss due to agricultural runoff into the river using techniques established in the first two experiments.

CHAPTER 2:

GENERAL METHODS AND MATERIALS

Site Selection

The Wilmot River is one of PEI's longest rivers at roughly 19 km; it has a watershed area of approximately 166 km^2 and has a high percentage of agriculture (Fig. 2-1). In July 2002, heavy rainfall was responsible for two separate runnoff events into the Wilmot River, resulting in the massive mortality of brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) (Gormley *et al.* 2005). Water samples analyzed by Environment Canada revealed high concentrations of azinphos-methyl (Gormley *et al.* 2005). In 2003, Jardine (2005) assessed the toxicity of the sediments at three sites along the Wilmot River. Results from that study indicated that Japanese medaka embryos exposed to Wilmot River sediments had delayed hatching times, increased developmental abnormalities, and were smaller at hatching than those in the reference groups. One of the sites used in Jardine's study was selected for this study because the sediments had consistently negative effects on developing medaka and were hypothesized to have high concentrations of agricultural chemicals or chemical metabolites.

Cleaning Procedures for Glassware and Utensils

All new glassware was soaked for four days in a solution of water and Aquet® laboratory detergent and then thoroughly hand-washed. Glassware was triple rinsed with glass distilled water and left to air dry. It was then wrapped in aluminum foil and placed in a muffle furnace at 450 °C for 4 h. Glassware that had been used for previous experiments was soaked in the Aquet® solution for four days, then washed thoroughly by

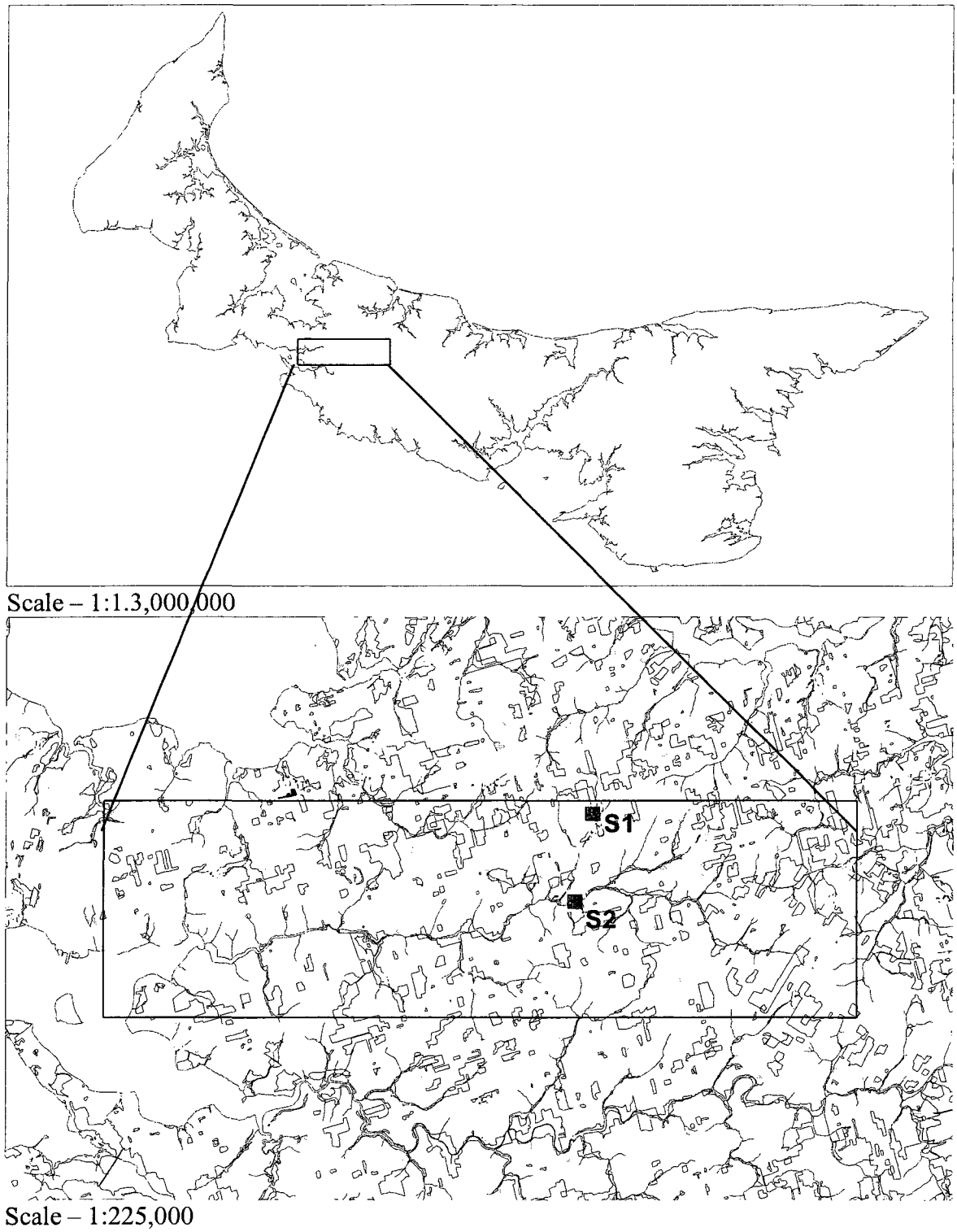


Figure 2-1. The Wilmot River, Prince Edward Island with sites S1 (Spring) and S2 (Above Arsenault's Pond) highlighted.

hand and rinsed with tap water. Glassware was then placed in a weak HCl (<1 %) bath for 4 h and then triple rinsed with glass distilled water. Once dry, glassware was wrapped in tinfoil and placed in a muffle furnace at 450 °C for 4 h. All utensils were also soaked in a solution of water and Aquet® detergent overnight, hand washed thoroughly, and triple rinsed with glass distilled water before letting air dry. At the sample sites, glassware and utensils were rinsed in river water before sediment collection.

Sediment Collection and Storage

At each stream site the top 5-10 cm of sediment were collected using a stainless steel spoon and emptied into a sieve with a mesh size of 2 mm. Sediments were rinsed with approximately 500 mL of river water before being placed into 1,000 mL glass canning jars. A total of 2 L of sediment was collected at each site. Sediments were then placed into an insulated container and kept at approximately 4 °C until returning to the laboratory. In the lab, sediments were either placed in the refrigerator (5 ± 2 °C), freezer (-18 ± 2 °C), or freeze dryer, depending on requirements for further experiments. The dry weight of each sample was obtained by drying five replicates of 1 g of sediment in an incubator at 70 °C for 3 h. Samples were weighed after 2 h and then again after 3 h to ensure all water had evaporated. The average weight was taken from the five samples and the percent moisture was calculated. This process ensured a standardized amount of sediment was used from each site in each experiment (30 g dry weight in each whole sediment exposure replicate).

Organic and Inorganic Carbon Analysis

Once the dry weight for each sample was obtained, the percent organic and inorganic carbon was determined. A 5 g dry weight equivalent of wet sediment for each site was placed in ceramic crucibles. They were then placed in a muffle furnace at 450°C for 4 h. Samples were weighed at this point to determine the percent organic carbon. They were then placed in the muffle furnace at 950°C for 2 h. Samples were then weighed to obtain the percent inorganic carbon of the sediment.

Study Organism

The Japanese medaka (*Oryzias latipes*) is an oviparous freshwater killifish belonging to the Cyprinodont family and is indigenous to areas of Japan, Taiwan, and southeastern Asia (Metcalfe *et al.* 1999). Adult medaka are 2-4 cm long and commonly found in rice paddies where they feed extensively on mosquito larvae (Kirchen and West 1999). In their natural environment the temperature varies from 5-35 °C, and therefore they can be easily maintained in unheated aquaria at room temperature (Kirchen and West 1999). Sexual dimorphism is externally evident with the anal fin of the male being a different shape than that of the female. Japanese medaka are useful test organisms for embryolarval toxicity tests because they are easily bred in captivity, their eggs possess exceptional optical clarity (Owens and Baer 2000), they have a rapid and well characterized period of embryolarval development, and they exhibit the basic patterns of vertebrate organogenesis (Shi and Faustman 1989). The natural breeding season of the Japanese medaka extends from mid-April to late September, with peak activity in June,

July, and August. Egg production is influenced by light, food, and temperature (Kirchen and West 1999); as a result, medaka are easily induced to breed in an artificial setting by adjusting these three variables.

Egg Collection

Between breeding cycles the temperature in the tanks was maintained at 20-21 °C with a 12:12 h light to dark cycle. Fish were fed once daily with Nutrafin® staple food and occasionally with newly hatched brine shrimp. To induce breeding, the light cycle was increased to a 16:8 h light to dark cycle, and the temperature in breeding tanks was increased to 24-26 °C. Fish were fed twice daily, once with newly hatched brine shrimp nauplii (*Artemia* sp.), and once with Nutrafin® staple food. Females were checked for eggs beginning two hours after the daily light cycle began. Eggs were manually removed from the abdomen of each female and placed into a glass petri dish containing embryo rearing medium [1% NaCl, 0.03% KCL, 0.04% CaCl, 0.163% $MgSO_4$ (Kirchen and West 1976)] and 1-2 drops of methylene blue to reduce the incidence of fungal infection. Eggs were then put into an environmental chamber at 25 °C for 4 h after which they were examined for fertilization using a dissecting microscope. Fertilization is characterized by the movement of oil droplets to the vegetal pole of the egg (Kirchen and West 1976). Unfertilized or dead eggs were removed from the dish and the remaining eggs were separated from each other manually using stainless steel scoopulas.

Endpoints

Hatching Success: All eggs were checked once daily one hour after the onset of the light cycle. Survivorship was recorded and dead embryos were removed from the jars. Dead embryos were easily distinguished by their blue colour caused by methylene blue in the medium passing through the chorion.

Heart Rate: At day five post fertilization, the heart rate of each embryo was taken by determining the time required for 20 beats and then converting this value to beats per minute.

Hatching Time: Hatching was defined as the full release of the larva from the egg. The day on which each larva hatched was recorded. Partially hatched embryos were discarded and considered dead.

Length at Hatch: On the day of hatching, a magnified photograph of each larva was taken using a PixieLink camera attached to a Zeiss dissecting microscope. The images were transferred to a computer and viewed using PixieLink software. Lengths were determined by measuring from the tip of the head to the end of the notochord directly on the screen. Measurements were then converted to mm using a conversion factor based on the magnification used.

*Developmental Abnormalities:*All newly hatched larvae were examined for a variety of developmental abnormalities (Table 2-1). These included pericardial edema (fluid accumulation around the heart), yolk sac edema (fluid accumulation in the yolk sac), lordosis (abnormal ventral curvature of the spinal column), scoliosis (lateral curvature of spinal vertebrae), kyphosis (abnormal dorsal curvature of the spinal column),

malformed/missing fins (pectoral fins missing or bent), and hemorrhaging (internal bleeding) (Boudreau *et al.* 2005, Hill *et al.* 2004, Silverstone and Hammell 2002, Villalobos *et al.* 2000, Rice *et al.* 1997).

Data Analysis

The mean hatching times and lengths for eggs in each jar were calculated. Using these values, the mean hatching times and lengths were calculated for the entire group. Thus each jar, rather than each embryo was counted as a replicate.

The distribution of all variables was checked for normality using the Minitab® Anderson-Darling test (1972-2005 Minitab Inc.). Parametric statistics were used when both the Anderson-Darling test and the Levene's test for equal variances each had a non-significant p-value ($p \geq 0.05$). Non-parametric tests were used when data were not normally distributed and the data could not be transformed. Parametric data were compared using one-way analysis of variance (ANOVA) and non-parametric data were compared with Kruskall-Wallis tests using Statistica® (1984-2003 StatSoft, Inc.). *Post hoc* testing of groups for which significant differences had been found using ANOVA's was carried out using Tukey's *post hoc* test. *Post hoc* testing of significant Kruskall-Wallis tests were carried out using a Dunn's multiple comparisons test. Hatching success and incidence of developmental abnormalities were compared using G-tests.

Table 2-1. Description of developmental abnormalities found in fish when exposed to various contaminants.

Developmental Abnormality	Description
Yolk sac edema	Fluid accumulation in the yolk sac
Lordosis	Abnormal ventral curvature of the spinal column
Kyphosis	Abnormal dorsal curvature of the spinal column
Scoliosis	Lateral curvature of spinal vertebrae
Malformed/missing pectoral fins	Pectoral fins missing or bent
Hemorrhages	Internal bleeding
Pericardial edema	Fluid accumulation around the heart

CHAPTER 3

COMPARING THE TOXICITY OF REFRIGERATED, FROZEN, AND FREEZE-DRIED SEDIMENTS USING JAPANESE MEDAKA (*ORYZIAS LATIPES*) EMBRYOLARVAL BIOASSAYS

INTRODUCTION

Most studies examining the toxicity of aquatic sediments require the collection of sediments from a variety of locations and subsequent testing in a laboratory. However, factors such as the potential change in toxicity due to sample processing, transport, storage, and manipulation must be considered when attempting to relate laboratory results to the natural environment. Extended storage of sediment is often necessary when field seasons are short and if retesting is required. This study investigated the effects of different sediment storage methods by comparing refrigerated, frozen, and freeze-dried sediments.

Limited information is available on how the toxicity and availability of sediment contaminants is influenced by sample storage prior to analysis. Storage methods should be designed to maintain structural and chemical qualities of sediment and pore water samples (U.S. EPA 2001). According to Environment Canada (1994), sediment samples should be kept refrigerated at 4 ± 2 °C and tightly sealed in the dark. While acknowledging that some types of sediments (e.g. sediments to be used in metals analysis) can be preserved by freezing, it was noted by Environment Canada that freezing permanently changes the structure of sediment and potentially alters the bioavailability of sediment-associated contaminants. U.S. federal guidelines also discourage freezing sediment as a storage method (Dillon *et al.* 1994) due to physical changes in sediment composition known to occur and on the altered availability of nutrients and contaminants within sediments during frozen storage. Not all studies have found that freezing

sediments increases their toxicity. Both Stenberg *et al.* (1998) and Dillon *et al.* (1994) found no difference in toxicity between sediments tested upon arrival to the lab and sediments that had been frozen at - 20°C and tested after 2, 4, 7, and 12 days and 20 weeks. However, it is generally agreed that sediments to be used for toxicity testing should be kept at temperatures just above freezing for no more than two weeks before testing (U.S. EPA 2001, ASTM 2000, Norton *et al.* 1999, Becker and Thomas 1995, Schuytema *et al.* 1989). For example, Schuytema *et al.* (1989) spiked freshwater sediments with both DDT and endrin and found that the toxicity of the sediments decreased in both treatments after 14 days of refrigeration. They concluded that data from studies using cold-stored and frozen-stored sediment may not be comparable.

Freeze-drying sediment is a less common method of sediment storage. Some evidence suggests that freeze-drying may result in sediments that are more toxic than fresh sediments from the same location. For example, Beiras and His (1995) compared the toxicity of fresh and freeze-dried sediments to Japanese oyster (*Crassostrea gigas*) embryos and found significant differences in their toxicity. The fresh sediments were not found to be toxic at any of the three concentrations tested, while the freeze-dried sediments were found to be highly toxic at the two highest concentrations. Geffard *et al.* (2004a, 2004b) also showed that freeze-dried sediments were more toxic to Japanese oyster embryos than either frozen or fresh sediments. They speculated that freeze-drying may have mobilized otherwise non-bioavailable toxicants.

In addition to the uncertainty regarding how sediments should be stored, the majority of studies that have assessed the toxicity of sediments have used aquatic invertebrates (e.g. Norton *et al.* 1999, Stemmer *et al.* 1990, Schuytema *et al.* 1989). Little research has been conducted to compare the toxicity of different sediment storage methods using aquatic vertebrates (Hallare *et al.* 2005).

This study had two objectives. The first objective was to determine whether exposure to sediments would have effects on survival, time to hatch, length at hatch, and rate of developmental abnormalities. The second was to determine if sediment toxicity was affected by different storage methods as assessed using Japanese medaka embryolarval bioassays.

METHODS

General details regarding the study organism, site selection, and data analysis from this study are provided in the General Methods section. Information specific to this investigation is provided below.

Sediment was collected from the Wilmot River on the morning of June 6, 2006. Upon return to the laboratory, water was decanted from each sample jar. Refrigerated sediment was immediately placed in a refrigerator at 4 ± 2°C, while subsamples of sediment to be frozen were divided into three glass jars and placed in a freezer at - 18 ± 2°C for 48 h prior to experiment set up. The remaining sediment was frozen with liquid nitrogen and placed on a freeze-dryer for 48 h.

The initial set-up for the exposure took place 48 h after the sediment collection and took place over three days. Medaka eggs were exposed to sediments using a static non-renewal bioassay. Nine replicates were set up for each of the treatments: a reference (sediment free), fresh (refrigerated), frozen, and freeze-dried. Preparation procedures were identical for all sediment treatments. Bernardin® 250 mL wide mouth mason jars and snap lids were used for all exposures. Each day one of the jars of sediment was removed from the freezer and thawed at room temperature. Replicates of each treatment were prepared the day before addition of embryos; each replicate was allotted 30 g dry weight of the respective sediment and 150 mL of embryo rearing medium. The contents of each replicate were mixed thoroughly for one minute and allowed to settle at room temperature. The larger sediment particles settled out first, leaving only fine particles to come in contact with the eggs. Immediately before eggs were placed in treatments, 6 cm

petri dishes were placed gently on the sediment. These were used to reduce potential predation of the eggs by invertebrates in the sediment and to facilitate observation of the eggs under a dissecting microscope. One egg was placed in each replicate of each treatment. This process was repeated until each replicate of each treatment had eight eggs. Prepared treatments with eggs were placed in an environmental chamber at 25 ± 2°C under a 16:8 h light to dark photoperiod.

Eggs were checked daily for survivorship and hatching. Newly hatched larvae were removed from the jars daily using disposable polyethylene pipets. The notochord of each larvae was measured to the nearest 0.1 mm and each was checked for developmental abnormalities (see General Methods and Materials for more information).

RESULTS

Problems with the reference group (reference) were encountered in this exposure and thus a control group from a subsequent experiment is also presented (reference comparison) as a comparison. The reference comparison group was set up using identical methods as the reference group of the present study except that the reference comparison had ten replicates with four eggs in each while the reference for the present study had nine replicates with eight eggs in each. These problems are discussed in more detail below.

Sediment Characteristics

The water content of the sediment was 27.2 %. Organic carbon made up 0.34% of the sample; there was no inorganic carbon detected.

Hatching success

All treatments began with nine replicates, each containing eight medaka embryos. The hatching success of medaka exposed to Wilmot River sediments differed significantly between treatments ($G = 77.21$, $df = 3$, $p < 0.05$) (Fig 3-1). All medaka in the reference group and the reference comparison hatched. Those exposed to refrigerated sediments had a hatching success of 75%, frozen sediment-treated embryos had a hatching success of 69.4%, and the freeze-dried sediment-treated embryos had a hatching success of 2.5%.

Time to hatch

There were significant differences in the time to hatch among the different treatment groups (Kruskall-Wallis $p<0.001$, Fig 3-2). Refrigerated, frozen, and freeze-

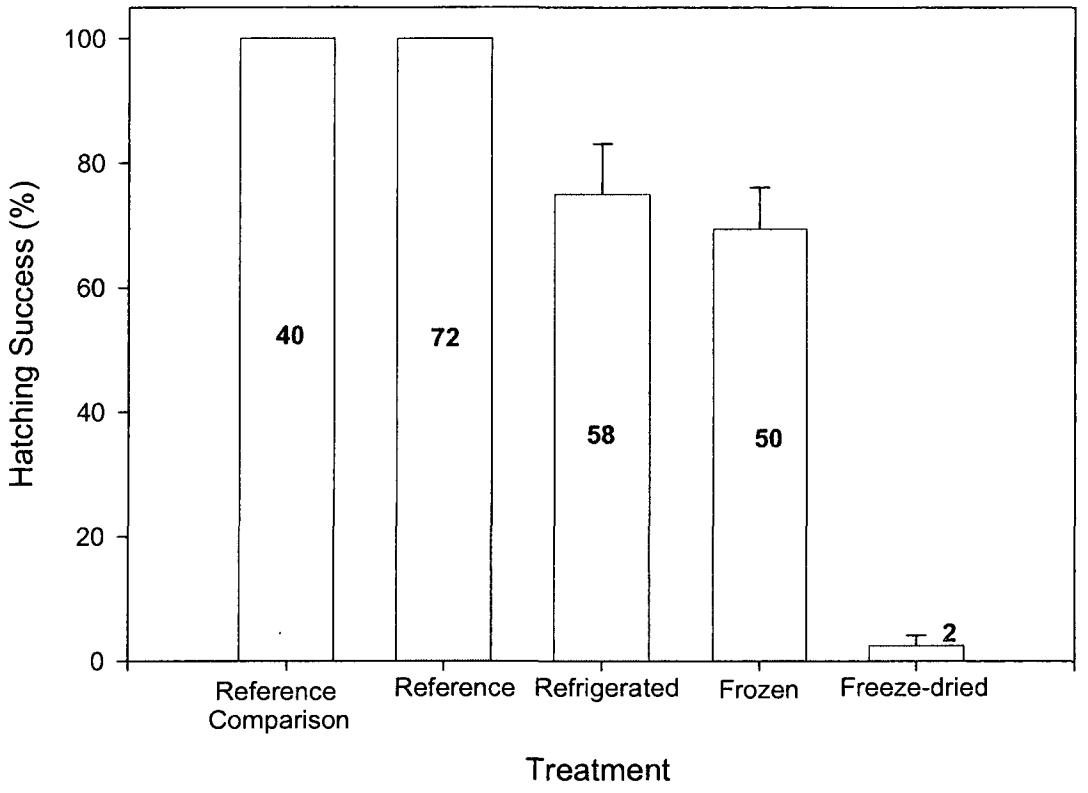


Figure 3-1. Mean hatching success (± SE) of Japanese medaka embryos exposed to reference comparison, reference, refrigerated, frozen, and freeze-dried sediments. Bold numbers represent the number of individuals that hatched in each treatment group.

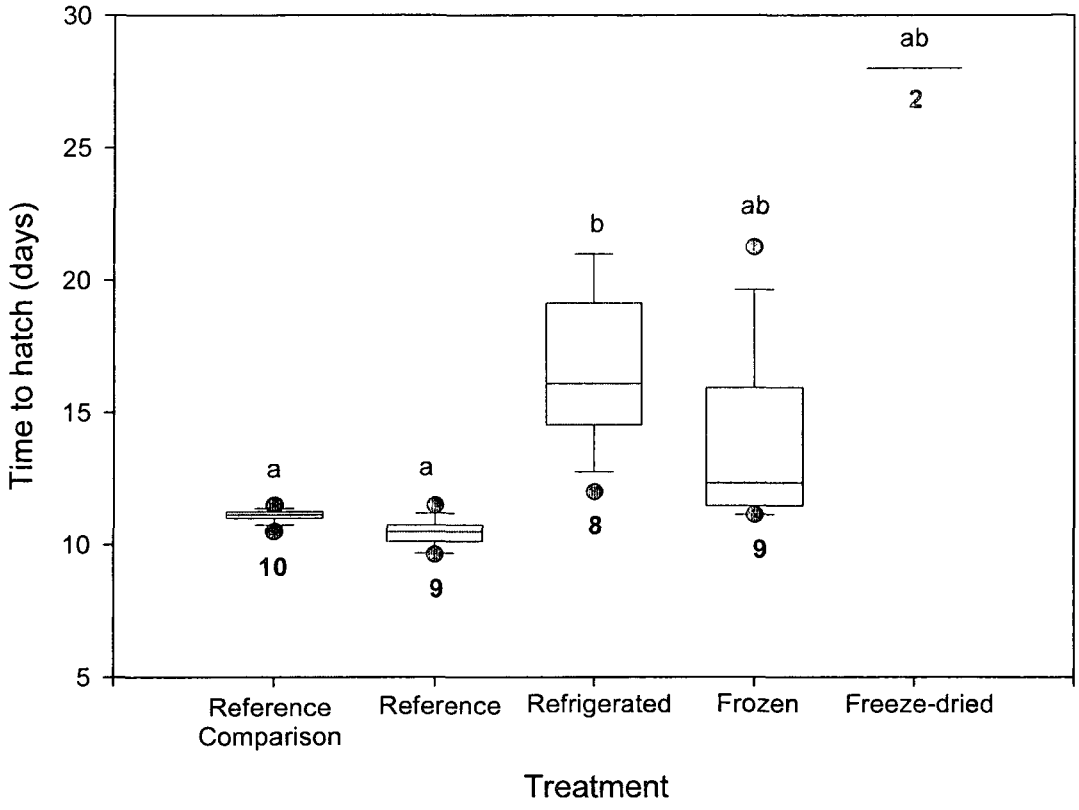


Figure 3-2. The time to hatch for Japanese medaka eggs exposed to a reference comparison, reference, refrigerated, frozen, and freeze-dried Wilmot River sediments. The box is delineated by the 25th percentile and the 75th percentile; while top and bottom whiskers represent the 90th and 10th percentiles respectively, and the center line represents the median. Individual points indicate data that fall outside the listed percentiles. Letters denote values of statistical significance between treatments at $p<0.05$. Bold numbers represent sample size.

dried sediment-exposed groups hatched significantly later than the reference and reference comparison groups (Dunn's, $p<0.05$ for each). Although not significantly different (likely because of the small sample size) embryos exposed to freeze-dried sediments hatched much later than the other groups.

Length at hatch

Although the reference and reference comparison groups did not differ in either hatching success or hatching time, those of the reference group from the present study were significantly smaller than those from the reference comparison group (Kruskall-Wallis, $p<0.0001$) (Fig. 3-3). The main reason for this was the high percentage of spinal abnormalities found in the reference group from the present study. For this reason, the reference comparison provides a more reliable comparison for this endpoint. Larvae from the reference comparison were significantly larger than those exposed to refrigerated sediments (Dunn's, $p<0.01$) but did not differ significantly from those exposed to frozen or freeze-dried sediments.

Developmental Abnormalities

Newly hatched fry showed a varying numbers of developmental abnormalities between the different treatment groups (Table 3-1). These included kyphosis (curvature of the spine resulting in a humpback), scoliosis (lateral curvature of the spine), and lack of a pectoral fin. Fish in the reference group consistently showed a very high rate of the spinal deformities kyphosis and scoliosis. None of the fish in the reference comparison had developmental abnormalities.

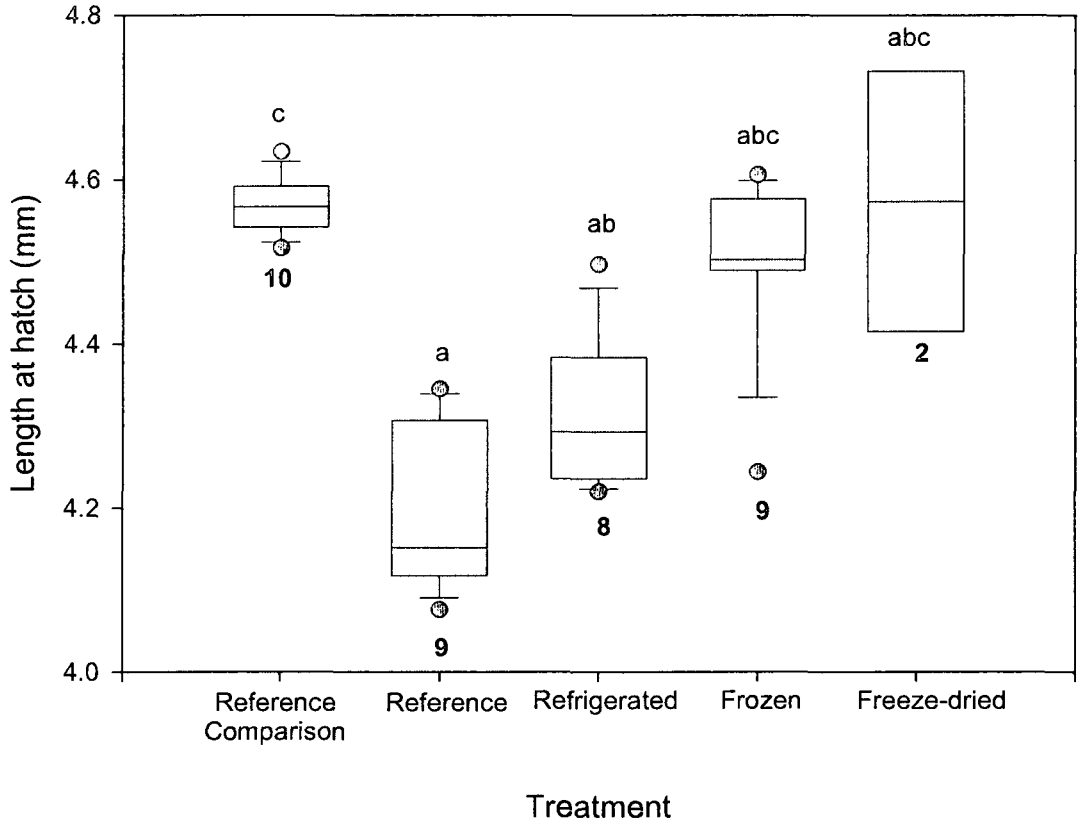


Figure 3-3. Length at hatching of Japanese medaka exposed to reference comparison, reference, refrigerated, frozen, and freeze-dried Wilmot River sediments. Refer to figure 3-2 for description of boxplot. Different letters indicate groups that differ significantly ($p<0.0001$)

Table 3-1. Total number of newly hatched Japanese medaka fry with developmental abnormalities after being exposed as eggs to Wilmot River sediments stored by various methods. The number of abnormalities was significantly different between groups ($G = 290.28$, $df = 4$, $p<0.05$).

Treatment	Number of replicates	Total number of eggs	Total number of successful hatches	Number of replicates with successful hatches	Total number of Developmental abnormalities
Reference Comparison	10	40	40	10	0
Reference	9	72	72	9	63
Fresh	9	72	45	8	3
Frozen	9	72	50	9	3
Freeze-dried	9	72	2	2	0

DISCUSSION

The first objective of this study was to determine if sediment exposure would affect any of the selected endpoints. In the present study, there were differences in the hatching success, time to hatch, and length at hatch between the reference (and reference comparison) and the refrigerated sediment. The results clearly indicate that exposure to sediments or light, affects development of Japanese medaka.

The second objective of this study was to test field-collected sediments for potential differences in toxicity between refrigerated, frozen, and freeze-dried sediments using the medaka embryolarval assays. Sediments for this experiment came from a site heavily impacted by agriculture on the Wilmot River, PEI. Jardine (2005) used this site for sediment toxicity experiments and showed consistently that sediments from this area were toxic to medaka embryos as suggested by a high incidence of severe developmental abnormalities in exposed fish. By comparing survivorship to hatch, time to hatch, length at hatch, and incidence of developmental abnormalities of medaka exposed to sediments stored by different methods, it is possible to document problems associated with storage methods.

Before discussing the results of this study, problems with the reference group must be reviewed. Over 85% of the fry in the original reference group hatched with a developmental abnormality (mainly spinal deformities) compared to 6.6%, 6%, and 0% in the refrigerated, frozen, and freeze-dried exposures respectively. Lall and Lewis-McCrea (2007) reviewed a variety of factors that can contribute to skeletal disorders in larval and juvenile fish. Biotic factors (nutrient deficiencies, stocking density, parasites, and

infections) as well abiotic factors (light intensity, pH, carbon dioxide, oxygen, temperature, radiation, and salinity) can affect the prevalence of skeletal abnormalities in fish. In the present study salinity, temperature, and oxygen levels were ruled out (through experimentation) as potential causes of deformities. After decreasing the light intensity in the incubator, the incidence of spinal deformities in the reference groups decreased to 0%. It is likely that the embryos in the sediment groups would have been protected from the intensity of the light and its radiation as the eggs in these groups were partially covered in sediment. While this problem made it difficult to establish the toxicity of sediment, it provides an interesting avenue of study in future investigations. It is for this reason that a reference group from a subsequent experiment was used to demonstrate potential toxicity of sediments from the Wilmot River subjected to different storage methods and exposed to Japanese medaka embryos.

While there have been other studies looking at the effects of storage method on contaminated sediments, effects of sediment storage methods on fish development have not been assessed extensively. The endpoints used here, survivorship to hatch, time to hatch, length at hatch, and incidence of developmental abnormalities have been used extensively to document toxicity of contaminants in developing fishes (Hallare *et al.* 2005, Rhodes *et al.* 2005, and Teather *et al.* 2000). Thus, I am confident that differences in toxicity, if they occurred, could be detected using these endpoints.

There were no differences in the hatching success, time to hatch, and length at hatch of embryos exposed to refrigerated and frozen sediments. This is unexpected as most studies have shown that freezing as a storage method alters sediment toxicity. In

other storage studies sediment has been spiked with a known toxicant, and therefore differences in toxicity between refrigerated and frozen sediments were more evident when present. For example, Schuytema *et al.* (1989) spiked sediment with both DDT and endrin and then froze them at – 20 °C. When the amphipod *Hyalella azteca* was exposed to frozen stored sediments it exhibited decreased survivorship compared to individuals exposed to refrigerated sediments. Further, Beiras *et al.* (1998) found that survivorship decreased when Japanese oyster (*Crassostrea gigas*) embryos were exposed to sediments which were stored at – 20 °C or in liquid nitrogen (- 196 °C) compared to sediment placed in a refrigerator before use. Other studies have found no differences in the toxicity of sediments after they had been frozen. Dillon *et al.* (1994) studied the effects of storage temperature and time on sediment toxicity using young mysid shrimp (*Mysidopsis bahia*). They found that after storing sediments at – 20 °C there was little change in toxicity to shrimp that were exposed to thawed sediments at intervals over 20 weeks. Similarly, Stenberg *et al.* (1998) studied the degradation rate of linuron in both frozen and refrigerated sediments. They found that over a 13 month period the linuron concentration in sediments stored at -20 °C did not significantly degrade. Stenberg *et al.* (1994) suggested that sediments which are home to microorganisms that have adapted to climates with freeze and thaw cycles will be more resistant to storage at -20 °C.

Freeze-drying greatly reduced hatching successs and delayed the hatching of medaka embryos. Freeze-drying has been consistently found to increase the toxicity of sediments when tested with invertebrates (Geffard *et al.* 2004a. Geffard *et al.* 2004b, Geffard *et al.* 2002, Beiras and His 1995). Geffard *et al.* (2004b) exposed Japanese

oyster larvae to refrigerated, frozen, and freeze-dried sediments and found that both freezing and freeze-drying considerably increased the toxicity of decanted sediments and their elutriates when compared to the toxicity of refrigerated sediments. This was shown by the increased incidence of abnormal larvae with the freeze-dried and frozen stored sediments. Beiras and His (1995) found that while the fresh sediments were not toxic, freeze-dried sediments were highly toxic to Japanese oyster embryos and were associated with reduced survivorship at the highest concentrations and abnormal development at lower concentrations. Beiras and His (1998) subsequently found that freeze-drying generally results in higher levels of dissolved and particulate organic carbon (DOC and POC) and of total ammonia. In addition, freeze-drying may kill microorganisms that naturally control ammonia production. Geffard *et al.* (2002) found that although ammonia concentrations rarely exceeded the toxicity threshold, an increase in ammonia concentration after freeze-drying may have resulted in synergistic effects with other contaminants. Another possible cause of increased toxicity is the reaction to freezing with liquid nitrogen. Freezing with liquid nitrogen (- 196 °C) has been found to dramatically distort the vertical profile of sediments (Rutledge and Fleeger 1988). This can alter the equilibrium between the sediment and associated chemicals in a way that releases toxic compounds previously bound to the particles making them bioavailable. Freeze-drying sediment may also have higher potential for causing cell rupture in microorganisms as freezing with liquid nitrogen occurs quickly. In the present study there were many small invertebrates such as chironomids found in the fresh sediments; therefore it is possible that freeze-drying ruptured cells, releasing potential contaminants

into the sample.

Similar to the present study, Hallare *et al.* (2005) placed zebrafish (*Danio rerio*) eggs on field collected sediments (mixed with varying amounts of artificial sediment) to evaluate the degree of sediment contamination. Developmental parameters such as survivorship to hatch, skeletal abnormalities, and time to hatch were also used as endpoints in their study. As the zebrafish embryos were exposed to increasing concentrations of sediments, increased mortality and increased incidence of developmental abnormalities were observed compared to the references. Premature hatching was observed in a few cases.

According to the findings in this study, freezing sediments for short periods of time, in this case two days, does not affect their toxicity to Japanese medaka eggs. If it is necessary to store sediments for long periods of time, further studies should be done to assess the potential impact of storage time. Freeze-drying sediments as a storage method is not recommended as it greatly increased the toxicity to medaka. If sediments do need to be stored for longer periods of time, freezing as opposed to freeze-drying is recommended. In addition, pesticide spiked references should be frozen along with the samples in order to determine if decreases in pesticide concentrations occur during frozen storage.

CHAPTER 4:

COMPARING THE TOXICITY OF ETHANOL, METHANOL, AND ACETONE USING JAPANESE MEDAKA (*ORYZIAS LATIPES*) EMBRYOLARVAL BIOASSAYS

INTRODUCTION

The choice of appropriate solvents for water insoluble chemicals is an important consideration in toxicological studies (Hallare *et al.* 2006, Hutchinson *et al.* 2006, Okumura *et al.* 2001). Organic solvents assist in the solubilization and dispersion of contaminants into the test media prior to exposure of the test organism. Ideally, carrier solvents should not affect test results by directly affecting the test organism. However, if this situation does occur, it is important to recognize the impact on the results of the study. Because carrier solvents may have toxic effects on their own, many of the guidelines include the maximum allowable concentrations and species of solvents for use in the experimental systems. For example, the U.S. Environmental Protection Agency (1975) recommends maximum acceptable limits of 0.05% solvents for acute toxicity (adverse effects of a substance resulting from a single exposure or multiple exposures in a short period of time) tests and 0.01% solvents for chronic toxicity (adverse effects of repeated exposure often at low doses over a long period of time) tests, while the American Society for Testing and Materials (1996) recommends that no test solution should exceed 0.05%. Since these values have been derived from studies on algae, adult invertebrates, and adult fishes, they may not represent the test solvent concentrations most appropriate for studies using fish embryos. While other research has assessed the toxicity of various carrier solvents to vertebrate models (González-Doncel *et al.* 2008, Hallare *et al.* 2006, Oxendine *et al.* 2006, Versonnen *et al.* 2004) the present study was repeated using ethanol, methanol, and acetone exposure to medaka, using the methods to be used over the course of this research project.

In this study, acetone, ethanol, and methanol were selected because they are commonly used delivery systems for non-water soluble chemicals in aquatic bioassays. Ethanol is naturally produced by fruit or cereal fermentation. It is also soluble in both aqueous and lipid environments and can easily cross biological membranes (Lockwood *et al.* 2004). Acetone occurs naturally as a metabolic by-product of plants and animals and is released into the atmosphere by volcanoes and forest fires. It is an organic compound that is mainly used as solvent for fats, oils, waxes, resins, rubbers, plastics, pharmaceuticals, and rubber cements (Hallare *et al.* 2006). Methanol is an organic solvent widely used in a number of industries such as manufacture of formaldehyde, pesticides, plastic, and textiles. It is used as an antifreeze, solvent, fuel, and as a denaturant for ethyl alcohol (Hutchinson *et al.* 2006).

Since carrier solvents may have toxic effects of their own, it is necessary to evaluate responses of the test organism of choice to avoid misinterpretation of results in further studies. The objective of this study was to assess the toxicity of ethanol, methanol, and acetone on the development of Japanese medaka embryos. Results of this study are required to decide the most appropriate solvent to be used as a carrier solvent for sediment extracts in subsequent studies involving medaka.

METHODS

General details regarding the study organism and data analysis are provided in the General Methods section. Information specific to this investigation is provided below.

This experiment was completed in two stages. During the first stage, medaka eggs were exposed to varying concentrations of ethanol while during the second stage eggs were exposed, using the same methods, to acetone and methanol. Initially five replicates were set up over two consecutive days for ethanol concentrations of 0.0% (reference), 0.001%, 0.01%, 0.05%, and 1.5% v/v. Bernardin® 250 mL wide mouth mason jars and snap lids were used for all exposures. Each replicate consisted of 150 mL of embryo rearing medium and varying concentrations of ethanol (reagent grade absolute). The contents of each replicate were mixed thoroughly for one minute. Immediately before eggs were placed in treatments, 6 cm petri dishes were placed in the jars to facilitate observation of eggs. One egg was placed in each jar of each treatment. This process was repeated until each replicate of each treatment had five eggs. Prepared treatments with eggs were placed into an environmental chamber at 25 ± 2 °C under a 16:8 h light to dark photoperiod. This same procedure was subsequently repeated for methanol (HPLC grade) and acetone (distilled in glass). For methanol and acetone an additional concentration of 0.0001% v/v was added and exposures were set up over three days.

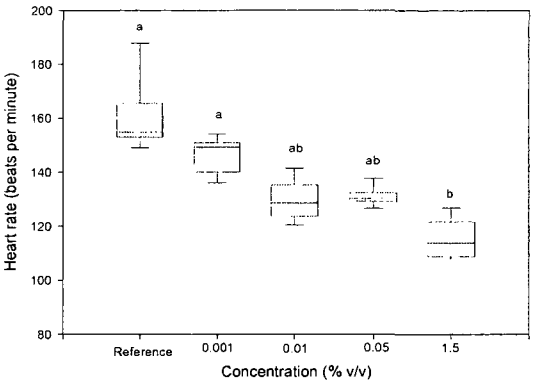
Eggs were checked daily for survivorship and hatching. On day five of each exposure, heart rate was calculated by determining the time required for the heart to beat 20 times and then converting this into beats/min. Newly hatched larvae were removed from jars using disposable polyethylene pipets. The notochord of each larva was measured to the nearest 0.1 mm and each was checked for developmental abnormalities.

RESULTS

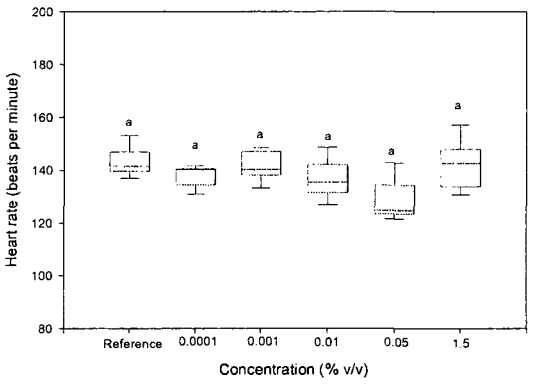
Heart Rate

There were significant differences in heart rates found among the different concentrations of each of the carrier solvents (Fig. 4-1). The 1.5% ethanol treated embryos had significantly lower heart rates than both the reference and 0.001% groups (Dunn's, $p<0.05$ for each). There were no significant differences found among those groups treated with different concentrations of methanol. Medaka exposed to 1.5% acetone had a significantly lower heart rates than those of the reference group (Dunn's, $p<0.05$). Notable differences were found when the carrier solvents were compared at each concentration (Fig. 4-2). At the 0.0001% and 0.001% v/v concentrations the acetone treated embryos had significantly slower heart rates than the reference (Tukey's HSD, $p<0.05$ for each). At 0.01% v/v both ethanol and acetone treated embryos had slower heart rates than the references (Tukey's HSD, $p<005$ for each). Once at the 0.05% and 1.5% v/v groups embryos treated with ethanol and acetone had significantly slower heart rates than the reference group (Tukey's HSD, $p<0.05$ for each), while the methanol treated embryos only had significantly lower heart rates than the reference group at 0.05% v/v (Tukey's HSD, $p<0.05$). The 0.001% v/v group was also analyzed by the non-parametric Kruskal-Wallis test as data were not normally distributed. In this case the groups treated with each of the solvents had significantly lower heart rates than the

A. Ethanol



B. Methanol



C. Acetone

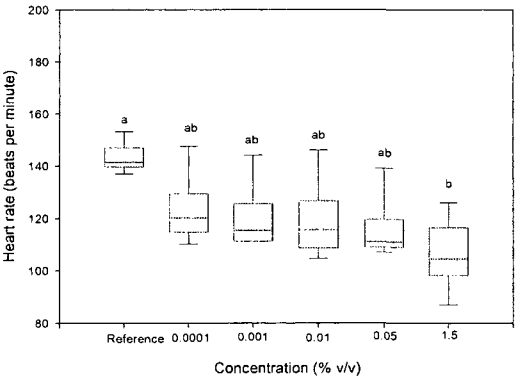
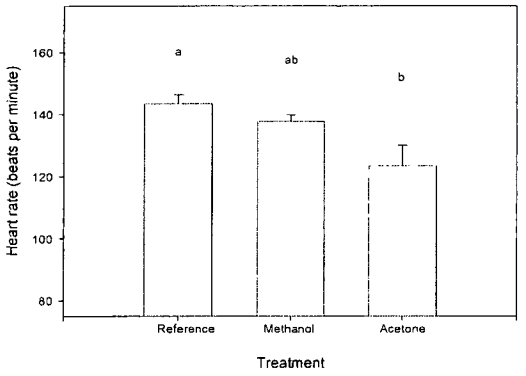
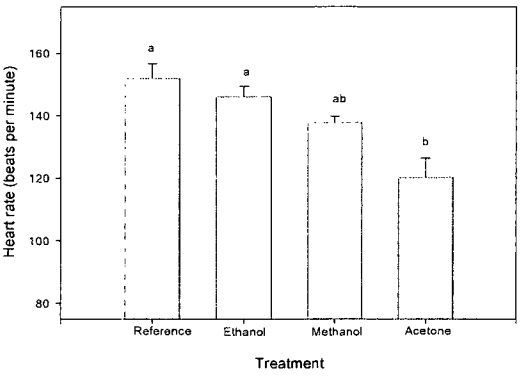


Figure 4-1. Heart rates of Japanese medaka embryos exposed to varying concentrations of A) ethanol, B) methanol, and C) acetone. Refer to figure 3-2 for description of boxplots. Different letters indicate groups that differ significantly ($p<0.05$).

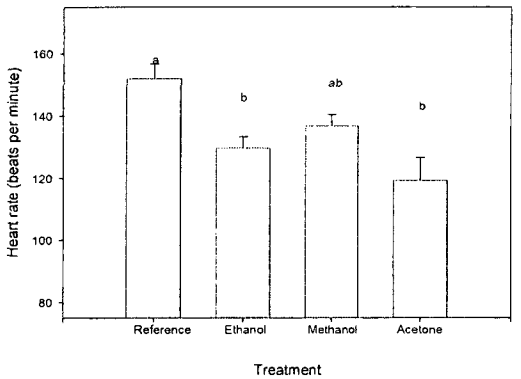
A. 0.0001% v/v



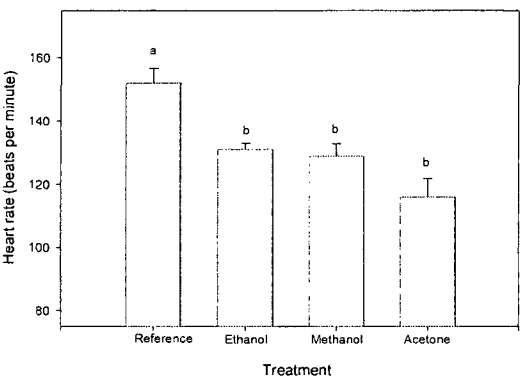
B. 0.001% v/v



C. 0.01% v/v



D. 0.05% v/v



E. 1.5% v/v

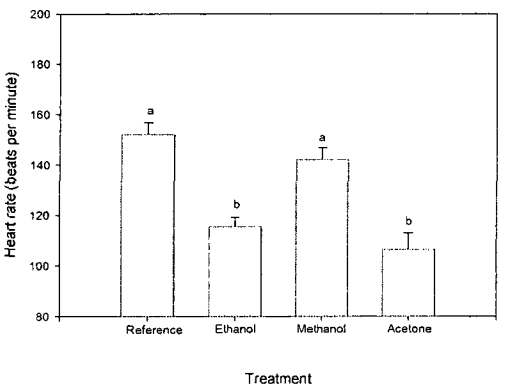


Figure 4-2. Comparison of Japanese medaka mean embryo heart rates (± SE) between three carrier solvents at varying concentrations. Different letters indicate groups that differ significantly ($p<0.05$).

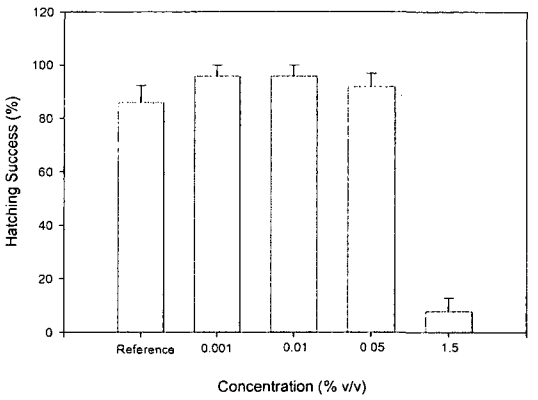
Hatching success

Hatching success varied between the different concentrations of each carrier solvents as well as between the different carrier solvents at the same concentrations (Fig. 4-3 and 4-4). There were significant differences found in the hatching success of medaka embryos in all three [ethanol ($G=12.5$, $df=5$, $p<0.05$), methanol ($G=16.9$, $df=6$, $p<0.05$), and acetone ($G=32.6$, $df=6$, $p<0.05$)]. Hatching success dropped dramatically at the 1.5 % treatment for both ethanol and acetone with only one individual surviving to hatch in the ethanol treated group and four surviving to hatch in the acetone treated group. When compared all three of the solvents at each concentration there were significant differences found in the hatching success of embryos at 0.001% v/v ($G = 9.6$, $df = 3$, $p<0.05$, 0.01% v/v ($G = 10.6$, $df = 3$, $p < 0.05$), 0.05 % v/v ($G = 8.9$, $df = 3$, $p<0.05$), and 1.5% v/v ($G = 32.6$ $df = 3$, $p<0.05$).

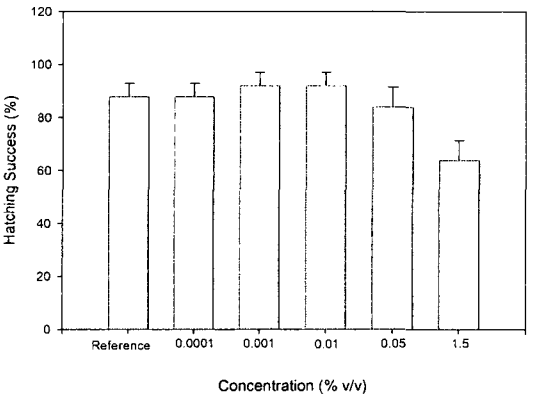
Time to hatch

Solvent concentration did not affect the time to hatch for any of the three solvents (Fig. 4-5). When responses to the solvents were compared at each concentration the only differences found occurred in the 1.5% v/v treated groups in which fry in both the ethanol and acetone treatments hatched significantly later than either the reference group or those treated with methanol (Fig. 4-6; Tukey's HSD, $p<0.05$ for each). This group was also analyzed with the non-parametric Kruskal-Wallis test (as the data were not normally distributed). This showed fry from the acetone treated group hatched significantly later than those in the reference group (Dunn's, $p<0.05$).

A. Ethanol



B. Methanol



C. Acetone

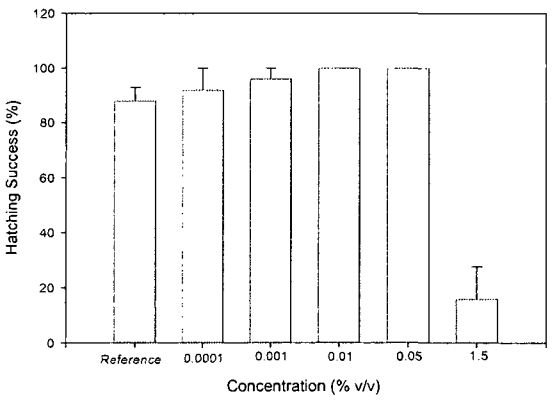
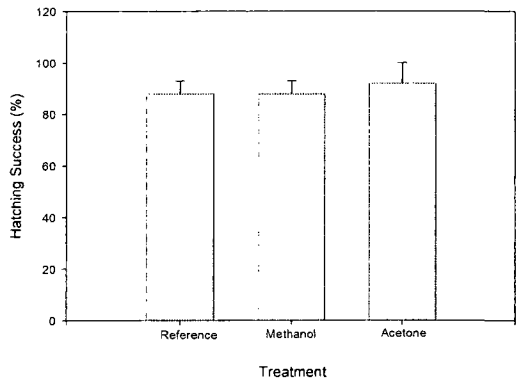
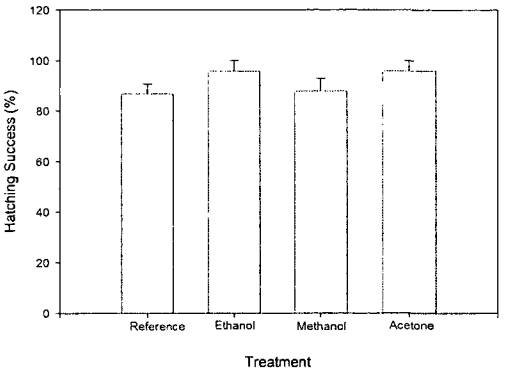


Figure 4-3. Mean hatching success (± SE) of Japanese medaka exposed to ethanol, methanol, and acetone. Significant differences were found in hatching success of embryos treated with varying concentrations of G=12.5, df=5, $p<0.05$), methanol (G=16.9, df=6, $p<0.05$), and acetone (G=32.6, df=6, $p<0.05$).

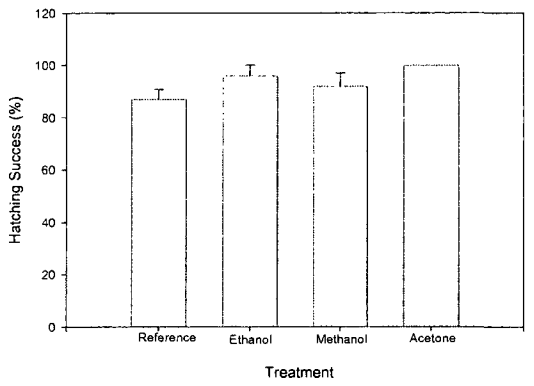
A. 0.0001% v/v



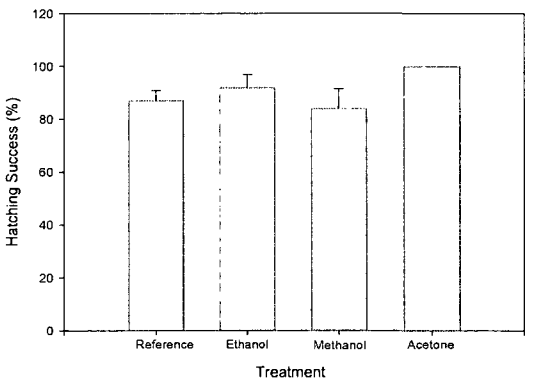
B. 0.001% v/v



C. 0.01% v/v



D. 0.05% v/v



E. 1.5% v/v

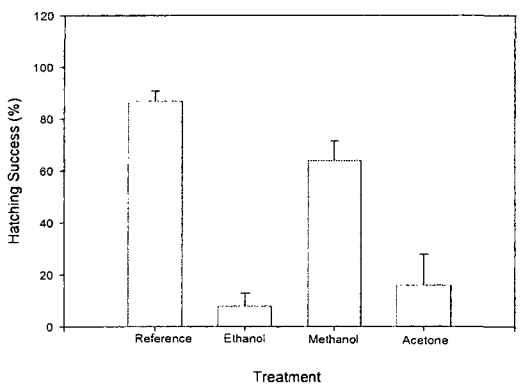


Figure 4-4. Comparison of mean hatching success (± SE) of Japanese medaka between three carrier solvents at varying concentrations. The 0.01% and 1.5% v/v treated groups showed significant differences in hatching success of embryos ($G = 10.6$, $df = 4$ and $G = 32.7$, $df = 4$, $p<0.05$), respectively).

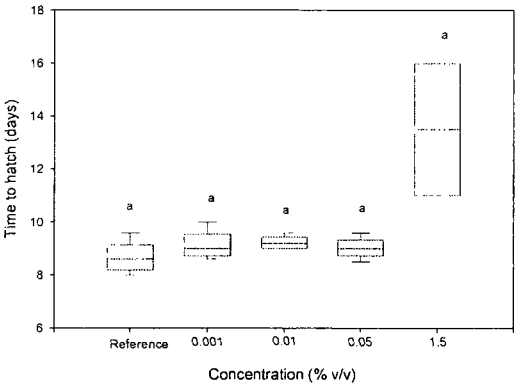
Length at hatch

Differences in length at hatching of larvae were found between embryos exposed to different concentrations of the three carrier solvents (Fig. 4-7). The fry in the ethanol reference were significantly larger at hatching than the 1.5% ethanol treated group (Dunn's, $p<0.05$). Fry from the 0.0001% and 0.001% methanol treatments were significantly larger at hatching than the 1.5% methanol treated group (Dunn's, $p<0.05$). There were no significant differences in hatching length found in the acetone treatments. All three carrier solvents were also compared at each concentration (Fig. 4-8). At 0.0001% v/v fry in the methanol treatment were significantly larger at hatching than those treated with acetone (Dunn's, $p<0.05$ for each). At 1.5% v/v fry from the reference group were significantly larger at hatching than those in the ethanol treatment (Dunn's, $p<0.05$).

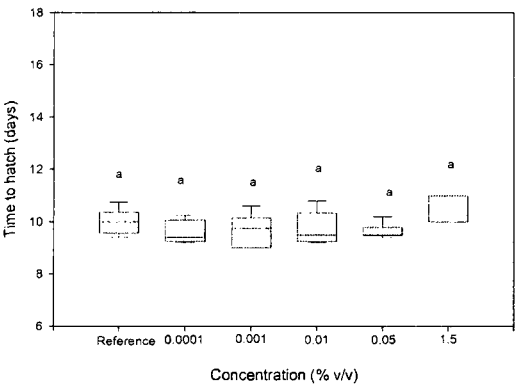
Developmental Abnormalities

Embryos in the reference group, ethanol, methanol, and acetone groups all showed differences in developmental abnormalities at hatching between concentrations (Table 4-1). Medaka in the reference group exhibited a high incidence of spinal deformities such as scoliosis and kyphosis. Embryos exposed to the carrier solvents predominantly

A. Ethanol



B. Methanol



C. Acetone

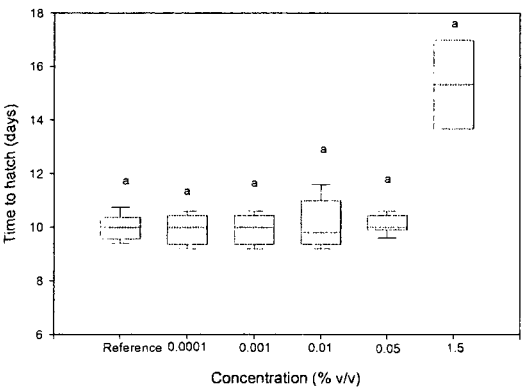
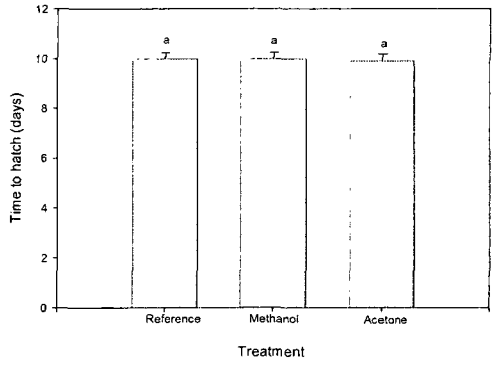
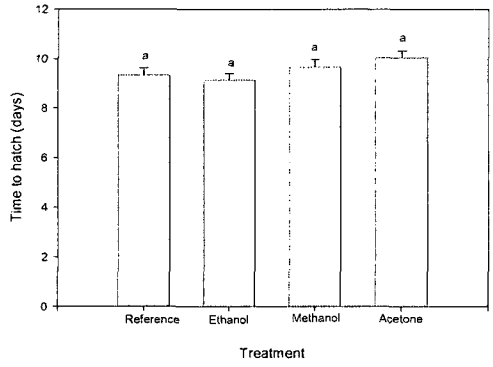


Figure 4-5. Mean time to hatch (± SE) of Japanese medaka exposed to a reference and varying concentrations of ethanol, methanol and acetone. Different letters indicate groups that differ significantly ($p<0.05$).

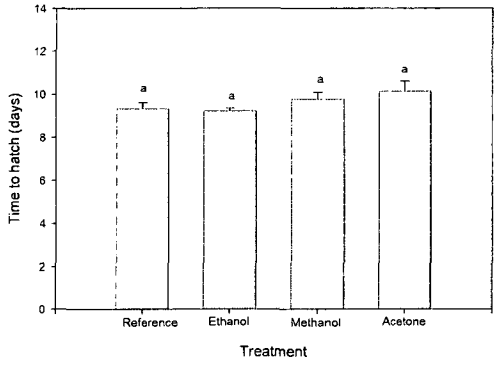
A. 0.0001% v/v



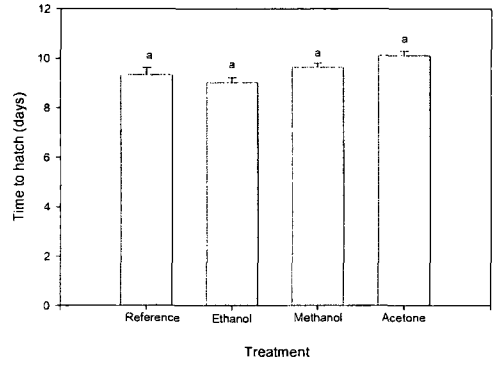
B. 0.001% v/v



C. 0.01% v/v



D. 0.05% v/v



E. 1.5% v/v

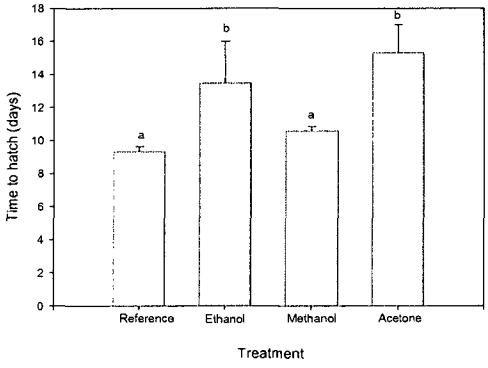
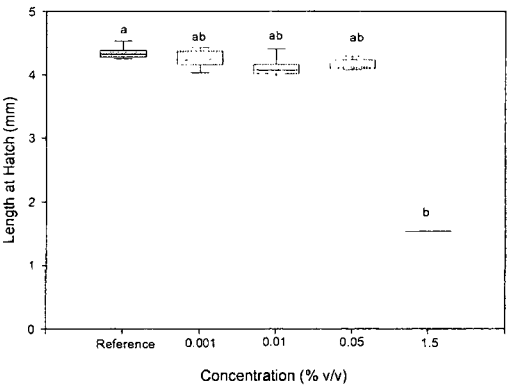
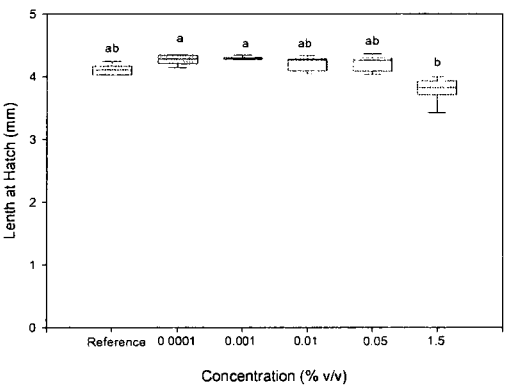


Figure 4-6. Comparison of the mean time to hatch (± SE) of Japanese medaka exposed to a reference and three carrier solvents at various concentrations. Different letters indicate groups that differ significantly ($p<0.05$).

A. Ethanol



B. Methanol



C. Acetone

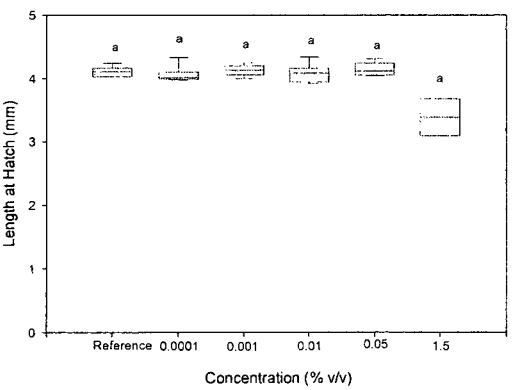
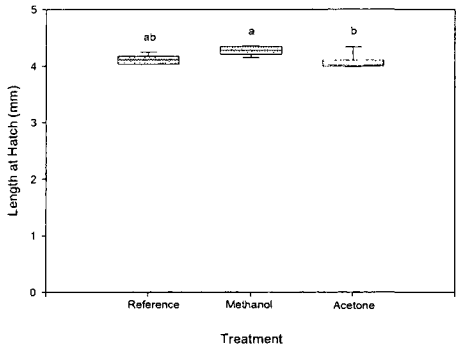
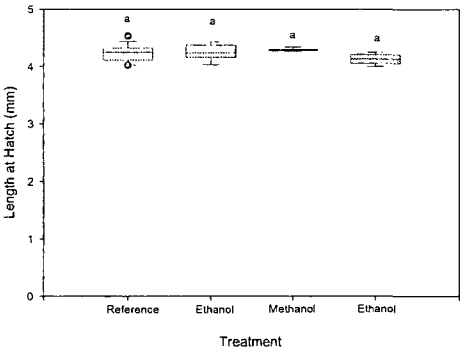


Figure 4-7. The length at hatch of Japanese medaka exposed to ethanol, methanol, and acetone. Refer to figure 3-2 for description of boxplots. Different letters indicate groups that differ significantly ($p<0.05$).

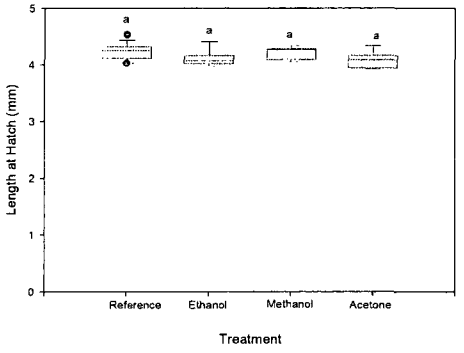
A. 0.0001% v/v



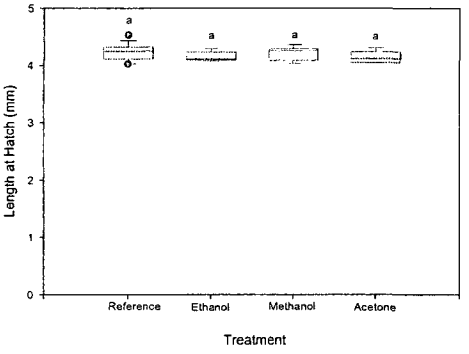
B. 0.001% v/v



C. 0.01% v/v



D. 0.05% v/v



E. 1.5% v/v

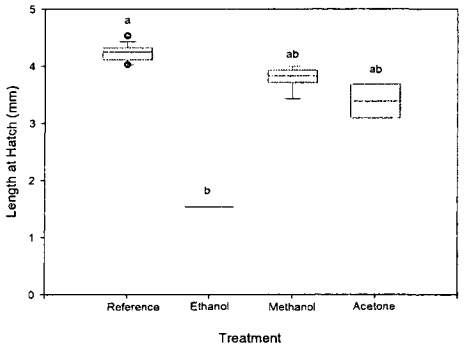


Figure 4-8. The hatching length of Japanese medaka exposed to a reference as well as ethanol, methanol, and acetone at varying concentrations. Refer to figure 3-2 for description of boxplots. Different letters indicate groups that differ significantly ($p<0.05$).

Table 4-1. The total number of developmental abnormalities in newly hatched Japanese medaka fry exposed to ethanol, methanol, and acetone as embryos. Significant differences were found between the different concentrations ethanol, methanol, and acetone ($G = 432.08$, $df = 4$, $G = 253.24$, $df = 5$, and $G = 672.35$, $df = 5$, respectively, $p < 0.05$).

Concentration (% v/v)	Total # of eggs	Total # successful hatched	# of Developmental abnormalities	% Developmental abnormalities
Ethanol				
Reference	27	23	7	30.4
0.001	25	24	12	50
0.01	25	24	19	79.2
0.05	25	23	14	60.9
1.5	25	1	1	100
Methanol				
Reference	25	22	4	18.2
0.0001	25	22	4	18.2
0.001	25	23	4	17.9
0.01	25	23	8	34.8
0.05	25	21	4	19.0
1.5	25	16	12	75
Acetone				
Reference	25	22	4	18.2
0.0001	25	23	21	91.3
0.001	25	24	20	83.3
0.01	25	25	16	64
0.05	25	25	14	56
1.5	25	4	4	100

showed spinal deformities upon hatching but also showed other abnormalities such as premature hatching, pericardial edema, partial hatching, yolk sac edema, missing pectoral fins, hemorrhages, and in one case a missing eye.

DISCUSSION

Many toxicants need to be dissolved in carrier solvents so that they can be effectively used in toxicological exposure studies. In the present study the effects of three common carrier solvents (ethanol, methanol, and acetone) to developing Japanese medaka embryos were assessed. A range of concentrations was used so that the maximum acceptable values for solvents recommended by the US Environmental Protection Agency and the American Society for Testing Materials were included. Medaka embryos exposed to varying concentrations of three carrier solvents exhibited various developmental problems during embryonic stages and at hatching. These included decreased heart rates, reduced hatching success, delayed time to hatch, decreased hatching size, and various developmental abnormalities.

The heart rate of medaka exposed to ethanol and acetone decreased with increasing concentration. Hallare *et al.* (2006) also found that the heart rate of zebrafish embryos decreased when exposed to ethanol and acetone. Embryonic circulation is essential in nutrient and oxygen delivery as well as removal of CO_2 and other metabolic wastes. The reason for reduced heart rates is not known. Disruption of the central nervous system and inhibition of acetylcholinesterase have been suspected to lead to a reduction in heart rate (Baratti *et al.* 1980). Decreased embryonic heart rates have been associated with decreased early growth and survival (von Westernhagen 1988).

Hatching success decreased at the highest concentration (1.5% v/v) for all three carrier solvents. The most drastic decrease was in the ethanol treated group where only one embryo (of 25) survived to hatch. Hallare *et al.* (2006) also found that there was a

significant reduction in survival in zebrafish embryos in the 1.5% v/v ethanol treated group but not the acetone treated group.

The only differences seen in the time to hatch in this study were when embryos were exposed to the highest concentration (1.5% v/v) of ethanol and acetone. Both these groups hatched significantly later than the reference group and the methanol treated embryos. Versonnen *et al.* (2004) found that zebrafish embryos in an ethanol solvent reference were found to hatch later than zebrafish in a chemical free reference group. Hallare *et al.* (2006) found that while zebrafish embryos exposed to ethanol took longer to hatch, those exposed to acetone did not take as long. In the present study the delay in hatching time is likely due to the initial delay in development that was observed at the highest concentrations of ethanol and acetone. Hatching time for references in the present study were similar to those found by Teather *et al.* (2000) who studied the early life-history parameters of Japanese medaka and found that 76% of larvae hatched between nine and fourteen days with a median hatching time of eleven days. Many other studies have found either delayed or advanced hatching times for larvae when exposed to various substances (Gormley and Teather 2003, Sano *et al.* 2005, Zha and Wang 2006a). For example, Zha *et al.* (2006a) found that after exposure to pentchlorophenol, adult medaka produced eggs that either hatched significantly earlier or later than the reference group depending on the concentration of the exposure. Differences in time of hatching could affect the long term survivorship of larvae. If exposed to chemicals that cause premature hatching, larvae could hatch before essential final stages of development have taken place. Alternatively hatching later could also affect larvae if others from the same cohort

were much larger. For example, midas cichlids (*Cichlasoma citrinellum*) show size differences in the first few weeks after hatching. By eight weeks some of the fish within the brood are large enough to prey on the smaller individuals (Valerio and Barlow 1986). Fry in the highest concentration (1.5% v/v) of both ethanol and methanol were significantly smaller than the reference at hatching. Oxendine *et al.* (2006) studied the effects of ethanol on Japanese medaka embryos and also found that embryos had decreased body length when exposed to ethanol concentrations of 0.1, 0.5, and 1.0% v/v.

In the present study the high incidence of developmental abnormalities in fry from the higher concentrations could explain the decrease in hatching length. The decreased length could also be due to significantly decreased heart rates at the higher concentrations that could decrease circulation of essential oxygen and nutrients which could contribute to decreases in embryo growth (von Westernhagen 1988). Alternatively, Johnson and Sloman (2007) suggest decreased length could be due to embryos shifting energy requirements due to responding to the toxic stress and could divert energy away from growth.

While a high number of spinal abnormalities arose in the reference group, a higher rate of various developmental abnormalities arose in embryos exposed to high concentrations of all three carrier solvents. These included spinal deformities, pericardial edema, missing pectoral fins, yolk sac edema, cranial malformations, and hemorrhages. These abnormalities can result in impaired growth and may affect their survivorship (Hilomen-Garcia 1997, Koumoundouros *et al.* 1997). Other studies have also found developmental abnormalities in fish exposed to carrier solvents including reduction in

pigmentation, pericardial edema, microphthalmia (small pointed eyes), cyclopia (eyes very close or attached), and malformed bodies (González-Doncel *et al.* 2008, Hallare *et al.* 2006).

Based on the results of this study, methanol was the least toxic to developing Japanese medaka embryos followed by acetone and then ethanol. The time to hatch for all methanol treated groups was not significantly affected and fry hatched with fewer developmental abnormalities than in the ethanol and acetone groups. González-Doncel *et al.* (2008) found similar patterns when studying sensitivity of the Japanese medaka early life stage bioassays to solvents. They found that methanol only induced delayed hatch and high rate of abnormalities at 2.0% v/v while ethanol produced high rates of abnormalities at 1.0% v/v and high incidence of mortality at 1.5% v/v. They concluded that among the three solvents, ethanol induced the most severe effects in newly hatched fry. The results from the present study suggest that methanol is the most appropriate carrier solvent to use for sediment extraction and subsequent extract exposures with Japanese medaka. The maximum concentration of 0.05% v/v recommended by the U.S. Environmental Protection Agency and the American Society for Testing and Materials was confirmed.

CHAPTER 5:

COMPARING THE TOXICITY OF WHOLE SEDIMENTS AND SEDIMENT EXTRACTS FROM TWO SITES IN AN AGRICULTURALLY IMPACTED RIVER USING JAPANESE MEDAKA (*ORYZIAS LATIPES*) EMBRYOLARVAL BIOASSAYS.

INTRODUCTION

Contamination of waters and sediments from agricultural activity is a consistent problem on Prince Edward Island (Mutch *et al.* 2002). Agricultural contaminants that enter streams through agricultural runoff or spray drift include pesticides, agriculturally related metals, and high levels of nutrients. Pesticides vary in their affinity to bind to sediments and in their toxicity to fishes. Some pesticides that have been commonly used on the Island such as endosulfan and azinphos-methyl can be toxic to fish even at very low concentrations. The effects of pesticides were especially evident after a series of fish kill events on PEI between 1999 and 2002 when 22 fish kill events occurred (Gormley *et al.* 2005, Mutch *et al.* 2002). Sublethal effects of pesticides are less obvious and may be manifested in reduced growth, decreased mobility, changes in hatching time, reduced reproductive ability, and increased number of developmental abnormalities. The eventual outcome of such effects is likely to be an overall decline in the population health of many local fish species.

Metals such as copper, zinc, and cadmium are also commonly used in agricultural practices. The sources of such metals include fertilizers, pesticides, livestock feed, livestock manure, and livestock medicine (de Vries *et al.* 2002). Metals can also affect fish by causing abnormal development, variation in hatching time, and reduced size (e.g. Brauner and Wood 2002, Dave and Xiu 1991, and Weis and Weis 1977).

Japanese medaka embryos exposed to Wilmot River sediments in a 2005 study (Jardine 2005) exhibited reduced hatching success and other sublethal responses such as decreased hatching length and increased incidence of developmental abnormalities. Since

that time, legislation that had been implemented shortly before the 2005 study have been strengthened and more vigorously enforced. This legislation prohibits planting an agricultural crop within 10 m - 90 m of a watercourse or designated wetland depending on the slope of the field and other factors such as type of agricultural activity. In 2002 there were amendments to this legislation affecting a winter cover time limit. In recent years seed piece treatments have also become a more popular method of applying pesticides, further reducing the risk of being transported to surface waters. Because of the new regulations concerning farming practices and the introduction of some new lower risk pesticides, the toxicity of Wilmot River sediments was once again investigated using Japanese medaka embryolarval bioassays in order to determine whether sediment toxicity has decreased.

The first objective of this study was to compare the toxicity of a downstream site (S2) surrounded by agriculture with the upstream spring site (S1) surrounded by forest. It was predicted that sediment collected from the impacted downstream site would contain more contaminants than the sediment collected from the upstream site. Embryos were also exposed to extracts of the upstream sediment, the downstream sediment, and from the sediment collected during the 2005 study to assess the effects of contaminants which may not be bioavailable when bound to the sediments.

The second objective was to compare the rate of developmental abnormalities from the downstream site (S2) with results from a previous study done on the sediments of the Wilmot River in 2004 (Jardine 2005). The most reliable endpoint in the previous study was consistently high numbers of developmental abnormalities in the

eggs exposed to sediments in areas with a high percentage of agriculture. In 2000, more than 77% of the land in the Wilmot River watershed was used for agriculture, with 36% of this being used for potato production (determined using MapInfo Professional Version 6.5 from 2000 Corporate Resource Inventory GIS layer obtained from the PEI Dept. of Environment, Energy, and Forestry). This comparison will help determine if new agricultural regulations and practices have reduced the amount and type of contaminants entering the Wilmot River. Recently acquired sediments (2007) from the Wilmot River were expected to be less toxic to Japanese medaka than those in the 2004 study. The present study involved pesticide and metals analysis of both the 2004 sediments and the 2007 sediments.

METHODS

General details regarding the study organism, site selection, and data analysis from this study are provided in the General Methods section. Information specific to this investigation is provided below.

Site Selection

Two sites were selected on the Wilmot River, Prince Edward Island. The first site (S1) was near the source of the river and surrounded by forest (46.42625° x 63.60187°). Sediments from this site would be expected to have been less impacted by agricultural activity (with the possible exception of high nitrate levels entering through the springs). The second site (S2) was located above Arsenault's Pond and was the site used in a previous study of sediment toxicity (Jardine 2005) and is surrounded by agricultural fields (46.40282° x 63.60981°).

Sediment Collection, Processing, and Storage

Sediments were collected from the Wilmot River on April 26, 2007. Sediments were rinsed through a 2 mm sieve with river water before transferring them to three 1 L glass jars. Upon return to the laboratory, water was decanted off the top of the sediment from each jar. Five replicates of 1 g of wet sediment were placed in an incubator at 70 °C to determine their dry weight. Sediments were then refrigerated at 4 ± 2 °C until the next day when the egg exposures began.

Sediments from the Jardine (2005) study were collected on July 24 2004. Sediment was collected and placed in 1L glass jars and placed in a cooler on ice. It was then transported back to the laboratory and rinsed through a 2 mm sieve with distilled

water. At this time sediment was refrigerated until use (within one week of collection).

Whole Sediment Exposures

Japanese medaka eggs were exposed to sediments using a static non-renewal bioassay. Ten replicates were used for whole sediment assays using sediment collected from the S1 and S2 sites plus a sediment-free reference. Procedures for set-up were identical for all replicates. Each replicate in the sediment exposure used 30 g dry weight of sediment and 150 mL of embryo rearing medium combined in Bernardin® 250 mL wide mouth mason jars, while the reference group was exposed to150 mL of embryo rearing medium only. All treatments were stirred for 1 minute and then left at room temperature for 4 h. The majority of the larger sediment particles settled out after this time, leaving only fine particles to come in contact with the eggs. Before placing eggs in each of the jars, petri dishes (diameter = 6 cm) were gently placed on the sediment. These were used to reduce predation of the eggs by invertebrates in the sediments and to facilitate easy observation of the eggs under a dissecting microscope. One egg was placed in each jar of each treatment. This process was then repeated four times until each jar had four eggs. All jars were then placed into an environmental chamber at 25 ± 2 °C under a 16:8 h light to dark photoperiod. Eggs were checked daily for survivorship and hatching. Newly hatched larvae were removed from the test jars daily using disposable polyethylene pipets. The notochords of all larvae were measured to the nearest 0.1 mm and each was checked for developmental.

Jardine used similar methods when setting up whole sediment exposures with the exception of the fact that assays were set up over a period of 5 days. This was due to egg

availability.

Sediment Extraction

Many agricultural contaminants are not water soluble and bind tightly to sediments making them unavailable to organisms in the surrounding water column. Soxhlet extraction of sediment removes contaminants bound to the sediments by using an organic solvent. Sediment extracts were done to allow analysis by liquid chromatography/mass spectrometry for pesticides and for further embryolarval exposures. Sediment was frozen using liquid nitrogen and then placed on a freeze-drying apparatus for 48 h. All glassware and sodium sulfate was muffled (450 °C) before use. Three replicates of 20 g of freeze-dried sediment mixed with sodium sulfate were placed on a Soxhlet extraction apparatus. Dichloromethane (150 mL) was added to flasks (part of the extraction apparatus) along with Teflon boiling chips. The dichloromethane was then heated to boiling and let cycle for 24 h. After 24 h the dichloromethane was transferred into 200 mL turbovap tubes and evaporated to 1 mL using a TurboVap® II concentrator. Methanol (150 mL) was added to the flasks and let cycle for 24 h. The methanol was added to the turbovap tubes and evaporated down to 1 mL. The sides of the tubes were rinsed with 4-5 mL of methanol and the contents vacuum filtered. Whatman 47 mm glass microfibre filters were triple rinse with methanol before the sample was added. After filtration, samples were placed in methanol rinsed 5 mL volumetric flasks. Samples were evaporated with nitrogen gas to 5 mL. A 1 mL aliquot was removed from each concentrated sample and placed in 2 mL autosampler vials for pesticide analysis. The remaining 4 mL (48 g dry weight equivalent) of each sample was evaporated dry before

the addition of 0.5 mL of methanol (distilled in glass).

Extract Exposures

To assess the toxicity of the sediment extracts a 14 day static renewal embryolarval bioassay with Japanese medaka was used. Serial dilutions of the extracts were done to assess four concentrations - 48 mg/mL, 4.8 mg/mL, 0.48 mg/mL, and 0.048 mg/mL dry weight equivalent of sediment. A solvent reference with 0.05% methanol and a reference with embryo rearing medium were also used. For each treatment there were three replicates with five eggs in each. Five mL autosampler vials were used. Each vial contained 5 mL of embryo rearing medium and 2.5 μl of the assigned concentration of each extract.

Eggs were checked daily for survivorship and hatching. Newly hatched fry were removed from the test jars daily using disposable polyethylene pipets, measured to the nearest 0.1 mm, and checked for developmental abnormalities.

Pesticide Analysis

Aliquots of 1 mL of extract representing an equivalent of 12 g dry weight of sediment were sent to The Aquatic Ecosystem Protection Research Branch at The National Water Research Institute in Burlington, Ontario for pesticide analysis by liquid chromatography/mass spectrometry (LC/MS). Each sample was filtered through a 0.2 μm syringe filter prior to analysis. Samples were spiked with 100 μl of 13C Atrazine as an internal standard and then analyzed by a Sciex API2000 (MDS Sciex, Concord, ON) LC-MS-MS system equipped with an atomospheric pressure photoionization (APPI, PhotoSpray) source and an Agilent 1100 HPLC (Agilent, Mississauga, ON). Sample

extracts were injected into a reverse phase column (Waters Symmetry C18, 3.5 μm, 100 mm x 2.1 mm). The solvent elution profile was 50/50 water/methanol to 0/100 water/methanol in 4 min and held for 3 min.

Metals Analysis

Sediments were also analyzed for cadmium, copper, and zinc using a modified version of EPA method 3050B Acid Digestion of Sediments, Sludges, and Soils (U.S. EPA 1996). An equivalent of 1 g dry weight of sediment was weighed out for the Wilmot River July 2004, Wilmot River April 2007, and Wilmot Spring samples as well as three quality control samples of TORT (lobster hepatopancreas reference materials for trace metals), bovine liver standard reference material and deionized water were used. Each sample was combined with 10 mL of 1:1 HNO_3 in 50 mL digestion tubes, covered with watch glasses and heated to 95°C for 15 minutes without boiling. Samples were then cooled and 5 mL of concentrated HNO_3 was added before being heated again for 30 minutes. If there was no sign of oxidation after 30 minutes, samples were left on the heat source for an additional 2 h. Samples were then cooled and 2 mL of H_2O and 3 mL of 30% H_2O_2 were added. Samples were returned to the heat source to start the peroxide reaction and were left for 2 h (without boiling). After 2 h the samples were cooled and 50 mL of deionized H_2O was added. Samples to be analyzed by graphite furnace atomic absorption (GFAA) were shaken and 1.5 mL aliquots were centrifuged at 3,000 rpm for 10 minute to clear the supernatant. Samples to be analyzed by flame atomic absorption (FAA) had 10 mL concentrated HCl added to them and were then heated to 95°C for 15 minutes without boiling. The FAA samples were then refrigerated until analysis; since

samples had settled out, aliquots were not centrifuged. All samples were analyzed with a Perkin-Elmer Aanalyst 800 Atomic Absorption Spectrometer.

RESULTS

Pesticide Analysis

A summary of LC/MS analysis for pesticides can be found in Table 5-1. Concentrations of metribuzin, metobromuron, and linuron were all higher in the S2 2005 sample while concentrations of imidacloprid and metalaxyl were higher in the S1 site from 2007. Concentrations of azinphos-methyl remained constant between all the samples.

Metals Analysis

Results from metals analysis are summarized in Table 5-2. Concentrations of zinc, copper, and cadmium were comparable between all the samples.

Whole Sediment Embryolarval Bioassays

Hatching success

There were significant differences in the hatching success of larvae from the different treatments (Fig. 5-1) ($G = 46.0$, $df = 2$, $p<0.05$). The reference group from the 2007 exposures showed the highest success with 100% of the embryos hatching while the S2 sample from 2007 showed the lowest hatching success with only 55% surviving to hatch.

Time to hatch

There were significant differences found in the hatching time of embryos exposed to the different treatments (Fig. 5-2). Fry in the S1 and S2 sediment treated groups hatched significantly later then the reference group (Tukey's, $p<0.001$).

Table 5-1. Results from pesticide analysis by liquid chromatography/mass spectrometry (LC/MS) on Wilmot River sediment extracts from above Arsenault's Pond (S2), and a spring on the Wilmot River (S1).

Pesticide	S2 2005 (pg/g dry weight)	S2 2007 (pg/g dry weight)	S1 2007 (pg/g dry weight)
Propamocarb	No Peak	No Peak	No Peak
Imidacloprid	153	193	450
Metribuzin	60	31	33
Metobromuron	148	60	26
Metalaxyl	8	13	27
Carbaryl	<0	No Peak	No Peak
Azinphos methyl	6	6	4
Linuron	2225	401	73

Table 5-2. Metal concentrations in sediments collected from above Arsenault's Pond (S2) and from a spring (S1) on the Wilmot River. The % RSD was ≤5% for all samples.

Sample	Metal concentration (mg/g dry weight)
Zinc (Zn 213.9)	
S2 2004	3.34×10^{-2}
S2 2007	3.10×10^{-2}
S1 2007	2.59×10^{-2}
Copper (Cu 324.8)	
S2 2004	7.60×10^{-3}
S2 2007	7.60×10^{-3}
S1 2007	5.50×10^{-3}
Cadmium (Cd 228.8)	
S2 2004	7.23×10^{-5}
S2 2007	7.86×10^{-5}
S1 2007	6.16×10^{-5}

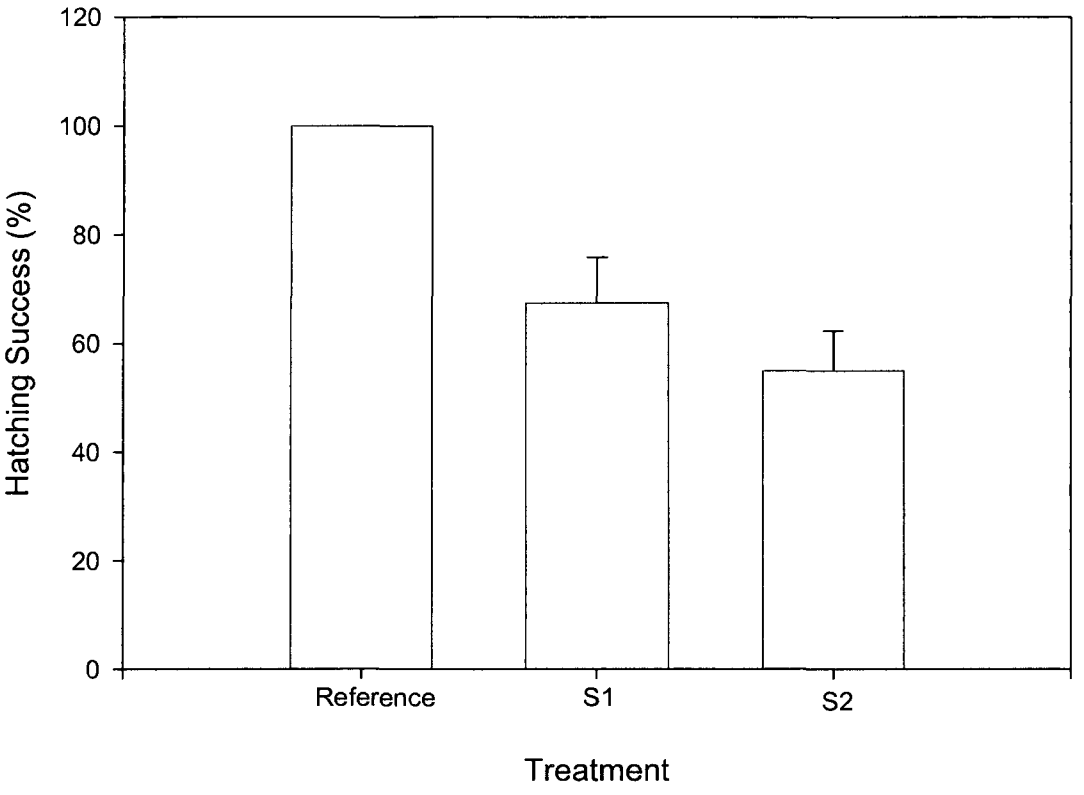


Figure 5-1. Mean hatching success (± SE) of Japanese medaka embryos exposed to Wilmot River sediments from a spring on the Wilmot River (S1) and from above Arsenault's Pond (S2). There were significant differences in the hatching success of fry from the different treatments ($G = 46.0$, $df = 2$, $p < 0.05$).

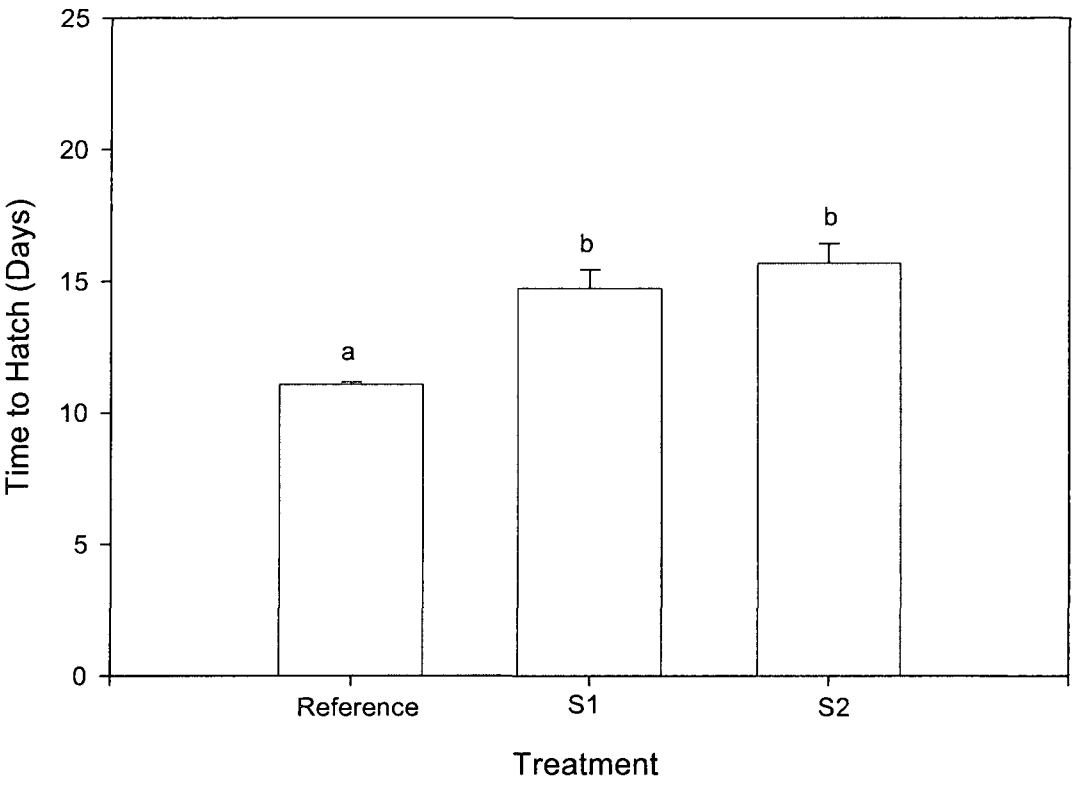


Figure 5-2. Mean hatching time (± SE) of Japanese medaka exposed to Wilmot River sediments from a spring on the Wilmot River (S1) and from above Arsenault's Pond (S2). Different letters indicate groups that differ significantly ($p<0.001$).

Length at hatch

There were significant differences found in the length at hatch of fry in the different treatments (Fig. 5-3). Fry from the group exposed to the agriculturally impacted site (S2) were significantly smaller than those in the reference group and those exposed to sediment from the spring (S1) (Tukey's, $p<0.05$).

Developmental abnormalities

There were differences found in the incidence of developmental abnormalities between the treatments. Larvae from all the treatments except the S1 group showed at least one developmental abnormality (Table 5-3). Embryos exposed to S2 sediments from the 2005 study (Jardine) showed the highest incidence of abnormalities upon hatching with 26 of 28 larvae having at least one developmental problem.

Wilmot River Sediment Extract Embryolarval Bioassays

Hatching Success

There were no significant differences in the hatching success of larvae between the different treatments (Fig. 5-4) ($G = -21.17$, $df = 13$, $p< 0.05$) or within the treatments (48 mg/mL: $G = -7.14$, $df = 4$, $p<0.05$, 4/8 mg/mL: $g = -10.71$, $df = 4$, $p<0.05$, 0.48 mg/mL: $G = -5.36$, $df = 4$, $p<0.05$, and 0.048 mg/mL: $G = -7.92$, $df = 4$, $p<0.05$).

Time to Hatch

There were no significant differences found in the time to hatch of larvae between any of the treatment groups (multiple Mann-Whitney U Tests, $p>0.05$ for each) (Fig. 5-5). There were also no differences found in the time to hatch of embryos exposed to extracts from different sites or years (multiple Mann-Whitney U Tests, $p>0.05$ for each).

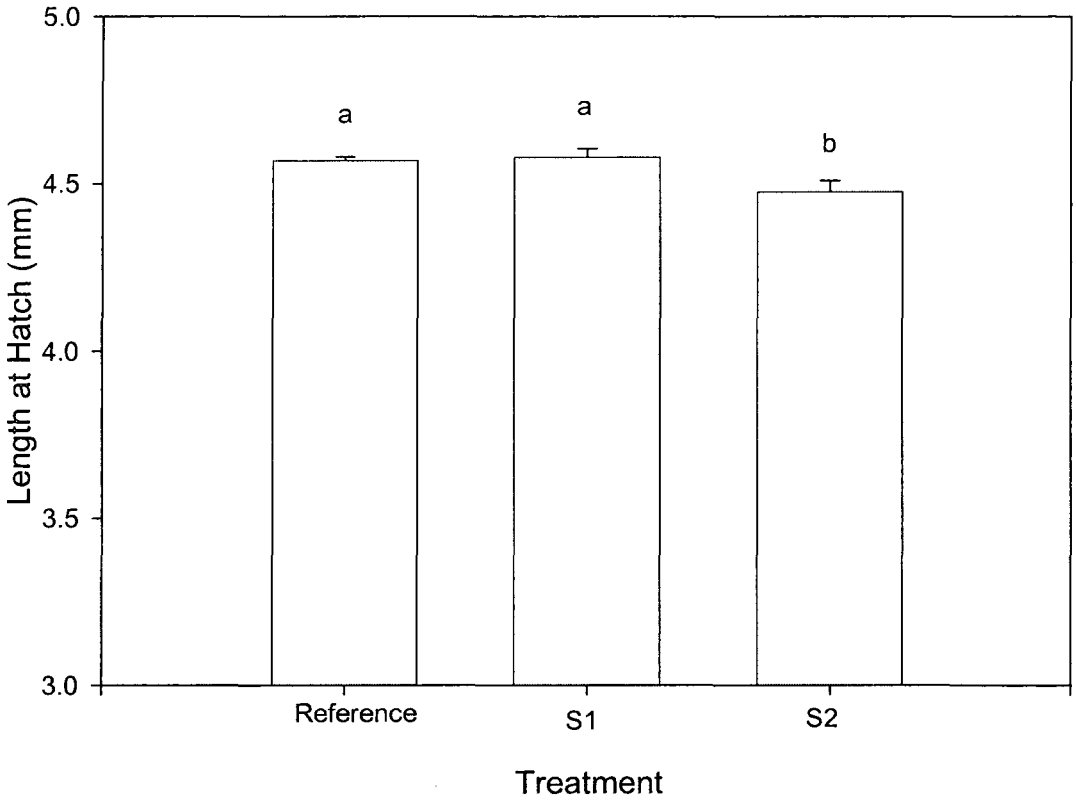


Figure 5-3. Mean hatching length (± SE) of Japanese medaka exposed to Wilmot River sediments from a spring on the Wilmot River (S1) and from above Arsenault's Pond (S2). Different letters indicate groups that differ significantly ($p<0.05$).

Table 5-3. Total number of newly hatched fry with developmental abnormalities after being exposed as eggs to three different treatments of Wilmot River sediments. Significant differences in the incidence of developmental abnormalities were found between the treatments ($G = 254.44$, $df = 4$, $p < 0.05$).

Treatment	Number of replicates	Total number of eggs	Total number of successful hatches	Total number of Developmental abnormalities	Percentage of Developmental abnormalities
Reference 2005	12	42	26	2	7.8
S2 2005	12	53	28	26	92.9
Reference 2007	10	40	40	1	2.5
S2 2007	10	40	22	2	9.1
S1 2007	10	40	27	0	0

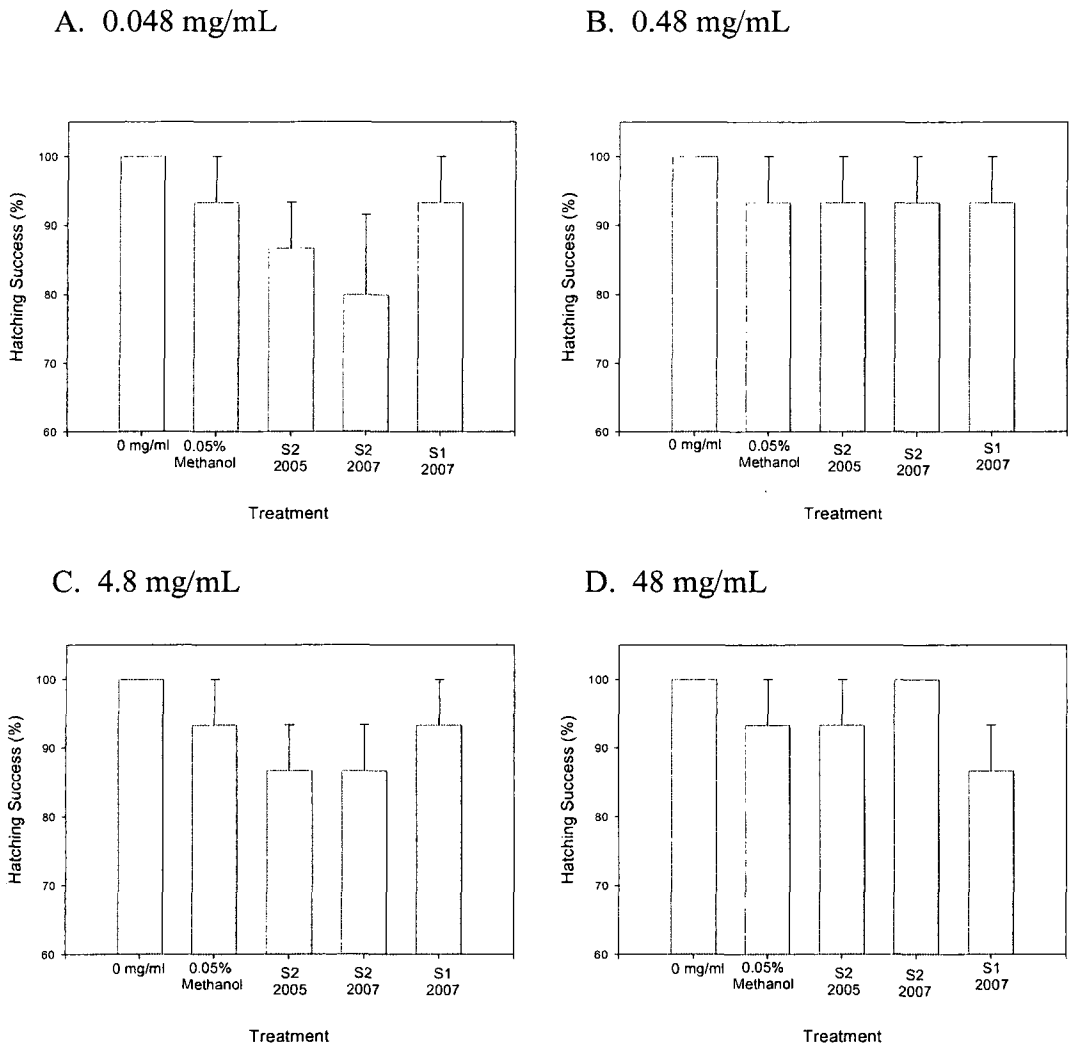
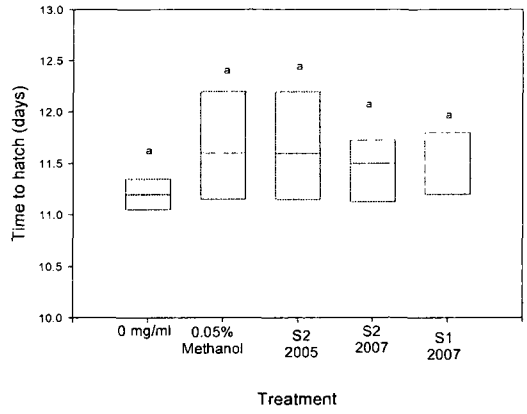
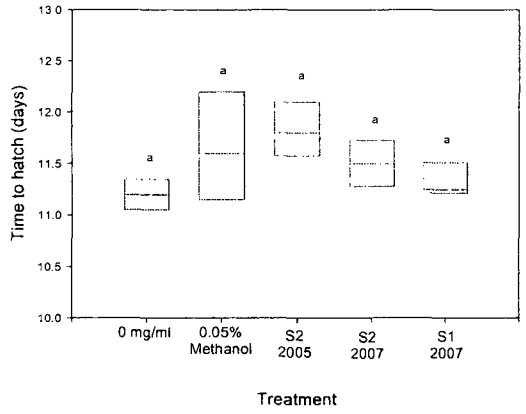


Figure 5-4. Mean hatching success (± SE) of Japanese medaka embryos exposed to four different concentrations of sediment extracts from Above Arsenault's Pond (S2) as well as from a spring (S1) on the Wilmot River. There were no significant differences in hatching success between the different treatments ($G = -21.17$, $df = 3$, $p > 0.05$) or within each treatment (48 mg/mL: G -7.14, $df = 4$, $p > 0.05$, 4.8 mg/mL: $G = -10.71$, $df = 4$, $p > 0.05$, 0.48 mg/mL: $G = -7.92$, $df = 4$, $p > 0.05$).

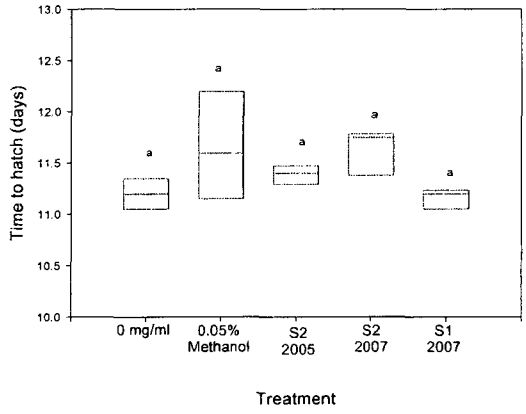
A. 0.048 mg/mL



B. 0.48 mg/mL



C. 4.8 mg/mL



D. 48 mg/mL

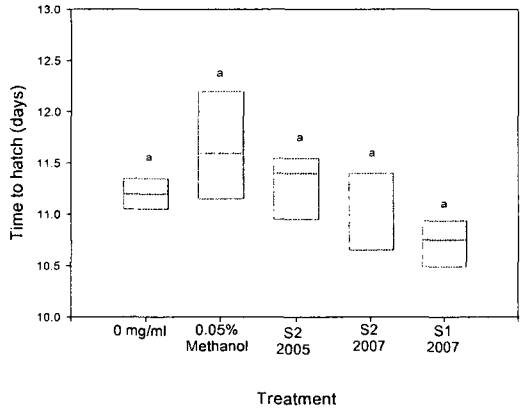


Figure 5-5. The hatching time of Japanese medaka embryos exposed to four concentrations of extracts from Above Arsenault's Pond (S2) as well as from a spring (S1) on the Wilmot River. Refer to figure 3-2 for description of boxplots. Different letters indicate groups that differ significantly ($p<0.05$).

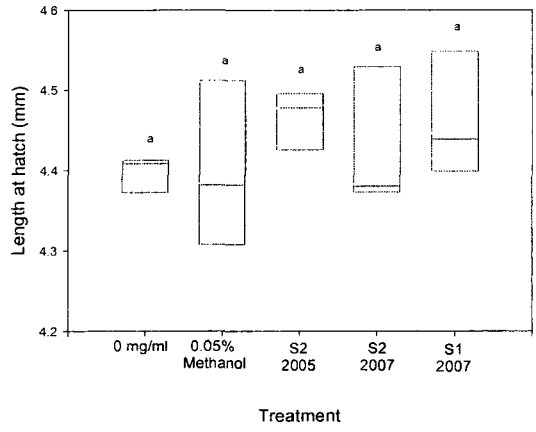
Length at Hatch

There were no significant differences found in the length at hatch of larvae between any of the treatment groups (multiple Mann-Whitney U Tests, $p>0.05$ for each) (Fig. 5-6). When the sites and years were compared there were no length differences (multiple Mann-Whitney U Tests, $p>0.05$ for each).

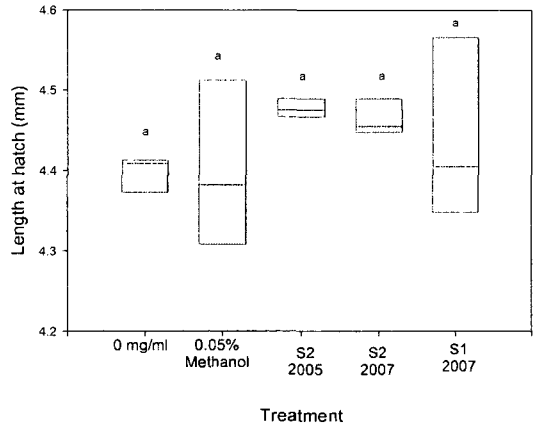
Developmental abnormalities

There were no significant differences in the incidence of abnormalities of larvae between the three treatments and reference groups (Table 5-4) ($G = 4.24$, $df = 13$, $p<0.05$).

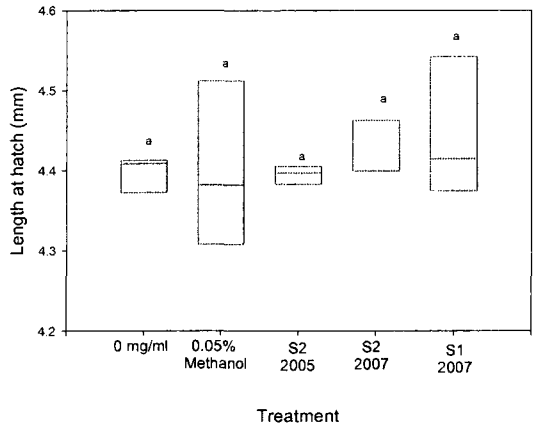
A. 0.048 mg/mL



B. 0.48 mg/mL



C. 4.8 mg/mL



D. 48 mg/mL

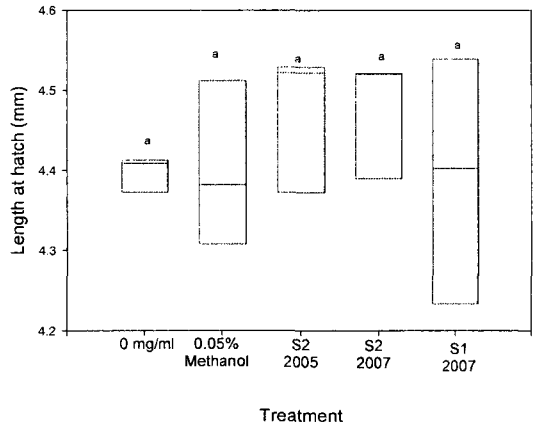


Figure 5-6. The hatching length of Japanese medaka exposed to four concentrations of extracts from Above Arsenault's Pond (S2) as well as from a spring (S1) on the Wilmot River. Refer to figure 3-2 for description of boxplot. There were no significant differences found between any of the treatments ($p>0.05$).

Table 5-4. Total number of newly hatched Japanese medaka fry with developmental abnormalities after being exposed as eggs to Wilmot River sediment extracts taken from above Arsenault's Pond from July 2004 (S2), above Arsenault's Pond from April 2007 (S2), and from a spring on the Wilmot River from April 2007 (S1). There were no significant differences in the number of abnormalities ($G = 4.24$, $df = 13$, $p > 0.05$).

Treatment	Number of replicates	Total number of eggs	Total number of successful hatches	Total number of Developmental abnormalities
0 mg/mL	3	15	14	0
0.05% Methanol	3	15	13	0
S2 2005 48 mg/mL	3	15	15	0
S2 2005 4.8 mg/mL	3	15	13	0
S2 2005 0.48 mg/mL	3	15	14	0
S2 2005 0.048 mg/mL	3	15	13	0
S2 2007 48 mg/mL	3	15	15	0
S2 2007 4.8 mg/mL	3	15	13	0
S2 2007 0.48 mg/mL	3	15	14	0
S2 2007 0.048 mg/mL	3	15	12	0
S1 2007 48 mg/mL	3	15	13	0
S1 2007 4.8 mg/mL	3	15	14	0
S1 2007 0.48 mg/mL	3	15	14	0
S1 2007 0.048 mg/mL	3	15	12	1

DISCUSSION

The first objective of this study was to compare the toxicity of a downstream site (S2) surrounded by agriculture with the upstream spring site (S1) surrounded by forest. The downstream site was used in a previous study (Jardine 2005) and results suggested some degree of contamination. It was predicted that sediment collected from the impacted downstream site would contain more contaminants than that collected from the upstream site. The second objective was to compare the rate of developmental abnormalities from the downstream site (S2) with results from a previous study done on the sediments of the Wilmot River in 2005 (Jardine 2005).

Results of pesticide analysis from both years showed differing concentrations of several pesticides analyzed (imidacloprid, metribuzin, metobromuron, metalaxyl, and linuron), with some being higher in 2005 and others higher in 2007. The concentrations of metribuzin, metobromuron, and linuron were all higher in the 2005 sample results. The pesticides tested for from both years were of a limited scope. Due to the high amount of agriculture on PEI and the various pesticides used, it was not feasible to test for all possible contaminants. Therefore, it is possible that there was a pesticide (or pesticides) responsible for the negative results found in the 2005 study that was not tested for in the analysis. The differences between Jardine's 2005 whole sediment exposures and the sediment extract exposures using 2005 sediment extracts was most likely due to the long term storage and handling of the samples. The sediment used for the extracts had been stored in the freezer for three years and had also been shipped between PEI and Ontario twice during this time. The concentration of pesticides in the stored sample most

likely experienced at least partial degradation during this time.

Sediments from the Wilmot River contained low concentrations of several pesticides including imidacloprid, metribuzin, metobromuron, metalaxyl, azinphos methyl, and linuron. This is not surprising due to the high percentage of agricultural land within the watershed. Two fish kills on the Wilmot River in July of 2002 were likely caused by azinphos-methyl (Murphy and Mutch 2005). Azinphos-methyl is an organophosphorus insecticide that acts as a cholinesterase inhibitor (Environment Canada 2004). It is highly toxic to fishes but has been shown to cause sublethal effects such as inhibitory responses in catalase activity and glutathione content in juvenile rainbow trout (*Oncorhynchus mykiss*) (Ferrari *et al.* 2007). In addition to azinphos-methyl, metalaxyl was also detected in the surface water at the time of the fish kills (Murphy and Mutch 2005). Metalaxyl is a fungicide which inhibits protein synthesis in fungi, and is considered non-toxic to fish and other wildlife (Environment Canada 2004). Imidacloprid is a chloronicotinyl introguanidine insecticide with contact and systemic action (Environment Canada 2004) and currently has the world's fastest growing sales for an insecticide (Raymond-Delpech and Matsuda 2005). Although no studies exposing fishes to imidacloprid have been published, Key *et al.* (2007) found it to be significantly more toxic to the larvae than the adults of grass shrimp (*Palaemonetes pugio*). Metrobromuron and metribuzin are pre-emergence herbicides with low toxicity to fish (Environment Canada 2004). While no studies were found that assessed the toxicity of metribuzin, Bonnemoy *et al.* (2004) found that toxicity of metrobromuron increased with increasing UV radiation. Linuron had the highest concentrations of all the pesticides

found in the Wilmot River sediment samples (2,225 pg/g, 401 pg/g, and 73 pg/g in the S2 2004, 2007, and S1 sites respecitively). Linuron is a substituted-urea herbicide with systemic activity and acts by inhibiting photosynthesis (Environment Canada 2004). Although it is classified as having low toxicity to fish, sublethal effects have been found. When exposing rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), and sockeye salmon (*Oncorhynchus nerka*) to sublethal concentrations of linuron, Tierney *et al.* (2007) found that the baseline amino acid and bile salt olfactory responses were impaired at 10 μg/L. For salmon species, amino acid and bile salt detection form the basis for important behaviors such as predator evasion and conspecific recognition, respectively (Tierney *et al.* 2007).

Concentrations of metals were comparable between each of the sediment samples. Toxicity due to metals in the present study is unlikely as the concentrations were constant between the samples and the degree of toxicity observed varied between them but still may have contributed to any mixture effects.

There were significant differences found between the reference group and those exposed to the upstream and downstream sediments as well as between the upstream and downstream sediments. The sediment treated groups had fry hatch later than those in the reference group. The fry in the group exposed to the downstream sediments (S2) were significantly smaller than fry in the reference group and fry exposed to sediment from the upstream spring (S1). There were significant differences in the number of developmental abnormalities. Numbers were comparable between the embryos exposed to upstream and downstream sediments from 2007 (S1 and S2), however the fry from Jardine's 2005

study (S2) showed a much higher incidence of abnormalities.

A decrease in hatching success was found in embryos exposed to sediments from the downstream site, above Arsenault's pond (S2) and from the upstream spring site (S1) in comparison to the reference group. When exposed to Wilmot River sediment extracts from both years, hatching success of medaka was not affected. Many studies have found that embryos and larvae exhibit decreased survivorship when exposed to agriculturally related chemicals (e.g. Viant *et al.* 2006 and Helmstetter and Alden 1995). Hallare *et al.* (2005) found that there was 0% survival in zebrafish embryos exposed to an extract at 60 mg/mL from sediments that were not toxic to the embryos when exposed in whole sediment assays. The extracted phase is usually more toxic as it contains contaminants that would be both bioavailable and non-bioavailable in the whole sediment phase. Strmac *et al.* (2002) also found that zebrafish embryos exposed to contaminated sediment extracts exhibited reduced survivorship when compared to the reference groups and to embryo exposure to sediment elutriate.

Embryos exposed to sediments from the S1 and S2 sites hatched significantly later than those in the reference but not from each other. Further, when exposed to extracts from both years, there were no significant differences in hatching time from the reference or between each year (2005 vs. 2007). This suggests that there was no difference in sediments from the two sites and also that sediment exposure itself, rather than agricultural contaminants, could have been responsible for delayed hatching. In the present study a reference with uncomtaminated sediment was not possible, nor was the formulation of reference sediment due to the unique nature of PEI sediment. Johnson *et*

al. (2007) suggested that interference with chorionase could not only affect the hatching success as previously discussed, but could also result in delayed hatching since hatching is often the result of a combination of enzymatic, mechanical, and osmotic mechanisms. Alternatively, delayed hatching may be attributed to a slower developmental rate.

Medaka exposed to the downstream sediment (S2) were significantly smaller at hatching than those in the reference and upstream spring (S1) groups. A reduction in body size can negatively affect the survival of young fish. According to Valerio and Barlow (1986) larger individuals are less likely to starve, particularly in conditions where little food is available. They are also more likely to be able to detect predators. Smaller individuals in the brood may also face the possibility of predation by larger members of their cohort (Valerio and Barlow 1986). A reduction in length has also been associated with exposure to pesticides commonly found in PEI streams. For example, Teather *et al.* (2005) found that exposure to the insecticide azinphos-methyl resulted in significantly smaller medaka at hatch.

There were far fewer developmental abnormalities found in the medaka exposed to the S1 and S2 sites from 2007 than those from the S2 site in the 2005 study. In the present study, medaka embryos exposed to Wilmot River sediments exhibited scoliosis and hemorrhages. Common developmental abnormalities found by Jardine in the 2005 study of Wilmot River sediments included pericardial edema, fin and eye deformities, and spinal deformities. The high number of deformities in that year suggests that aquatic sediments may have been more heavily contaminated. Although abnormalities can occur naturally, some deformities are indicative of exposure to contaminants (e.g. blue sac

disease). In other studies, increased incidence of developmental abnormalities has been associated with exposure to contaminants (Teather *et al.* 2001 and Gray and Metcalfe 1999). Although certain contaminants have been found to induce predictable abnormalities in fish, making associations between certain pesticides and deformities is difficult.

Given the mixture of the contaminants found in the sediments from the Wilmot River (refer to Table 5.1 and Mutch *et al.* 2002), the effects found in the present study may be attributed to non-definable additive or more than additive interactions of the contaminants with each other (Strmac *et al.* 2002). In their natural environment, fish are not exposed to a single toxicant but often to a mixture of contaminants present in varying amounts. In other words, the impacts of mixtures may be different from the effect of each chemical separately and are therefore unpredictable. Teather *et al.* (2005) showed that medaka exposed to an environmentally relevant combination of endosulfan, chlorothalonil, and azinphos methyl exhibited reduced swimming activity and males had significantly larger livers than control medaka or individuals which had been exposed to each pesticide separately.

The techniques assessed here represent a substantial contribution to our knowledge of aquatic sediment analysis. The medaka embryolarval bioassay has proved to be a useful tool in assessing sediment toxicity to developing fish.

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