Current-Use Pesticides Affect Development of Early Life Stages and Timing of Alevin Emergence in Sockeye Salmon (*Oncorhynchus nerka*)

by

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Abstract

The effects of two pesticides on Pacific sockeye salmon (*Oncorhynchus nerka*) exposed from fertilization to emergence were evaluated in a gravel-bed flume incubator, designed to simulate a natural streambed environment. Eggs were exposed to a commercial formulation of atrazine at 25 or 250 µg/L, and chlorothalonil at 0.5 or 5 µg/L, to examine effects on developmental success and timing, physical growth parameters, and biochemical indicators of growth. High chlorothalonil exposure reduced survival to hatch and increased finfold deformity incidence. All treatments resulted in reduced alevin condition factors at the time of emergence. Atrazine exposure resulted in premature hatch, while chlorothalonil exposure resulted in delayed hatch compared to controls. All treatment groups experienced premature emergence, highlighting the importance of using a gravel-bed incubator to examine this subtle but critical endpoint. These alterations in developmental success, timing and growth may alter survival of early life stages of sockeye salmon in the wild.

Keywords: Sockeye salmon; atrazine; chlorothalonil; emergence; development; growth

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Table of Contents

List of Tables

List of Figures

List of Acronyms

ai	Active ingredient
AL	Atrazine low (25 µg/L)
AH	Atrazine high (250 µg/L)
BC	British Columbia
CF	Craniofacial
CH	Chlorothalonil high (5 µg/L)
CI	Confidence interval
CL	Chlorothalonil low (0.5 µg/L)
dpf	Days post-fertilization
FF	Finfold
GDP	Gross domestic product
GSI	Graduated severity index
LC50	Concentration that kills 50% of organisms tested
MS-222	Tricaine methane sulphonate
OC	Organochlorine
PMRA	Pesticide Management Regulatory Agency
QA/QC	Quality assurance/quality control
SFU	Simon Fraser University
SK	Skeletal

Glossary

Alevin	Sockeye life stage just after hatch with the yolk sac still present
Davison's Solution	Solution used to preserve alevins, consisting of 22% formalin, 33% ethanol, 34% distilled water and 11% glacial acetic acid
E50	Time at 50% emergence
Emergence	Salmonid life stage when alevins have absorbed yolk sac and leave gravel substrate into water column to find exogenous food source
Fry	Salmonid life stage at which sockeye have emerged from the gravel into the water column, and are less than a few months old
Glycolysis	Metabolic pathway to convert glucose into pyruvate
H50	Time at 50% hatch
Heath stack	Vertically-stacked incubation trays, often used in incubation and rearing of salmonid eggs and alevins
LC50	The concentration at which 50% of organisms in a toxicity test die
Na+/K+ ATPase	Antiporter enzyme located in plasma membrane of all animal cells – pumps sodium out of cells while pumping potassium into cells
Photosystem II	Protein complex found in the thylakoid membrane of plants used in photosynthesis
Plastoquinone	Involved in the electron transport chain in photosynthesis
Redd	Series of depressions dug into gravel substrate by a female salmon in which her eggs are deposited
Smolt	A juvenile salmon that has completed freshwater rearing and is physiologically able to migrate into the marine environment
Smoltification	The physiological changes whereby a fry becomes a smolt
Swim-up	Salmonid early life event, when alevins swim up through gravel out into water column

1.

General Introduction

1.1. Fraser River Sockeye Salmon (*Oncorhynchus nerka*)

As the third most abundant species of Pacific salmon comprising the genus *Oncorhynchus*, sockeye salmon inhabit coastal waters and inland rivers of western North America from California to Alaska, as well as the far west-Pacific and Bering Sea waters of Russia and Japan [1]. The Fraser River, in British Columbia (BC), Canada, supports the largest single-river abundance of sockeye salmon in the world, providing significant environmental and societal value to the region [1,2]. Flowing south from the headwaters in the Rocky Mountains for 1600 km through BC, the Fraser River collects water from a 223,000 km^2 watershed, an area only slightly smaller than that of the United Kingdom, eventually draining from a broad flood plain into the Strait of Georgia [1]. The economic benefits of the commercial Fraser River sockeye fishery have historically held an important role in the industrial development of British Columbia and continue to provide opportunities and employment to a diverse group of Canadians involved in the fishing, canning and export of this resource [2]. Sockeye products are exported all over the world to over 60 countries [2]. At the height of recent sockeye production, in the mid-1990s, over 42.5 thousand tonnes of sockeye products were exported to generate an annual return of over $195 million dollars [2]. The recreational fishery, though it represents only a small proportion of the sockeye harvested, draws tourists from around the world and contributes as much as 40% of the Gross Domestic Product (GDP) value of all fisheries in BC, including aquaculture [2]. The value of sockeye salmon to Aboriginal community members who relate their “cultural, physical and spiritual wellbeing” to the fishery extends far beyond what can be reflected in dollars [2].

Identification of notable declines in Fraser River sockeye abundance and productivity over the last two decades by fisheries managers and scientists, in addition to a record low Fraser River sockeye return in 2009, prompted the federal government to establish the Cohen Commission, led by the Honourable Justice Cohen [2]. The collection of evidence through public submissions, scientific reports and evidentiary hearings began in 2010 to fulfill the mandate of the Commission: to encourage broad cooperation among stakeholders, investigate the causes of the decline and improve the future sustainability of the fishery [2]. As anadromous fishes with a life history involving both stream to ocean and return migrations, salmonids are exposed to innumerable stressors at multiple life stages. These stressors can be biological (pathogens, predators, etc.), physical (temperatures, currents, etc.) and/or anthropogenic (fishing practices, aquatic contaminants, etc.) [3]. Cohen's final report, released in 2012, outlined a wide range of potential stressors that could affect Fraser River sockeye, ranging from climate change, overfishing, disease prevalence, aquaculture practices to aquatic contaminants [4].

Sockeye salmon exposure to a wide range of contaminants was outlined as a potential cause or contributing factor in the decline of Fraser River sockeye; however, the presence, concentration and potential effects of these contaminants are largely unknown [4]. An extensive list of potential point sources of contamination was generated in the final report, and included industrial activities such as pulp and paper mills and mines, contaminated sites, wastewater treatment facilities, salmonid enhancement facilities and runoff from both forest management areas and agricultural operations [4]. Over 200 compounds were examined, ranging from persistent flame-retardants to metals, nitrogen-rich compounds and petroleum hydrocarbons. Although pesticides represent another major class of contaminants that could impact Fraser River sockeye, little is known both about their presence and levels in sockeye streams and rivers, as well as the potential effects of these compounds.

1.2. **Pesticides**

A wide variety of synthetic pesticides have been developed for use in urban, forestry and agricultural settings to minimize impacts of target pest species of plants, insects, moulds and fungi, and vertebrate pests [5]. In Canada, regulation of these products is divided

amongst federal, provincial and municipal governments through development and enforcement of acts, regulations and guidelines [5]. At the federal level, Health Canada's Pest Management Regulatory Agency (PMRA) controls the import, registration, sale, manufacture and use of pesticides under the *Pest Control Products Act*, with the main objective to "prevent unacceptable risks to human health and the environment from the use of pesticide products" [5]. Pesticide types and classes continue to evolve as researchers tinker with chemical properties to decrease persistence and transport within the environment, minimize effects to non-target organisms and increase effectiveness of the compound to eliminate pest species. As new pesticides continue to be developed, the challenge for regulators becomes not only to assess the impacts of past and current compounds on the environment and non-target species, but also the potential impacts of these new compounds continuously being introduced into the market.

When organochlorines (OCs), the first class of pesticides to be used in significant quantities, peaked in use from the 1940 to 1960s, there was little to no regulation of these products [6,7]. With mounting evidence that these OCs were not only causing effects in local wildlife populations, but were also persistent in the environment, subject to long-range transport and bioaccumulation in organisms, phase-out strategies and bans were implemented worldwide. Even with these restrictions in place, OCs are still detected in a multitude of environmental media around the globe today and continue to be permitted for use in some regions due to their low cost and high efficacy. With the need for continued high crop yields driven by a large human population demand, many new classes of pesticides were developed to take the place of OCs, including organophosphates, carbamates and triazines [6,7].

Agricultural pesticides are of particular concern to aquatic life as they are seasonally applied to cropland and can easily enter waterways from soil erosion, surface runoff, spray drift and atmospheric deposition [5]. Migration of pesticides to nearby surface and groundwater bodies is a common problem influenced by many factors, such as the quantity and chemical properties of the pesticide applied, site-specific factors including topography and soil composition, as well as local meteorological factors like precipitation and wind [5]. Implementation of management strategies, such as application during appropriate weather conditions and setting aside buffer zones between adjacent waterways are implemented in an attempt to minimize the movement of pesticides into

surface waters and exposure to non-target organisms [5]. The potential for exposure of aquatic organisms to these pesticides is further complicated by the breakdown of these compounds into metabolites or degradation products, of which their interaction with other compounds and potential effects are often not well-understood [6]. Furthermore, pesticides are not applied as a pure active ingredient; they are applied as formulations of surfactants, dyes, catalysts and intensifiers to increase the effects of the active ingredient or increase their dispersion on the applied area [6]. Often labeled as “inert” ingredients, these components can account for up to 99% of the formulation and some have been found to be more harmful than the active ingredient and cause sub-lethal effects to non-target organisms, such as endocrine disruption [6-9]. Since inert ingredients are considered part of the trade secret formulation of a pesticide, they do not have to be listed or reported [7], making their regulation difficult.

Designed to be lethal to their target pest organisms, pesticides can cause unintentional sublethal effects in non-target organisms depending on the toxicity of the chemical and the exposure scenario [5]. Non-target species in aquatic ecosystems may experience effects from this pesticide exposure as organisms often share common systems and pathways including receptors and enzymes [10,11]. Understanding how these pesticides affect aquatic organisms presents a significant challenge as both the number of pesticides and the number of potentially exposed organisms are substantial.

1.3. **Pesticides in Fraser River Sockeye Salmon Habitat**

A variety of pesticides are used in a number of applications in BC for control of weeds, fungi and insects in urban, agricultural and forestry practices [6], though little is known about their presence in nearby aquatic ecosystems. Though pesticide usage is not well documented at any level of government, provincial sales numbers in BC are maintained, and, in 2003, totalled over 4.6 million kg of active ingredients [5]. Though the majority of these pesticides are used in the forestry sector as anti-microbial products for wood treatment (71.7% of total sales), the remaining sales consist of insecticides (8.8%), fungicides (6.5%), herbicides (6.1%) and a variety of other pesticides (6.9%) [5,12]. The majority of these non-forestry pesticides are used in agriculture, which in BC includes a variety of crops, the most abundant being forage crops of spring and durum wheat,

followed by fruits and vegetables including apples, blueberries, potatoes, ginseng, cranberries, grapes, raspberries, cherries and corn [6]. Agricultural production often occurs in fertile soils where water is readily available for crops and livestock, enabling potential contamination of these waterways with agricultural pesticides and increasing the importance of regulation and mitigation measures.

Pesticides can enter Fraser River sockeye habitat via over-spraying, runoff, erosion of contaminated soils and seepage from contaminated groundwater from private properties, cropland and forestry areas [13]. Concentrations in environmental media tend to vary seasonally [77]. From 2003-2005, Environment Canada conducted the first national aquatic surveillance program focused on monitoring current-use pesticides in aquatic systems across the nation [5]. In BC, the focus was on two large agricultural and, therefore, high pesticide-use regions: the Lower Fraser River Valley and Okanagan Valley. In these regions, pesticides and/or their degradation products were detected at 100% of all sites sampled, including those acting as reference sites [5].

Several reports written in the last decade have ranked lists of pesticide sales, frequency and concentration of aquatic detection, along with correlations of an increase in use with a decrease in Fraser River sockeye escapement [5-7,14]. A number of pesticides in use in BC were deemed to be of high or medium risk to aquatic life, and, some, even more specifically, as a priority for examination of their effect on Fraser River sockeye salmon. On these lists, the herbicide, atrazine, and fungicide, chlorothalonil, both consistently made appearances ranking with either high or medium priority.

1.3.1. Atrazine

Atrazine is a widely used herbicide in North America to control broadleaf weeds and grasses in both agricultural and industrial areas and historically was used to control submerged vegetation in slow-moving waters [15]. In Canada, atrazine is used for weed control on crops such as corn and lowbrush blueberries, and on non-cropland, but globally is recognized most often for its extensive use on corn crops [16]. Atrazine effectively kills weeds by competing with plastoquinone at its binding site in the process of electron transport in photosystem II, thus inhibiting photosynthesis and plant energy production [17,18]. Due to its high use, especially in high-density corn cropland,

persistence in environmental media and relatively high mobility in soils, atrazine has been frequently detected in surface and ground water around the globe. This includes consistent detection in Arctic and sub-Arctic surface water, suggesting a potential for long-range transport [19,20]. Atrazine is relatively persistent in surface waters with a half-life ranging from 41 days to 237 days in aquatic environments [18,21].

Through Environment Canada's monitoring program from 2003-2005, atrazine was recognized as one of the most frequently detected herbicides, with some of the highest concentrations in groundwater and was found in 75% of samples taken in the Lower Fraser Valley and 71% of samples in the Okanagan Basin. These consistently high levels of detection are notable in light of the fact that its use had declined by more than 50% in the years leading up to the study [5,22]. The use of atrazine has been declining as environmental concerns escalate, leading to a complete ban on the triazine herbicide in the EU due to its persistent contamination of groundwater in 2004 [23]. Interestingly, Italy and Germany had already banned atrazine in 1991 and were followed by Sweden, Finland and Denmark by 1994. Despite concern in Europe, the United States, a major corn producer, and Canada continue to renew registration of atrazine for use [23]. British Columbia recently reapproved atrazine for use in April 2012 [24].

In the contaminant-related technical report on the potential effects of contaminants on Fraser River sockeye submitted to the Cohen Commission, atrazine was listed as one of the contaminants of the highest priority associated with agricultural activities [14]. This report cited a study by Verrin et al. (2004) [6], which combined priority lists from six agencies including the PMRA, Environment Canada, Fisheries and Oceans Canada, the Toxics Work Group and World Wildlife Fund. With regards to pesticides of concern to aquatic ecosystems in BC, this group listed atrazine in the category of moderate priority [6].

1.3.2. Chlorothalonil

Chlorothalonil is a non-systemic foliar fungicide used to control fungal pathogens including late blight in a number of fruit and vegetable crops including cabbage, broccoli, cauliflower, carrots, celery, cucumbers, potatoes as well as in turf, ornamental and conifer farming [25]. In fungi, chlorothalonil combines with thiols, particularly glutathione,

interfering with the production of cellular energy by inhibiting glycolysis [5,26,27]. Use of chlorothalonil in BC increased dramatically throughout the noted decline of the Fraser River sockeye salmon returns – from 1991-1999, chlorothalonil sales increased by 616% [6,22]. In the Environment Canada monitoring program, chlorothalonil was detected in 89% and 81% of samples taken in the Lower Fraser Valley and Okanagan Basin, respectively [5]. Contamination of surface water can occur through spray drift at the time of application or after application through runoff and erosion with a half-life in aerobic aquatic environments ranging from 2 hours up to 6-8 days [28]. In BC, chlorothalonil had the highest concentration of any pesticide recorded in precipitation [5] and has also been detected in remote Arctic regions, suggesting potential for long-range transport [20]. In contrast to atrazine, relatively little research has been done on the effects of chlorothalonil on aquatic organisms, with LC50 values reflecting the acutely toxic nature of the chemical but few studies which examine long-term sub-lethal effects.

In the contaminant-related Technical Report submitted to the Cohen Commission, chlorothalonil was also listed as one of the contaminants of the highest priority associated with agricultural activities [14]. The Environment Canada report following the 2003-2005 monitoring program identifies chlorothalonil as one of five pesticides that with current application practices, “could result in impacts in the aquatic environment” [5]. In a report dedicated to identifying contaminants that may be affecting sockeye salmon in the Fraser River system, chlorothalonil ranked at the top of the priority list [7]. The aforementioned report produced by Verrin et al. (2004) [6] prioritized chlorothalonil in the highest category due to its potential effects on aquatic ecosystems in BC.

1.4. The Potential Effects of Atrazine and Chlorothalonil on Sockeye Salmon

Contaminants that have found their way into surface water can cause a number of effects on aquatic ecosystems. Effects can be as severe as direct mortality of fish and other aquatic organisms, or be more subtle, manifesting in sublethal effects, altering an organism’s ability to develop, reproduce, grow, effectively find prey, avoid predators and cope with a multitude of other environmental and anthropogenic stressors [5]. The effects are often different and vary in severity in organisms at different life stages, with

certain stages consistently demonstrating a greater sensitivity than others. Typically, early life stages of fish development tend to be the most sensitive to contaminant exposure, including both embryonic and larval stages [29].

Several studies have been performed examining the effects of pesticides on salmonids at a variety of life stages and from the cellular to population level. In Atlantic Canada, aerial spraying was correlated with a drop in returns of spawning salmon adults [30]. Pesticide exposure can affect behaviour of salmonids by interfering with olfaction, a vital system directing spawning, anti-predator and migratory behaviour [31-33]. Pesticides can also evoke a stress response [10] or depress immune system functioning [34]. These studies and most research on pesticide-induced effects on salmonids have been examined on fish at the fry, smolt and juvenile stages, with effects of pesticides on earlier life stages of salmonids remaining largely unknown. A survey of the literature reveals only a handful of other studies have examined the effects of current-use pesticides on the egg and alevin stages of salmonids, though none of this research has been performed on sockeye salmon. Research assessing the effects of three pesticides on eyed eggs and alevin of Chinook salmon showed that a 96 hour exposure was correlated with a decrease in survival and metabolic changes in eggs and alevin [35]. A more recent study found no effects to hatching success, survival, deformities and growth of Pacific Coho salmon exposed to pulses of a mixture of pesticides throughout development [36]. To ensure effective protection of salmonids, a strong understanding of the effects of pesticides on all life stages, especially those which are likely to be most sensitive, must be achieved.

1.4.1. Potential Effects of Atrazine on Salmon

The effects of atrazine on biota have been studied extensively with the outcomes of this research generating much debate. As atrazine's mechanism of action involves the inhibition of photosynthesis, non-plant species will not be affected via this specific pathway. Instead, atrazine has been suggested to disrupt the endocrine system as it has been found to inhibit androgen receptor binding in mammals and induce aromatase activity – an enzyme that converts androgen to estrogen [37]. The majority of atrazine research performed on aquatic organisms has been done on amphibians [18]. Declines in hatching success of salamander eggs [38] and metamorphic success of frogs [39]

accompany observations of sub-lethal developmental effects including altered sex ratios [39], increased incidence of hermaphroditism and demasculinization [40] and alterations in organogenesis and metamorphosis timing in multiple frog species [41,42]. Atrazine has also been shown to alter water-conservation behaviour in salamanders [43] and increase frog susceptibility to parasite infection [44], both important aspects of survival for these amphibians. However, these results have been called into question as other similar studies, like one by Coady et al. (2005) [45], has shown no indication of the same effects and a review by Solomon et al. (2008) [18] rejects the validity of many of these studies based on the experimental procedures employed.

Some indications of atrazine-induced endocrine disrupting effects exist in the research on fish. Trends noted include decreases in testes weight, maturity and sperm production in fathead minnows exposed to environmentally relevant concentrations of atrazine [46]. Additionally, atrazine has been correlated with declines in fathead minnow egg production [47] and elevated brain aromatase activity in zebrafish [48]. Beyond these potential endocrine disrupting effects, atrazine has been associated with disruption of osmoregulatory function in mummichog [49] and changes in swim behaviour of both goldfish [50] and zebrafish [51].

Specifically in salmonids, atrazine exposure has demonstrated apparent endocrine disrupting effects including altered sex steroid hormone levels in Atlantic salmon [52] and increased plasma vitellogenin in juvenile rainbow trout [53]. Decreased growth rates have been observed in rainbow trout [54] and Atlantic salmon smolts exposed to atrazine [55]. Ionoregulatory effects, such as a decrease in gill Na+/K+ ATPase activity and decreases in plasma ions in Atlantic salmon [55,56], as well as alterations to kidney tissue in rainbow trout [57] have been observed. However, Matsumoto et al. (2010) [58] found no such ionoregulatory effects in Atlantic salmon smolts at similar atrazine concentrations. Atrazine exposure has also been correlated to an increase in plasma cortisol levels in both rainbow trout [59] and Atlantic salmon [55], in addition to interference with proper immune system functioning [60]. In recent years, increasing attention has been put on the effects of atrazine on salmonid olfaction. Olfaction is a critical salmonid system, functioning in alarm response, reproduction, smoltification, and imprinting, homing and migration back to the natal stream for spawning [61]. It has been

shown that environmentally relevant concentrations of atrazine can affect olfaction in rainbow trout [61] and Atlantic salmon [52,56,62].

In the literature there are no reports of atrazine effects on salmonid early life stages – few studies report effects of atrazine on early life stages of any fish species, a data gap identified in atrazine review papers as a need for further examination [63,18]. Zebrafish have shown a decrease in survival, retardations in organogenesis and an increase in developmental deformities [64,15] when exposed at high (mg/L) atrazine concentrations unlikely to be encountered in the environment. Fathead minnow eggs exhibited a higher respiration rate when exposed to 150 µg/L atrazine after just 2 hours [65] and fathead minnow larvae developed spinal curvatures when exposed to 20 µg/L atrazine for 7 days [66]. A decreased growth rate and protein content was observed in red drum larvae exposed to as low as 40 µg/L atrazine, in addition to an increase in swim speed and hyperactive swim behaviour [19,67].

Contradictory results, questionable experimental procedures and authors' potential bias have fuelled the debate of atrazine's role as an endocrine disrupting compound and its potential for causing significant effects to aquatic organisms [18,63]. To provide further insight into this examination of the effects of atrazine, additional research is warranted, especially in the less-studied realm of fish early life stages and development.

1.4.2. Potential Effects of Chlorothalonil on Salmon

Chlorothalonil exerts its effect on fungi by binding to glutathione and disrupting cellular respiration [5,26,27]. Since glutathione's role in cellular respiration is vital to virtually every organism, it is not surprising that chlorothalonil can exert effects on a multitude of non-target organisms at low exposure concentrations. Compared to atrazine, chlorothalonil is much more acutely toxic to non-target organisms.

Most available data includes acute toxicity values reported by the manufacturer as part of the registration process. A survey of peer-reviewed literature indicates little is known about the sublethal effects of chlorothalonil on any class of aquatic organisms. Respiratory effects have been observed, including increased respiratory activity in the Australian freshwater fish *Pseudaphritis urvillii* [54], as well as extensive gill damage

accompanying increased respiratory activity in rainbow trout [68]. More recently, chlorothalonil has shown potential for interfering with immune system function, as observed by Shelley et al. (2009) [34] with the stimulation of an innate immune response in rainbow trout cells, and by McMahon et al. (2011) [69] with an increase in corticosterone levels in tree frog tadpoles. Only two studies could be found examining the sublethal effects of early life stages of aquatic organisms. Fathead minnows exposed to ≥ 6.5 µg/L experienced decreases in the number of eggs per spawn, egg hatchability and fry survival (as cited in CCME 1999b [25]). More recently, mummichog embryo hatchability was reduced at ≥ 32 µg/L chlorothalonil, with growth increased at this concentration and survival decreased at the 100 µg/L concentration [70].

In acute studies, salmonids have consistently been found to be more sensitive than other fish species to chlorothalonil exposure [71,72,54]. A major data gap exists regarding the sublethal effects of chlorothalonil on early life stages of the majority of fish species, including salmonids.

1.4.3. Fraser River Sockeye Salmon Early Life Stages

A Fraser River sockeye salmon life span consists of about four years, which, near the beginning, starts with a migration out to the ocean and ends as the mature adult returns to its natal stream to spawn and die. Female sockeye locate a suitable location for spawning based on stream flow, groundwater upwelling and gravel size where they deposit from 500 to over 1000 eggs into depressions they create in the gravel, called redds [73]. Males simultaneously fertilize the eggs as they are deposited into multiple redds, and the female will then cover the eggs with gravel from the streambed. Fertilized eggs develop in the gravel for several months, protected from being washed downstream, ice formation and predation [73]. Alevins hatch from the eggs in the early spring, but remain protected in the gravel until their food source, a yolk sac suspended from their belly, has been fully absorbed. Approximately eight months post-fertilization, Fraser River sockeye will emerge from the gravel as fry and migrate up or downstream to locate exogenous food sources in a nursery lake. Fry live in nursery lakes for often one, but sometimes two years, before undergoing smoltification: a physiological process that enables these smolts to transition from freshwater to saltwater as they migrate downstream to the ocean. From the mouth of the Fraser River, most juvenile sockeye

migrate north through the Strait of Georgia, to Queen Charlotte Sound and beyond to the waters off Alaska, where they grow and transition from feeding on plankton to other fish and squid [74]. Generally, in their fourth year adult sockeye mature and return to their natal stream in the Fraser River watershed to spawn and continue this cycle.

The most precarious stage of this intricate life history consists of the incubation, alevin and fry stages: estimates indicate that of the approximate 3000 eggs a female sockeye lays, it is likely that only between 10-20%, or approximately 300-500 of these individuals survive to the fry stage [75]. Many factors contribute to this low survival rate including predation, redd disturbance, desiccation or freezing due to low water levels, suffocation from excessive sedimentation or reduced oxygen levels, and pathogen infection [75]. Due to their immobility in the embryo and larval stages these salmon are at the mercy of the environment surrounding them, unable to evade exposure to these stressors, as well as to any aquatic contaminants. Effects caused by contaminants during these critical developmental stages can impart life-long impacts which may ultimately affect fitness and survival of the salmon.

1.4.4. "Swim-Up": A Critical Stage in Sockeye Salmon Development and Survival

Ideally, examination of the effects of a contaminant of interest should be studied in a salmonid's natural environment. Unfortunately, performing toxicological testing in the field is fraught with challenges, even for organisms with a relatively short, simple, spatially static life cycle. When complicated further by examining an organism with a long and complex life cycle, like a salmonid, this field study becomes essentially impossible. In the laboratory, salmonid early life stages are traditionally studied as eggs and alevins in trays of Heath stacks. Eggs can be easily monitored as they reach developmental milestones, such as eyeing, hatch and full absorption of the yolk sac (often refered to as emergence) in addition to other endpoints such as success and timing of reaching these developmental stages and examination of alevin for deformities.

In reality, these eggs would be buried in a redd under up to 20-30 cm of streambed gravel. Once hatched, the alevin remain in the gravel until their yolk has been absorbed and they need to leave the gravel, enter the water column and travel to a nursery lake in

search of food. The process of moving up through the gravel is referred to as “swim-up” or emergence, with swim-up success being a key reproductive endpoint vital to the survival of the salmonid [76]. Though variations of gravel-bed incubators have been used to examine emergence of salmonids none have incorporated the continuous addition of an exogenous contaminant for the purpose of toxicological testing.

One important aspect of this present study was to examine the success and timing of sockeye salmon swim-up out of the gravel substrate when exposed to pesticides throughout their embryonic and larval development. To do this, two gravel incubator systems were constructed by the Machine Shop at Simon Fraser University based on a design outlined by Pilgrim et al. (2013) [76], but modified to allow for the separation of treatment groups receiving different pesticide treatments.

1.4.5. General Overview of this Research

In the present study, sockeye salmon were exposed in a flow-through system to environmentally relevant low and high concentrations of atrazine and chlorothalonil, from fertilization through to emergence. Hatch success and timing were monitored in eggs in one incubator system while swim-up success and timing were monitored in a second gravel-substrate flume incubator, as described above. Growth at these developmental stages was examined and supplemented with biochemical analyses of total protein and triglyceride levels and a deformity analysis of emerged alevin.

Understanding the potential effects of pesticides in aquatic environments is incredibly important to ensuring effective protection of these ecosystems. Salmon are often used as an indicator species in BC due to their cultural and economic significance. In light of the recent declines, the importance of examining effects of these pesticides on sockeye salmon specifically is further reinforced.

At this time, based on the available data, directly linking the decline of Fraser River sockeye salmon to exposure to any contaminants, including pesticides, is not possible; however, ruling out any effect of contaminants due to a lack of data would be inappropriate and irresponsible [7,4]. Undertaking research to provide a greater understanding of the effects of pesticides on multiple life stages of sockeye salmon can

only be beneficial in understanding the role of these pesticides amongst the multitude of other stressors these fish encounter throughout their life cycle. Though atrazine and chlorothalonil are only two of dozens of pesticides these fish are exposed to in the Fraser River system, they are consistently detected in BC waters and have been demonstrated to be of moderate and high concern to sockeye salmon in the Fraser River system [14,7,6].

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2.

Current-use pesticides affect development of early life stages and timing of alevin emergence in sockeye salmon (*Oncorhynchus nerka*)

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2.1. Abstract

The effects of two currently used commercial pesticide formulations on Pacific sockeye salmon (*Oncorhynchus nerka*) exposed from fertilization to emergence were evaluated in a gravel-bed flume incubator, designed to simulate a natural streambed environment. Eggs (fish) were exposed to a commercial formulation of atrazine at 25 ['Low' or 'AL'] or 250 µg/L ai ['High' or 'AH'], and chlorothalonil at 0.5 ['Low' or 'CL'] or 5 µg/L ai ['High' or 'CH'], to examine effects on developmental success and timing, physical growth parameters and biochemical indicators of growth. Survival to hatch was reduced in the CH group (55% compared to 83% in controls), accompanied by a 24% increase in finfold deformity incidence. All treatments resulted in reduced alevin condition factors by 2.9-5.4% at the time of emergence, with triglyceride levels elevated in some chlorothalonil-exposed sockeye. Atrazine exposure caused premature hatch (AH time to 50% hatch (H50)=100 days post-fertilization [dpf]), while chlorothalonil exposure resulted in delayed hatch (CH H50=108 dpf) compared to controls (H50=102 dpf). All treatment groups experienced premature emergence (time to 50% emergence (E50): control E50=181 dpf, CL E50=175 dpf, CH E50=174 dpf, AH E50=175 dpf, AL E50=174 dpf), highlighting

the importance of using a gravel-bed incubator to examine this subtle but critical endpoint. These alterations in developmental success, timing and growth may affect survival of early life stages of sockeye salmon in the wild.

2.2. **Introduction**

The Fraser River in British Columbia (BC), Canada, supports the world's largest single-river abundance of sockeye salmon (*Oncorhynchus nerka*) [1]. Notable declines to this run over the past two decades, followed by a record low return in 2009, prompted an investigation into the causes of the decline and to develop strategies to ensure future sustainability of the run [2]. Throughout their complex life cycle, sockeye are exposed to a wide variety of stressors at multiple life stages. Possible contributors to these declines included biological stressors such as pathogens and predators, physical stressors such as temperature, current and salinity changes, as well as anthropogenic pressures including fishing practices and chemical contamination of aquatic systems [3].

Little information exists on the presence, concentration and effects of anthropogenic chemicals such as pesticides in relevant watercourses or their potential effects on various sockeye life stages. A nation-wide Canadian aquatic surveillance program for pesticide presence in aquatic environments, focusing on two major agricultural regions in BC (Lower Fraser River Valley and Okanagan Valley), detected pesticides and their degradation products in 100% of sites sampled, including those meant to serve as reference sites [4].

In BC alone, approximately 4.6 million kilograms of pesticide active ingredients were sold in 2003. Though the majority of these compounds are used in the forestry sector as wood preservatives or anti-sapstains (e.g. creosote, chromated copper arsenate), the second most common use is in the agricultural sector for application to wheat forage crops or crops of fruits or vegetables (e.g. glyphosate, mancozeb) [5]. For the current research, BC-specific pesticide sales volume and trends and detection in BC waterways [4], combined with an understanding of potential risks to aquatic life [5] and inference of possible links between pesticide exposure and declines in Fraser River sockeye salmon [6], resulted in the selection of two pesticides, atrazine and chlorothalonil, that

consistently ranked as compounds of high or medium priority with respect to their potential effects on sockeye salmon.

Atrazine is used for broadleaf weed control on crops such as corn and lowbush blueberries [7] and was detected in 71-75% of waterways sampled in BC [4]. Banned from use in the European Union (EU) in 2004 due to persistent groundwater contamination, atrazine was also briefly banned in BC, but in 2012 was reapproved for use on agricultural crops [8].

Chlorothalonil, a non-systemic foliar fungicide, is used to control fungal pathogens in various fruit and vegetable crops and tree farming practices [9]. A dramatic increase of 616% in chlorothalonil sales through the 1990s mirrored a decline in Fraser River sockeye runs in time period [5]. As chlorothalonil was also consistently detected in 81-89% of waterways sampled from 2003-2005 [4], chlorothalonil is a pesticide of high priority in its relation to its potential risk to sockeye salmon in BC [6].

The earliest life stages are the most precarious for sockeye salmon; of approximately 3,000 eggs laid by a female, only 10-20% will survive to the fry stage [10]. As sockeye embryos and alevins develop in streams, buried within gravel redds, they are relatively immobile and vulnerable to contamination of the watercourse [11]. These early life stages also tend to be the most sensitive to contaminant exposure [12], though the majority of research on the effects of pesticides on salmonids is performed on fry, smolts and juveniles. A handful of studies have examined the effects of pesticides on salmonid development [13,14], though none have looked specifically at sockeye salmon. One of the first major fitness tests in the early lives of these salmon is the process of swimming-up out of the gravel into the water column; however, it is often overlooked in salmonid early life stage toxicological testing despite being a critical developmental step vital to the survival of the salmonid [15]. Though variations of gravel-bed incubators have been used for examining swim-up success, none have incorporated the continuous addition of an exogenous chemical for the purpose of toxicological testing.

In the present study, sockeye salmon embryos and alevins were exposed to environmentally-relevant concentrations of commercially formulated atrazine and chlorothalonil from fertilization through to emergence to assess the effects on success

and timing of development, as well as both physical and biochemical parameters of growth.

2.3. **Methods**

2.3.1. Sockeye gamete collection and fertilization

Four male and four female mature spawning sockeye salmon (*Oncorhynchus nerka*) from the late-run Weaver Creek population were captured by beach seine in the Harrison River, BC. Fish were euthanized by cerebral concussion and eggs (~3,000 per female) and milt were extracted, stored in containers filled with oxygenated air and transported to Simon Fraser University (SFU). Fertilization procedures took place within 24 h of gamete collection.

Four unique offspring sets were created from four independent fertilizations to reduce variability caused by genetic variation as well as to assess potential differences in the occurrence or magnitude of effects observed in offspring from these specific crosses. A dry fertilization protocol, shown to be effective in other incubation studies on sockeye salmon from Weaver Creek [16], was employed for each of the crosses. Sixteen grams of eggs (~90-100 eggs) were combined with 0.15 mL of milt and activated with 30 mL of dechlorinated municipal water or a water-pesticide solution, as described in the exposure section. After 2 min, approximately 200 mL of additional water or pesticide solution was added and a further 10 min allowed for water hardening of the fertilized eggs. Eggs from each container were counted, gently poured into three netted, cylindrical baskets and placed in a corresponding exposure chamber. Each exposure chamber housed eggs from all four crosses. Fertilizations from one of the four crosses were not viable (less than 10% total fertilization) and therefore only three crosses were included in the experiment. Additionally, unsuccessful fertilizations of some eggs from cross 3 did not allow for this cross to be considered in assessments of emerged alevin in the flume incubators.

2.3.2. Incubation set-ups

In order to monitor egg development and survival from fertilization to hatch, two incubation systems were employed. Fertilized eggs were either placed in baskets in open bins (to determine pesticide effects on hatch and growth without the need to emerge from gravel), or in a flow-through, gravel-bed flume incubator (which simulated a natural streambed environment).

The flume design was modified from Pilgrim et al. (2013) [15]. Each flume (250 cm long by 40 cm wide and 30 cm deep) contained 5 large compartments separated by plexiglass dividers to isolate each treatment and allow for multiple pesticide concentrations to be used in the same flume (Figure 1). Each of these larger compartments contained 5 sub-channels, separated by stainless steel mesh dividers. Drainage occurred through an unoccupied central sub-channel; the four remaining sub-channels were used to house eggs and alevins.

The gravel-bed flume incorporated upwelling of water from multiple holes in pipes running the length of the bottom of each sub-channel. Upwelling water exited through the front and top of the center channel. Two sizes of gravel rock, 10 mm and 25 mm, were used to bury eggs based on sizes reported for salmon redd sites [17] and used in a similar apparatus [15]. The gravel was combined in a 1:1 ratio, disinfected with a 1% Ovadine solution, rinsed thoroughly with dechlorinated water and placed in the flumes to a height of 5 cm. Flumes and gravel were flushed for several days with dechlorinated water to remove any potential residue and air pockets in the pipes and gravel.

Eggs from each cross was separated by stainless steel mesh divided but received the same pesticide treatment. In the bin incubators, fertilized eggs remained in baskets and were monitored every other day. For gravel bed incubators, fertilized eggs were placed in baskets on top of the gravel until they reached the eyed stage. At this stage, eggs were poured out and carefully covered with gravel by hand to a depth of 15 cm, based on lab studies [15] and field observations [17]. Black plastic covered flume and bin incubators to prevent sensitive embryos and alevins from light exposure [18]. After hatch, the black plastic was removed from the tops of the flume incubators and room lights were set on timers, adjusted weekly to reflect natural photoperiod changes.

2.3.3. Water quality and pesticide exposures

Dechlorinated municipal water at ambient (external) temperature (4.5-12.3°C) was used in both incubation set-ups. Water temperature and dissolved oxygen concentrations were measured and recorded every other day for the duration of the experiment. Ammonia concentrations were monitored at each developmental stage, while pH was measured weekly.

Flow rates to each bin or flume compartment were continuously monitored and adjusted every 48 h to maintain a relatively constant flow rate of 750 mL/min. Gate valves were manually adjusted every second day, however, flow rates in the gravel flumes were expected to vary between 700-800 mL/min and between 650-850 mL/min in the bin incubators. Even at the lowest flow rate in these ranges, complete replacement of water in each of the bins for flume chambers occurred at minimum once an hour.

Atrazine and chlorothalonil exposure solutions were made using commercially available formulations (Terralink Horticulture, Abbotsford, BC). Exposure concentrations were chosen based on environmental data from reported concentrations in North American watercourses [7,9,19-24]. AAtrex® Liquid 480 (43.7% atrazine) was diluted with dechlorinated municipal water and delivered by Masterflex peristaltic pumps into the corresponding exposure chamber (in both bin and gravel set-ups) continuously and at a constant flow rate. The pesticide stock solution was added to the chamber water inflow to obtain concentrations of 25 µg/L and 250 µg/L active ingredient (ai). Stock solutions of Bravo® 500 (40.3% chlorothalonil) were used to generate 0.5 µg/L and 5 µg/L ai. Fish were continuously exposed from the initiation of fertilization to emergence in a control and four treatment groups: low atrazine (AL), high atrazine (AH), low chlorothalonil (CL) and high chlorothalonil (CH). Stock solutions were refreshed every 48 h. Concentrations were below water solubility values and thus the use of a vehicle was not required. All exposures were performed in duplicate. To determine actual pesticide concentrations, water samples were taken from flume exposure chambers approximately midway through the exposure period (at 95 dpf) and analyzed by Maxxam Analytics (Burnaby, BC).

2.3.4. Hatch and emergence success and timing

Eggs in the bin incubators were examined for mortality using red light every second day until the eyed stage. When eye pigmentation was clearly visible and well-defined in the majority of eggs (at 60 days post-fertilization [dpf]), eggs in bins were thoroughly examined and those that were not eyed counted and removed. Eggs were monitored every other day until the initiation of hatch, when monitoring frequency was increased. Once alevin yolk sacs were absorbed (approximately 165 dpf), bin incubations were terminated and alevins euthanized with an overdose of buffered tricaine methane sulphonate (MS-222), weighed, measured for length and stored in Davidson's solution for future deformity analyses [25]. A sample of alevin were flash-frozen on dry ice and stored at -80°C until biochemical analyses were performed.

Following the burial of eyed eggs under gravel in the flume incubators, compartments were monitored every other day for emerging alevins that had successfully completed the swim-up process. Emergence began at approximately 148 dpf and compartments were monitored daily until no new fish emerged for at least 5 days, indicating the completion of emergence (approximately 199 dpf). Captured emerged alevins were kept in baskets on top of the gravel in their respective compartments until experiment termination at 199 dpf. All alevins were euthanized with an overdose of buffered MS-222 before being weighed, measured for length and stored in Davidson's solution for future deformity analysis.

2.3.5. Growth parameters

In the bin incubators, at 50% hatch, alevins (n=10) from each cross in each treatment group were euthanized as above, with wet weight and total length and recorded. Alevins were flash frozen on dry ice and stored at -80°C until biochemical analyses were performed. At the termination of the bin incubations, weight and length of all alevins were recorded. At this time point, alevins (n=10) from each cross in each treatment group were sampled, frozen as above, and stored at -80°C until biochemical analyses were performed. All remaining alevins were stored in Davidson's solution for future deformity analysis. In gravel bed incubators, all euthanized alevins collected at emergence were weighed, measured and stored in Davidson's solution; a sample of

alevins (n=10) from each cross and treatment group were frozen as above and stored at -80°C for biochemical analysis.

2.3.6. Deformity analysis

Preserved alevins from bin and flume incubators were examined under a dissecting microscope for skeletal (SK), craniofacial (CF) or finfold (FF) deformities, with the severity of deformities categorized based on a graduated severity index (GSI) of 0-3; with 0 representing no apparent deformity and 3 representing a severe deformity that would significantly impair survival, as outlined in Rudolph (2006) [26]. Quality assurance practices were employed, including the establishment of the GSI before examination of samples began, use of a blind labeling system and a quality check performed by an external observer who re-examined at least 10% of all alevins assessed [27].

2.3.7. Biochemical analyses

Frozen alevins were thawed on ice, homogenized in 1 mL of Standard Diluent Assay Reagent (Cayman Chemical Company) and then centrifuged at 1,000 *x g* for 10 min at 4°C. Protein levels were measured [28] using bovine serum albumin as a standard while triglyceride levels were measured in the supernatant using a Triglyceride Colorimetric Assay Kit (Cayman Chemical Company, Item No. 10010303).

2.3.8. Statistical analyses

Differences between control and pesticide treatment groups for hatch and emergence success (% embryos hatched or alevins emerged) were compared with a standard logistic regression model to examine overall treatment effects. If significant results were found ($p<0.05$) post-hoc contrasts were performed using a Bonferroni correction. Time to hatch and time to emergence data were analyzed with a logistic regression model, using percent hatch or emergence as the response variable. This model was used to perform inverse predictions to identify time to 50% hatch (H50) or time to 50% emergence (E50), reported with the 95% confidence interval (CI). Predicted mean H50 or E50 values were then compared using a completely randomized analysis of variance (ANOVA) followed by a Tukey's post-hoc test ($p<0.05$). Growth and biochemical parameters were

compared using a two-way ANOVA with interaction, with fixed effect factors of treatment and cross, followed by a Tukey's post-hoc test ($p<0.05$). Though some cross-treatment interactions were observed, only overall treatment effects will be presented. All analyses were performed in JMP® 10.0.0 and graphs created in Prism 6.

2.4. **Results and Discussion**

2.4.1. Water quality and pesticide exposures

To mimic natural stream conditions, water temperature fluctuated with external ambient temperatures, ranging from 4.5-12.3°C and remaining within generally recommended temperatures for the incubation of eggs of salmon [29]. Dissolved oxygen levels were consistently high, averaging 12.0 mg/L or 102% (range 9.9-14.3 mg/L, 88-114%) while average pH was 7.04 (range 6.48-7.48). In both the bin and flume incubators, water flow varied slightly and therefore pesticide concentrations varied within each of the exposure treatments: minimum, maximum and average predicted pesticide concentrations, based on measured flows for the 199-d exposure period are outlined in Table 1. Water pressure changes occurred in the bin set-up, resulting in short-lived concentration spikes.

Nominal concentrations of atrazine used in this study (25 and 250 µg/L ai) were selected based on those that have been observed in streams and rivers in North America, with concentrations up to 500 µg/L suggested as ecologically relevant [7,19-21]. Nominal concentrations of chlorothalonil (0.5 and 5 µg/L ai) were chosen from limited North American and European reports, with concentrations up to 1.38 µg/L reported in the literature [9,22-24]. Measured concentrations of the active ingredient ranged from 49-63% of nominal, with atrazine concentrations at 15.8 µg/L (nominal 25 µg/L) and 141 µg/L (nominal 250 µg/L), and chlorothalonil at 2.5 µg/L (nominal 5 µg/L) and below the laboratory detection limit of 1 µg/L (nominal 0.5 µg/L). Commercial formulations of atrazine and chlorothalonil appeared to behave similarly in terms of measured compared to nominal concentrations.

2.4.2. Hatch and emergence success

For the first time, effects on embryo and alevin development have been observed in sockeye salmon exposed to environmentally relevant pesticide concentrations. Hatching success (% eggs hatched) was reduced in the high chlorothalonil (CH) treatment group (43.7%), compared to the control group (79.9%) ($p<0.0001$, Figure 2). Due to changes in water pressure, this group of fish was exposed to a handful of pulses of chlorothalonil at concentrations higher than those that have previously been measured in the environment; however, these events were short-lived and could resemble pesticide pulses entering watercourses in the environment resulting from heavy rains and run-off events, a phenomenon some researchers have attempted to replicate in laboratory toxicological tests [14]. Emergence success (% alevins of total buried that emerge from gravel) was not affected in any treatment group compared to the controls.

2.4.3. Growth parameters

Growth parameters, often mass, length and condition factor, represent one of the ultimate indicators of fish health as many biological processes that could be affected by contaminants are encompassed within this endpoint [30]. Wiegand et al. (2001) [31] postulates that embryos, with a limited energy reserve in the yolk, may experience reductions in growth resulting from additional contaminant detoxification requirements.

At hatch, alevin length, weight and condition factor were not significantly different between any treatment group compared to controls (Table 2). Alevin growth at emergence was examined in the both bin and flume incubators. Body lengths were increased in both CL ($p=0.001$) and CH ($p=0.045$) groups in the bin incubators at emergence, but were not affected in any chlorothalonil groups in the flume incubators. Body weight was also increased in the CH group in the bin incubators at emergence ($p=0.0218$), but was reduced in both CL (<0.0001) and CH treated alevins ($p<0.0001$) in the flume incubators. In a previous study, mummichog body lengths and weights were found to be increased after exposure to chlorothalonil, though this was attributed to a lower stocking density of exposed fish compared with the control [32]. Since 55% of sockeye in the CH group in the bins did not successfully hatch, a lower density of these sockeye could also have affected their growth. Atrazine-exposed sockeye in the current

study showed consistent decreases in body weight: in AH fish from the bin incubators ($p<0.001$), as well as AL ($p<0.001$) and AH ($p<0.001$) groups in the flumes (Table 2). Reduced growth in pesticide-exposed fish has been commonly observed [e.g., 33-35]. In a recent review paper, Rohr and McCoy (2010) [20] noted 15 of 17 studies in the literature reported significant reductions or considerable trends to reductions in amphibian size at metamorphosis after exposure to atrazine specifically.

Condition factors integrate body weight and length measurements (weight/length^3) to provide a more sensitive indicator of sublethal disturbances, with a decline in condition thought to be directly related to a decline in energy reserves [30]. Though condition factor was reduced in only the AH treatment group in the bin incubators (94.6% of control, $p<0.0001$), condition factors of exposed sockeye in the flume incubators were all reduced at the time of emergence (AL, 96.2% of control, $p<0.0001$; AH, 94.7% of control, $p<0.0001$; CL, 97.7% of control, $p=0.0002$; CH, 95.3% of control, $p<0.0001$; Figure 3). This finding is striking, as even alevins exposed to environmentally relevant, low pesticide concentrations experienced a reduced condition factor following emergence, which is another incredibly important period for survival affected by alevin size and condition [36]. Fry size at emergence can affect a number of survival factors [37] including swimming ability [38], downstream migration rate [39], migration timing [40] and future growth [41]. Smaller, weaker fry would likely have a smaller energy reserve and be less likely to find and defend a territory to begin exogenous feeding [36,42]. Therefore, the fitness of the emerging salmonid will influence survival during this critical phase, which could result in fluctuations in numbers of an entire population [36,43,34,44].

Observing significantly reduced condition factors in only one of the four treatments in the bin incubators could reflect the importance of examining the energy-demanding process of swimming up through the gravel and its effect on the condition of these salmonids. Alternatively, the alevins from the flume incubators were assessed for growth parameters just over three weeks after those from the bins (at 199 dpf vs 165 dpf in the bins), perhaps indicating that as time progressed exposed alevins depleted more energy stores than control fish as their detoxification processes were maintained, further decreasing the condition of these alevins [30].

2.4.4. Deformity analysis

A deformity analysis was conducted to examine potential disruptions in sockeye morphological development. Atrazine exposure has previously been shown to cause developmental deformities in both fathead minnows [45] and zebrafish [47,31], though examination of deformities in salmonids, to the best of our knowledge, has not previously been conducted. A literature survey reveals that the effects of chlorothalonil on the incidence of developmental deformities have not been previously examined in any fish species. Alevins from both the bin and flume incubators were assessed under dissecting microscope for the presence of skeletal (SK), craniofacial (CF) or finfold (FF) deformities, further categorized using a GSI of 0-3. Skeletal curvatures were relatively common among alevins, with a minor curvature of 15-44° (level 1 on the GSI) affecting 17-27% of all fish in both incubators within all groups, including controls (Figure 4). In the flume incubators, aside from these minor skeletal curvatures, deformities were rare in control and any treatment group (incidence SK>1=0.00%, CF=0.15%, FF=0.08%) with no significant differences observed in incidence of SK, CF or FF deformities for alevins in any treatment group when compared to the controls.

Among fish developing in the bin incubators, no significant differences were observed in the incidence of SK or CF deformities for alevins in any treatment group compared to the controls; however, FF deformities at emergence were higher ($p<0.0001$), at 27.6% in the CH group compared to no such deformities observed in the controls (Figure 4). These deformities manifested mainly as reduced and/or missing anal fins and caudal fin twists. Concentration spikes in the bin set-up during the early embryonic stages of development may have been the cause of the increased incidence of FF deformities observed in alevins of the CH group. The lower incidence of deformities observed in the flume incubators could have resulted from the lower overall average chlorothalonil concentration due to more consistent flow, or, because deformed fish did not successfully emerge from the gravel and were thus not analyzed for deformities, as was postulated in Pilgrim et al. (2013) [15]. Of note, four fish were observed with duplicative deformities, two with two-heads and two others with two-bodies, one of each from AL and AH groups, in both bin and flume incubators (those observed in flume incubators were found buried within the gravel upon experiment termination as they did not successfully complete swim-up).

2.4.5. Biochemical analyses

Whole body protein levels have been postulated to reflect long-term growth of the fish up until that particular time of sampling [46-48], while levels of triglycerides, the main energy storage form in fish, may indicate potential future growth [46,49]. Through a comparison of a number of biochemical indices used to measure condition of juvenile fish, Weber et al. (2003) [46] determined that triglyceride levels were the most sensitive indicator of condition when compared to protein levels as well as several other indices.

In the current study, protein concentrations in whole body homogenate were not consistently affected by any of the treatments at hatch or emergence (Table 3), while triglyceride levels were significantly elevated in CH alevins at hatch ($p=0.0007$) and both CL ($p=0.0084$) and CH ($p=0.0019$) alevins at emergence (Figure 5). Initially, this appears counterintuitive, as chemical detoxification processes require energy and would, in theory, deplete energy stores such as triglycerides [51,30]. However, these elevated triglyceride levels in exposed fish have been observed before, such as in northern pike exposed to uranium mining effluents [52] and adult zebrafish exposed to sublethal levels of 2,3,7,8-TCDD throughout development [53]. The elevation in triglyceride levels is not well-understood, though a handful of mechanisms have been suggested [53]. A reported decrease in locomotor activity of fish exposed to chronic levels of contaminants [54] could result in the conservation of energy stores such as triglycerides [53]. Further examination into the mechanisms behind these elevated triglyceride levels are required.

2.4.6. Hatch timing

In addition to these effects on survival and growth, more subtle alterations to hatch and emergence timing were observed in this study. Premature hatch occurred in the high atrazine group, with time to 50% hatch (H50) values lower than those of the controls (95% CIs for average values across crosses and replicates: Control H50=101.5-102.5 dpf, AH H50=99.5-100.4 dpf [$p=0.011$]; Figure 6). In contrast, time to hatch was increased in the CH treatment group when compared with the control (CH H50=107.0-108.1 dpf [$p<0.0001$]).

Hatch timing has been found to be a phenotypically plastic trait, with a number of factors capable of altering hatch timing in fish species, including oxygen stress [55], infectious

disease outbreak [56], low water flows [57] and even predation cues [58]. Contaminant exposure has been found to alter hatch timing in fish as well, with premature hatch observed found that Atlantic herring embryos exposed to a bitumen-emulsion fuel [59] and Pacific herring exposed to petroleum hydrocarbons [60-62]. Pesticide exposure has resulted in delayed hatch in Japanese medaka exposed to endosulfan [35] and zebrafish exposed to a carbaryl insecticide [63].

In a recent review paper on the effects of atrazine to aquatic organisms, 2 of 3 studies examining amphibian hatch timing showed no effect resulting from atrazine exposure [20,64], while the third study reported a delay in hatch [20,65]. Metamorphic timing in frogs has also been found to be affected by atrazine exposure, with both increased and decreased time to metamorphosis recorded [20,65-74]. Fewer studies have examined atrazine effects on fish hatch timing, though timing alterations were not observed in fathead minnows [45] or zebrafish [47] exposed to atrazine. In the current study, premature hatch resulting from atrazine exposure may appear as an adaptive response, as affected larvae can escape the confines of their egg and move away from the source of contamination, as they have been observed to do in a low oxygen or low flow environment as described above [59,75]. However, the ability of atrazine to induce premature hatch could also be related to its endocrine disrupting properties. Rainbow trout embryos, exposed from the eyed stage to 1 μg/L exogenous 17β-estradiol (E2), the main estrogen compound in fish, experienced significant reductions in time to hatch compared to the controls [76]. This result suggests that E2 levels can affect growth-related pathways in early life stages, which could result in premature hatch through a currently unknown mechanism [76].

In contrast to atrazine, chlorothalonil has received much less research attention and has not shown any indication of endocrine disrupting properties. Only two other reports describing the effects of chlorothalonil on fish early life stages were found in a literature survey, noting decreases in egg hatchability of fathead minnows and mummichog, though no effects on hatch timing were noted [9,32]. The mechanism for delayed hatch in chlorothalonil-exposed fish is unknown, however, similar delays in hatching have been observed in embryos exposed to pesticides [63,35]. It has been suggested that this delay may result from pesticide interference with the hatching enzyme, chorionase,

which breaks down the chorion surrounding the embryo [35,77], but this pathway has not been confirmed.

2.4.7. Emergence timing

Sockeye embryos and alevins develop while buried under 15-30 cm of gravel, to both protect them from predators and ensure they do not wash downstream [17]. Emergence is a critical life-history event defined by the swim-up of alevins out of the gravel once they have completely absorbed their yolk sac and need to switch from endogenous to exogenous feeding. Swim-up is an important test of salmonid fitness [15], with the timing of this major early life event being ecologically significant as it must occur at times of adequate food supply and minimal predation risk [37,78]. Emergence timing can be highly synchronized among alevins within a redd, likely as an anti-predator tactic [79,80]. Despite emergence being a key life stage for salmonids, it is rarely examined in toxicological tests. A literature review reveals only a handful studies which have examined contaminant-induced alterations in emergence timing. The timing of emergence was examined in pink salmon alevins exposed to weathered crude oil, though alevins were placed in gravel-bed incubators after hatch [81]. Pilgrim et al. (2013) [15] examined emergence success and timing of alevins from adult spawners who had been exposed to selenium. The majority of toxicity testing on early life stages of salmonids has been performed on salmonid embryos and alevins in Heath trays or open tanks without gravel substrate, with emergence timing defined by the approximate date of yolk sac absorption. Without the use of gravel substrate during development, assessment of the subtle but critical sub-lethal effect of emergence timing would be missed.

Exposure to both pesticides, at both low and high concentrations, resulted in premature emergence, with the initiation of emergence in the control group alevins lagging several days behind all exposed alevins (95% CIs for average values across crosses and replicates: Control E50=179.1-182.1 dpf, AL E50=172.7-175.3 dpf [p=0.0073], AH E50=173.5-176.2 dpf [p=0.013], CL E50=174.1-176.8 dpf [p=0.021], CH E50=172.9-175.6 dpf, [p=0.0085]; Figure 7). Though the mechanism that triggers swim-up of alevins out of the gravel is not well-understood, alevins are known to have the ability to alter their emergence behaviour, emerging earlier in response to low oxygen levels, changes

in temperature or the presence of predators [82,83]. It is also possible that detoxification processes result in a quicker depuration of limited energy resources [30], causing a premature emergence of the alevins as they leave their gravel substrate to find exogenous food sources. Regardless of the cause, without examining the effects of these pesticides on sockeye early life stages in gravel-bed flume incubators to replicate a natural streambed environment, these subtle changes in emergence timing, that affect survival of individual salmonids and potentially population viability, simply cannot be measured. The development of alevin in gravel-bed incubators allows for the natural swim-up process to occur, and any alterations in this critical behaviour caused by contaminant exposure to be observed.

2.5. **Conclusion**

The present study aimed to examine the effects of two currently-used pesticides in an environmentally relevant way: by examining the effects of the a commercial formulation instead of an isolated active ingredient; by exposing sockeye to concentrations of these pesticides that are similar to those they may encounter in the aquatic environments in North America; and by including the endpoint of swim-up success and timing, as alevins must complete this early life fitness test to enter the water column in their natural stream environment. The use of a gravel-bed flume incubator system allowed for the examination of the timing and success of the critical life stage of emergence, which has been consistently overlooked in classic open-water toxicological testing. With regards to government regulation of these compounds, a direct effect on growth and mortality was observed. Additionally, the timing of a critical life stage, emergence, was altered in a way that may ultimately effect the survival of these sockeye. Both the environmentally relevant low and high concentrations of commercial formulations of atrazine and chlorothalonil adversely affected the development of sockeye salmon and ultimately impacted the survival of early life stages of these salmonids.

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2.8. **Tables**

Table 1. Nominal and measured concentrations of pesticides in exposure chambers from both bin and gravel incubation set-ups.

	Pesticide Concentration (μg/L)				
	Target	Predicted Based on Water Flow [a]			Measured[b]
		Average	Minimum	Maximum	
Bins					
ALB1	25	27.3	22.6	98.7	
ALB2	25	28.2	18.8	93.8	
AHB1	250	273.5	210.7	1562.5	
AHB2	250	296.5	195.3	1875.0	
CLB1	0.5	0.52	0.44	0.99	
CLB2	0.5	0.61	0.44	3.13	
CHB1	5	5.2	4.2	10.7	
CHB2	5	5.5	4.4	46.8	
Flumes					
ALG1	25	25.3	21.6	31.8	
ALG2	25	24.4	21.0	31.8	15.8
AHG1	250	260.6	203.8	468.8	
AHG2	250	252.2	223.2	329.0	141
CLG1	0.50	0.50	0.44	0.63	ND[c]
CLG2	0.50	0.52	0.39	0.69	
CHG1	5	5.1	4.4	7.5	
CHG2	5	5.1	4.0	5.7	2.5

[a]Predicted concentrations calculated from flow of pesticide stock solution into exposure compartment (pump speed remained constant, therefore flow of stock solution was constant) and measured water flow rates through exposure compartments. Water pressure varied within the set-ups, therefore, individual compartment flow rates were measured and readjusted every 48 h.

[b]Pesticide concentrations measured in water samples taken February 13, 2013.

[c]Not detected (ND). Concentration below lab detection limit for chlorothalonil, or <1.0 μg/L.

Table 2. Weights and lengths of sockeye alevin at hatch and emergence in all treatment groups.

Note: SE = standard error; n=number of alevin measured; Hatch=alevin measured at hatch; EB=alevin from the bin incubators measured at emergence; EF=alevin from the flume incubators measured at emergence; *=significantly different from control group (two-way ANOVA with Tukey's post-hoc test, $p<0.05$)

	Control			Atrazine Low (AL)			Atrazine High (AH)			Chlorothalonil Low (CL)			Chlorothalonil High (CH)		
	Mean	***SE***	***n***	***Mean***	***SE***	***n***	***Mean***	***SE***	***n***	***Mean***	***SE***	***n***	***Mean***	***SE***	***n***
Length (mm)															
Hatch	20.0	0.8	29	20.0	0.7	30	20.4	0.7	29	19.9	0.8	30	20.7	1.1	16
EB	31.3	0.7	350	31.3	0.7	489	31.4	0.7	434	31.6*	0.6	345	31.6*	0.8	216
EF	31.5	0.7	226	31.4	0.6	405	31.4	0.6	374	31.4	0.6	384	31.7	0.7	390
Weight (g)															
Hatch	0.158	0.012	29	0.158	0.008	30	0.158	0.010	29	0.160	0.010	30	0.168	0.010	16
EB	0.206	0.009	350	0.205	0.010	489	0.198*	0.010	434	0.207	0.009	345	0.215*	0.011	216
EF	0.214	0.009	226	0.203*	0.012	405	0.200*	0.014	374	0.205*	0.013	384	0.207*	0.013	390

Table 3. Protein and triglyceride levels measured in whole body homogenate of sockeye alevin at hatch and emergence.

	Control			Atrazine Low (AL)			Atrazine High (AH)			Chlorothalonil Low (CL)			Chlorothalonil High (CH)		
	Mean	***SE***	***n***	***Mean***	***SE***	***n***	***Mean***	***SE***	***n***	***Mean***	***SE***	***n***	***Mean***	***SE***	***n***
Protein (mg/mL/g bw)															
Hatch	145.9	22.2	28	146.5	41.8	29	142.8	20.4	26	153.1	17.0	29	147.7	19.8	15
Emergence	34.3	6.1	15	32.5	6.5	15	35.0	7.0	15	35.1	5.0	15	32.2	5.1	15

Note: SE = standard error; n=number of alevin measured; Hatch=alevin measured at hatch; EB=alevin from the bin incubators measured at emergence; EF=alevin from the flume incubators measured at emergence; *=significantly different from control group (two-way ANOVA with Tukey's post-hoc test, $p<0.05$)

2.9. **Figures**

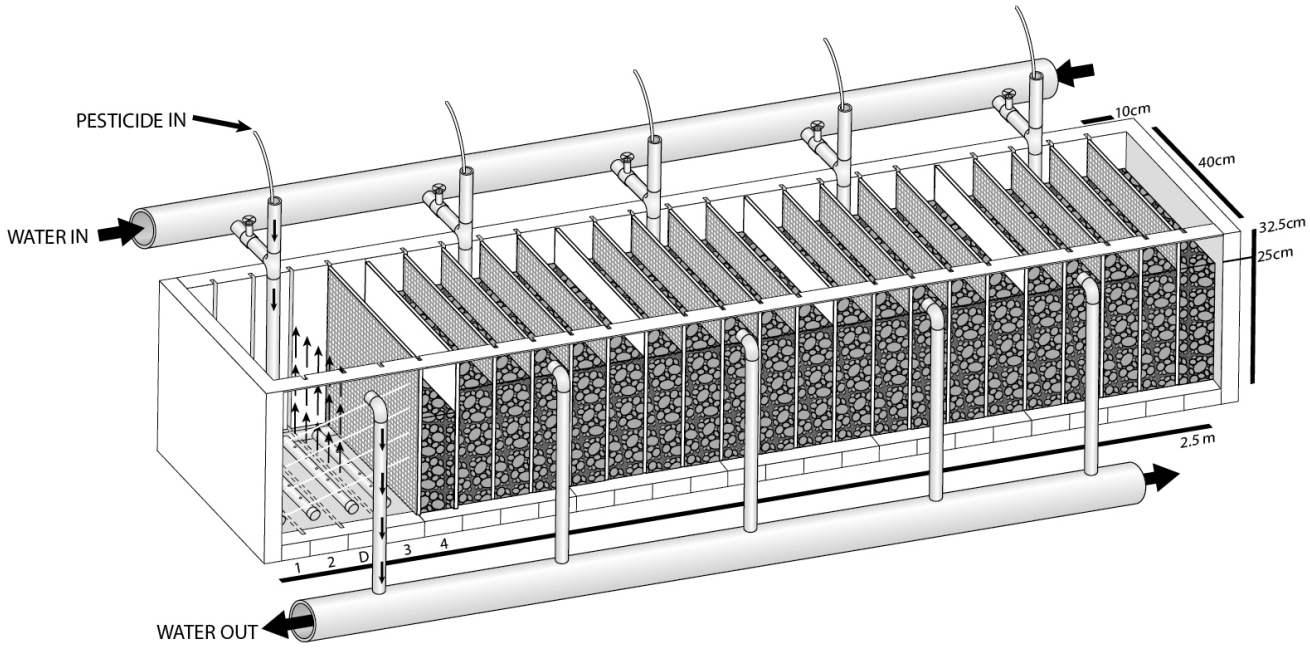

Figure 1 ***Gravel-bed flume incubator system used to examine effects of pesticide exposure on success and timing of sockeye salmon alevin emergence.***

Figure produced by Stephen DeMuth of SFU Creative Design (April 2014).

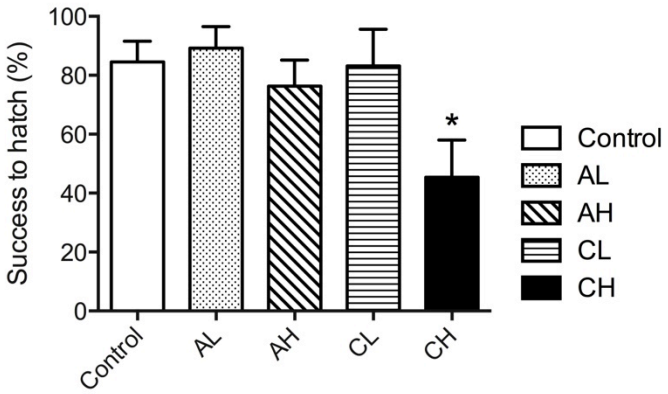

Figure 2. ***Sockeye eggs exposed to high chlorothalonil (CH) from fertilization have reduced hatch success.***

Hatch success of all treatment groups (low atrazine [AL], high atrazine [AH], low chlorothalonil [CL] and high chlorothalonil [CH]) represented by mean ± standard error. Statistically significant differences from controls indicated (*)(CH, $p<0.0001$; standard logistic regression followed by contrasts using Bonferroni correction).

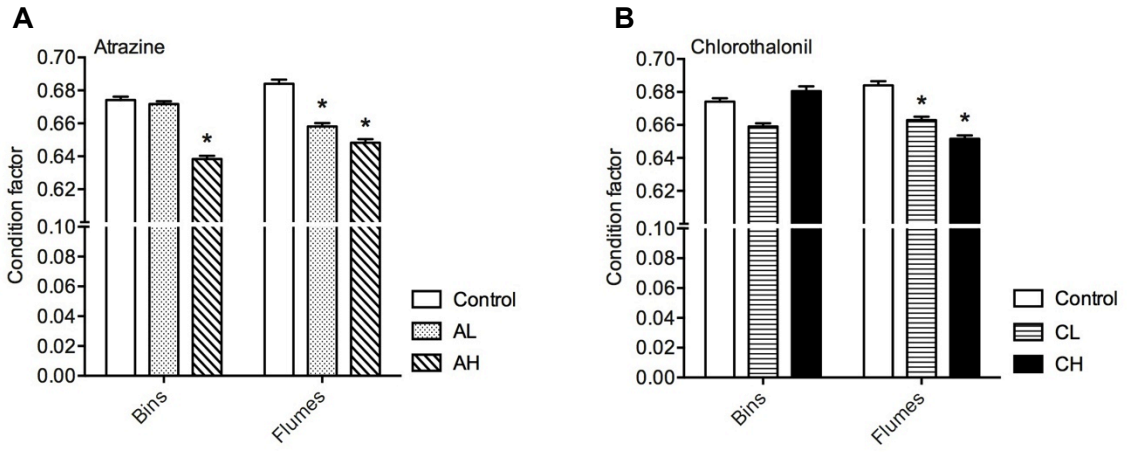

Figure 3. Sockeye alevin condition factor was reduced in all treatment groups (low atrazine [AL], high atrazine [AH], low chlorothalonil [CL] and high chlorothalonil [CH]) when exposed from fertilization to emergence.

In the bin incubators, condition factor was reduced in AH group only ($p<0.0001$), but was reduced in all treatment groups in flume incubators (AL $p<0.0001$, AH $p<0.0001$, CL $p=0.0002$, CH $p<0.0001$; two-way ANOVA with Tukey's post-hoc test, $p<0.05$). Condition factor values presented for atrazine- (A) and chlorothalonil-exposed (B) sockeye as mean ± standard error. Statistically significant differences from controls indicated (*).

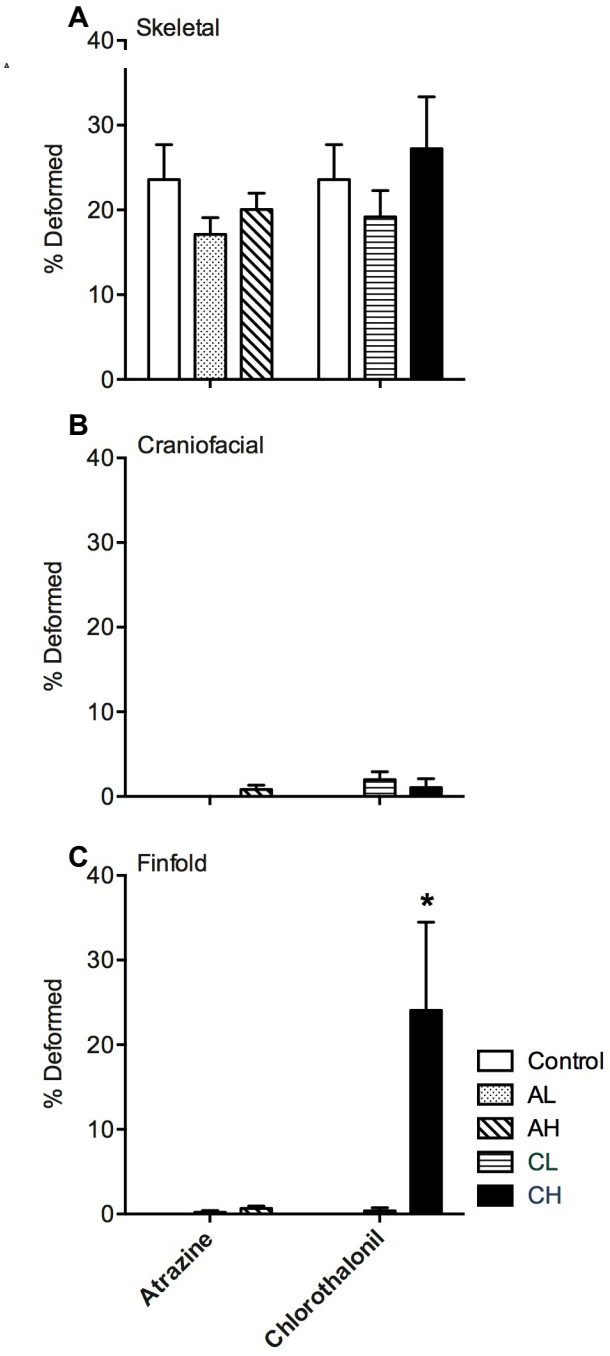

Figure 4. Sockeye alevin finfold (FF) deformity rates increased in high chlorothalonil (CH) treatment group at the time of emergence.

Rates of skeletal (SK; A), craniofacial (CF; B) and FF (C) deformities in a total of 1682 alevins examined from the bin incubators, presented as means ± standard error. Statistically significant differences from controls indicated (*) (FF in CH group, $p<0.0001$; two-way ANOVA with Tukey's post-hoc test).

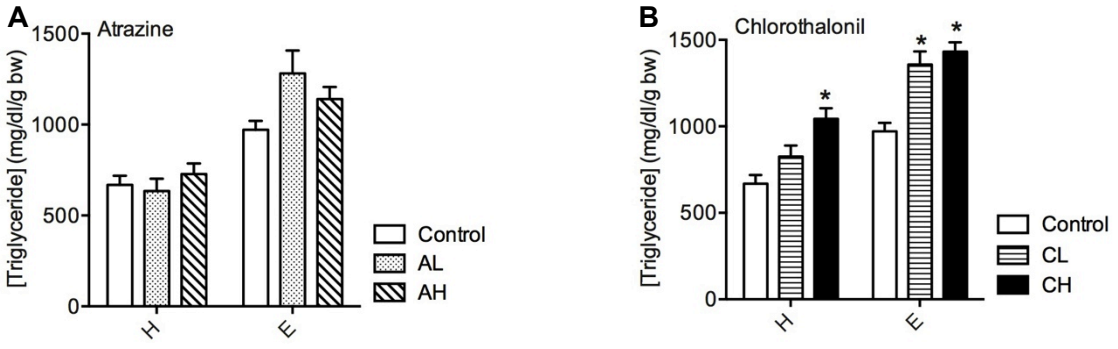

Figure 5. Sockeye alevin whole body triglyceride concentrations increased in low (CL) and high (CH) chlorothalonil groups at hatch and emergence.

Triglyceride concentrations in whole body homogenate, per gram alevin body weight, are presented for atrazine (low, AL, and high, AH, concentrations) (A) and chlorothalonil (B) treatment groups as means ± standard error at both hatch (H) and emergence (E). Statistically significant differences from controls indicated (*) (CH at hatch, p=0.0007; CL at emergence p=0.0084; CH at emergence p=0.0019; two-way ANOVA with Tukey's post-hoc test).

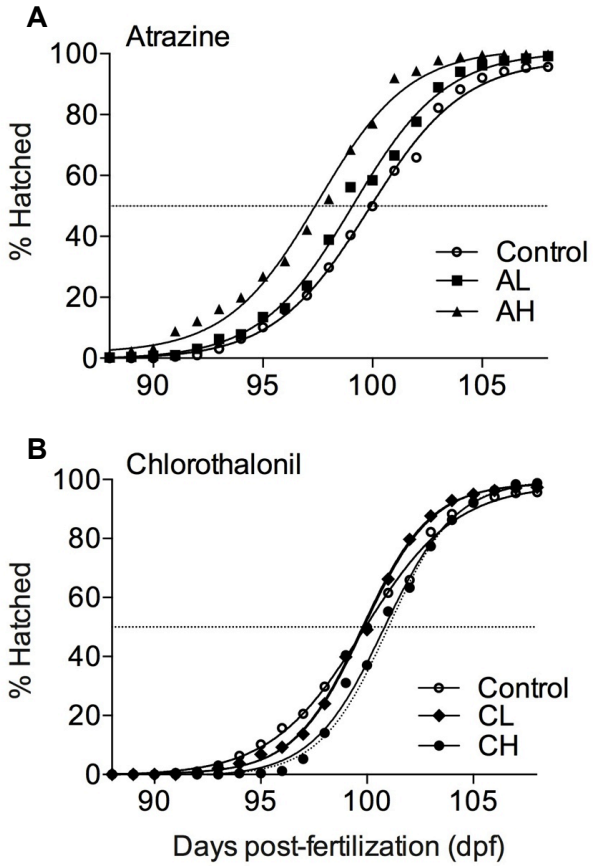

Figure 6. Time for sockeye eggs to reach 50% total hatch was decreased in high atrazine (AH) and increased in high chlorothalonil (CH) treatment groups.

Time (in days post-fertilization [dpf]) for sockeye eggs to reach 50% hatch (H50) was reduced in those exposed to a high atrazine concentration (AH 95% CI H50=99.5-100.4 dpf, p=0.011) and increased in those exposed to a high chlorothalonil (CH 95% CI H50=107.0-108.1 dpf, p<0.001) concentration when compared to the controls (control 95% CI H50=101.5-102.5 dpf). Points represent the percent hatched (presented as the number of eggs hatched of the total that did hatch), overlaid with a logistic regression model used to inversely predict the H50 (represented by the horizontal line) for each treatment (control, atrazine low [AL], AH, chlorothalonil low [CL] and CH).

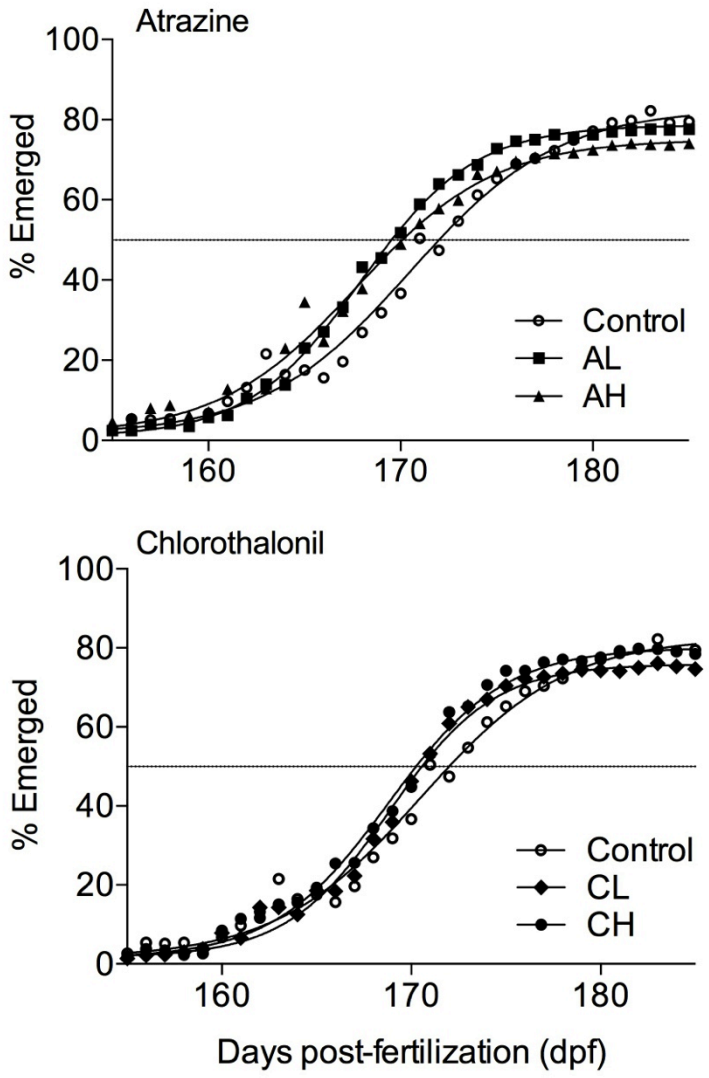

Figure 7. Time for sockeye alevin to reach 50% emergence was reduced in all treatment groups (low atrazine [AL], high atrazine [AH], low chlorothalonil [CL]) and high chlorothalonil [CH]).

Time (in days post-fertilization [dpf]) for sockeye alevins to reach 50% emergence (E50) was reduced in all treatment groups compared to the controls (control 95% CI E50=179.1-182.1 dpf; low atrazine [AL] E50=172.7-175.3 dpf [p=0.0073]; high atrazine [AH] E50=173.5-176.2 dpf [p=0.013]; low chlorothalonil [CL] E50=174.1-176.8 dpf [p=0.021]; high chlorothalonil [CH] E50=172.9-175.6 dpf [p=0.0085]). Points represent the total percent of alevins that swim-up on a given day (presented as a percentage of the total number of eggs buried in each group) overlaid with a logistic regression model used to inversely predict the E50 (represented by the horizontal line) for each treatment.

3.

Conclusions and Future Research

For the first time pesticide exposure has been shown to affect the early development and growth of sockeye salmon. Overall fitness at the time of emergence, represented by condition factors, was reduced in alevin exposed to environmentally-relevant concentrations of both commercial atrazine and chlorothalonil formulations. Flow-through gravel-bed incubators were used to assess alterations in emergence timing and success, which are important factors affecting the survival of newly emerged sockeye fry in their natural stream environment. These parameters are often overlooked in classic toxicological testing protocols. Without the employment of this type of environmentally-realistic incubation system, the potential effects of these pesticides on swim-up timing would not have been observed and further survival implications of premature emergence would not be considered in assessing their impacts on salmon. The use of commercial formulations of both atrazine and chlorothalonil does not allow for the isolation of effects caused specifically by the active ingredient, however, these formulations are those being used and contaminating aquatic environments following application.

Both the low and high concentrations of two very different pesticide formulations caused the premature emergence of alevins. Examining the effects of other contaminants, in addition to mixtures of contaminants, on sockeye salmon emergence timing is important and necessary to gain further insight into potential effects that are occurring within their natal streams. Characterization of concentrations and temporal variations of these compounds in Fraser River sockeye salmon spawning streams would allow for an even more meaningful and environmentally-relevant toxicological assessment to be undertaken. In the bin and flume incubator systems used, the employment of a more sophisticated water pressure regulation device would ensure a consistent water flow and constant pesticide exposure concentration. However, in nature, pesticide concentrations are likely to vary and enter aquatic ecosystems in pulses.

In laboratory toxicological testing, rainbow trout are commonly used as a representative salmonid; however, it is also important to examine the differences in sensitivities between salmonid species and to ensure that rainbow trout are suitable as a representative test species. Understanding the impacts of long-term pesticide exposure on the fitness and survival of sockeye beyond the alevin stage would further assist in understanding long-term implications of the current results of altered developmental timing and reductions in condition factor at emergence.

In light of recent declines to Fraser River sockeye salmon stocks, the importance of collecting evidence regarding potential contributing factors has never been greater. Although the effects of contaminants were considered by the Expert Advisory Council of the Cohen Commission to be unlikely contributors to the decline, limited data on contaminant levels and the complex nature of sockeye life history and potential effects at multiple life stages make conclusions about the role of contaminants highly uncertain [1, 2]. Fisheries and Oceans Canada was unable to rule out the effects of contaminants on Fraser River sockeye declines simply because of a lack of data and indicated that contaminant exposure may be contributing to sockeye declines in a way that is more subtle and difficult to identify [1].

While much of the research has been performed on juvenile and smolt life stages of salmonids, the current research contributes to one of many relevant data gaps, by examining the effects of two current-use pesticides on early life stages and development of sockeye salmon. Attempts to maximize environmental realism in laboratory setting were made in all components of the incubation, including the use of flow-through gravel-bed flumes, exposure to commercial formulations of pesticides, long-term exposure to relevant concentrations from fertilization through to emergence and the use of the actual species from the stock at risk, instead of a surrogate test species. While the current study has revealed exposure in these conditions to commercial formulations of atrazine and chlorothalonil result in adverse effects to sockeye salmon development and growth, more research is required to continue to fill the gap in knowledge on the effects of contaminants, including pesticides, on all developmental stages of sockeye salmon, including sensitive early life stages.

3.1. **References**

[1] Cohen Commission. 2012.The Uncertain Future of Fraser River Sockeye. Volume 2: Causes of the Decline.

[2] Ross PS, Kennedy CJ, Shelley LK, Tierney KB, Patterson DA, Fairchild WL, Macdonald RW. 2013. The trouble with salmon: relating pollutant exposure to toxic effect in species with transformational life histories and lengthy migrations. *Can J Fish Aquat Sci* 70:1252-1264.

4. Appendices

Appendix A.

Supplemental Information

Table A1. Temperature, pH, dissolved oxygen and ammonia levels in dechlorinated water used in incubation set-ups over the course of the incubation period.

Water Quality Parameter	n	Min	Max	Mean	Standard Deviation
Temperature	101	4.5	12.3	6.8	2.1
pH	24	6.5	7.7	7.0	0.2
Dissolved oxygen (%)	96	88	114	102	4
Dissolved oxygen (mg/L)	96	9.9	14.3	12.0	0.83
Ammonia	3	All ammonia level readings “ideal”			

Note: n=number of measurements taken, min=minimum measurement over exposure period, max=maximum measurement over exposure period