Whole System Perspective: Steelhead, *Onchorhynchus mykiss*, Glutathione S-Transferase as a Biomarker of Chlorpyrifos Exposure

by

Rachael G. Pecore

A PROJECT

submitted to

Oregon State University

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in partial fulfillment
of the requirements for the
degree of

Honors Bachelors of Science in BioResource Research (Honors Scholar)
and
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Presented May 30, 2001
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AN ABSTRACT OF THE THESIS OF

Rachael G. Pecore for the degree of Honors Baccalaureate of Science in BioResource Research presented on May 30, 2001. Title: Whole System Perspective: Steelhead, *Onchorhynchus mykiss*, Glutathione S-Transferase as a Biomarker of Chlorpyrifos Exposure.

Abstract approved:

Ray D. Williams

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Jeff Jenkins

Organophosphate insecticides may produce sublethal, adverse effects on salmonid growth, reproduction, maturation, swimming, and feeding. Chlorpyrifos, an organophosphate insecticide, was detected by the Oregon Department of Environmental Quality in the Hood River and tributaries during March 1999 at levels exceeding state water quality standards in the range of 0.011-0.482 μg/L. This timing overlaps with the early life stage development of winter steelhead populations listed as threatened on the Endangered Species Act. Hatchery steelhead were exposed in lab experiments to chlorpyrifos levels within the range detected in Hood River. Glutathione s-transferase activity in the liver was measured as a potential biomarker of exposure to complement biomarker measurements of acetylcholinesterase activity in the brain. Glutathione s-transferase was significantly inhibited after 24 hour exposure, and there was no dose-response after 96 hour exposure. Correlating internal exposure to a sublethal effect, and then a sublethal effect to a declining population, will take many years of research. There are many potential causes of the population decline, interacting and accumulating on a temporal scale of over 150 years and a spatial scale from the Hood River to seas fished by Japan, Canada, and the USA. Drawing relational diagrams in conjunction with standard research encourages the search for component interactions, causal flows, patterns, loops, temporal and spatial consequences and leverage points within the whole system affecting the steelhead population decline.

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Rachael Geneva Pecore

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Contribution of Authors

Sandahl, Jason F.	Technical support: experimental design and implementation of steelhead exposure and liver extraction, critical review of materials and methods, data, and manuscripts.

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Introduction

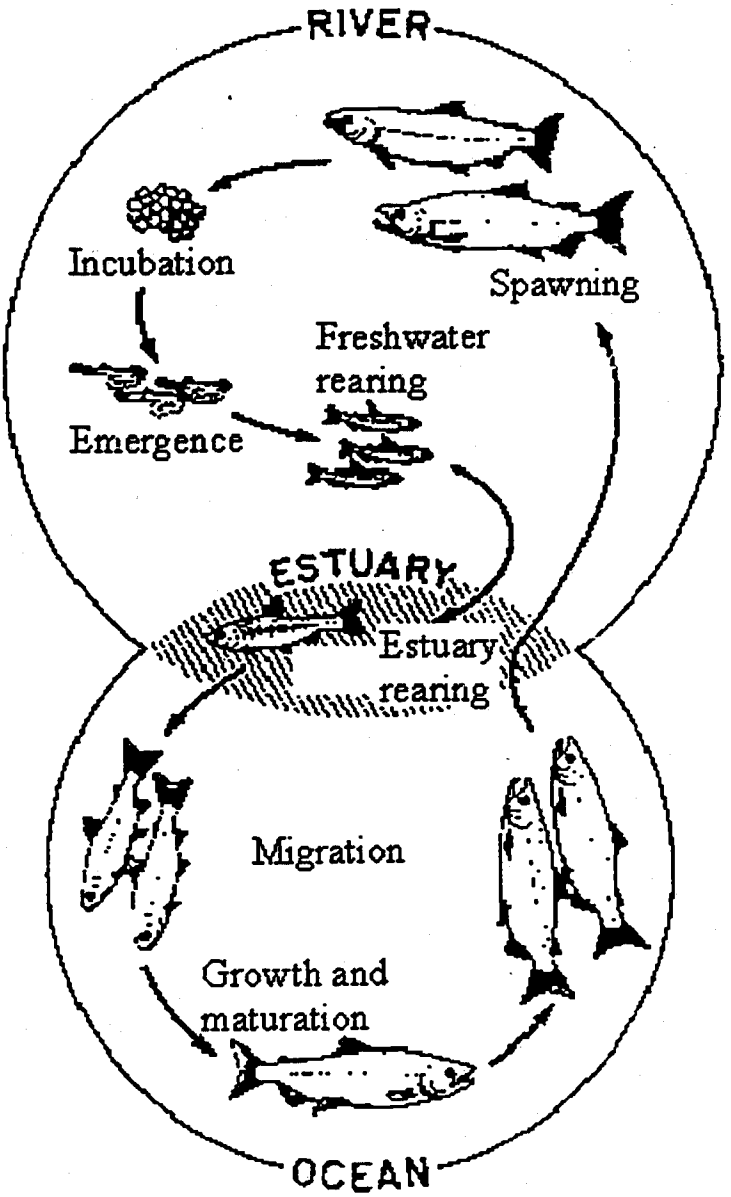
Once upon a time under a winter-coated stream, lay 7,000 eggs nested safely between the gravel of a recent spawning ground (Figure 1). Not long ago, a female salmon had prepared the nest with a swish of her tail and permitted her chosen mate to fertilize them. Their duty as parents completed, life soon faded from them as their bright scales had on the journey upstream (Figure 2).

Housed in interstitial spaces among gravel bits, oxygen flowed freely over the eggs and predators overhead remained oblivious of their presence. The young salmon, or alevins, subsisted for a few weeks off their yolk sacs but were soon driven by hunger to sample the waters above. Emerging from the redd, a 2-3 inch body termed fry, was no match for the streamflow and was swept downstream, over a riffle where the roller coaster ride ended abruptly in a cool, oxygenated pool behind a dead tree. Among the foliage and organic matter trapped behind the log, insects abounded like sweets at a fair and were given preference by the fry over other food items. With its eye on the shadow of a large stonefly above, the fry wriggled up towards the light but darted just in time from the lethal lunge of a frog. No matter, benthic invertebrates suited the fry just as well.

Becoming brave in its hunt, the fry skirted the edge of the pool and was swept further downstream into another pool. The temperature was outside the comfortable 12-16°C range so the fry dove deeper narrowly avoiding the jaws of a grown trout. Turbidity rendered vision useless, but the fry navigated with its keen sense of smell. There were

Figure 1. Potential Factors for Winter Steelhead Decline in the Lower Columbia Watershed

Figure 2. Salmon Life Cycle (Snapshot of Oregon, 1998)



few aquatic invertebrates in the pool which was just as well since the fry wasn't hungry (NMFS 1996).

One day there was a clamor above and the fry was surrounded by thousands of frantic, disorientated salmonids poured from a large tube. Large beaks struck from above and the clamor subsided as predators and water flow dispersed the newly introduced hatchery fish. Insects were scarce that day so the fry moved downstream.

The days passed, winter rains were eased by the spring glacial and snow melt followed by summer droughts. The fry's body adjusted to changes in flow, temperature, and chemical constituents, expending energy avoiding walleye, squawfish, herons, and other predators and gathering energy from aquatic invertebrates. It had been over a year of stream life in Oregon's tributaries, and it was time to explore the rivers beyond. The fry was entering salmonhood, its body began to turn silvery in color and its spots began to fade as pimples fade with maturity. The fry had become a smolt.

Spring melt and rains carried the smolt downstream towards the Columbia with heightened intensity, and its fitness was measured by countless dangers. Once, the smolt was nearly diverted into an adjacent farmer's field but was saved by the slap of a screen against its body. The experience was repeated when passing noisy cities where large quantities of water were diverted. Travel was slowed by large bodies of still, warmer water where predators and disease lurked under the jet skis and summer fun of humans at the reservoir. Many other smolts, stranded by immune systems weakened by the migration stresses, were lost to disease and infection.

Preferring cooler, well-oxygenated water, the smolt stayed low until a huge cement wall made navigation useless. Swimming toward the surface, the smolt was

subjected to an intense flow, and was nearly swept through the lethal dam turbines but for the interception of a screen. The smolt was shunted through tubes resembling an elaborate water park and released below the dam where they were crowded into barges and trucks to be carried past remaining dams. Smolts that were not intercepted by the bypass system and flung over spillways, were lucky this year as a "flip lip" had been installed reducing turbulence and therefore dissolved gases. Stunned survivors had reduced cases of nitrogen bubbling, similar to the bends, but in that state they were easy prey. In drought years, water became precious and instead of being diverted over spillways many smolts were hurled through the lethal, injurious or stressful spinning blades of the turbines (NMFS 1996).

Survivors congregated in a strange, new medium where familiar smells of the river were twice daily replaced by salty constituents. Some smolts were weighed down by heavy metals in their system and found the transition to saltwater difficult. The estuary was stocked with salmonid foods and many smolts extended their girth and length for a short while before heading out beyond the island of hungry Caspian terns into the wide ocean beyond. In the ocean the salmon dispersed, chinook and chum stayed close to shore while coho and steelhead powered on 2,000 miles offshore towards the gulf of Alaska. In the sea the salmon were at the mercy of ocean circulation patterns regulated by the Pacific decadal oscillation, el niño/la niña, the aleutian low and coñastal upwelling.

Offshore, squid, shrimp, herring and pelagic worms were added to their diet, and to avoid the diets of sea lions, orcas, and humans, they formed schools. El niño and intense aleutian lows encouraged periods (8-14 months) of low upwelling and found phytoplankton and zooplankton scarce, resulting in low food production for steelhead

(Mantua and Pearcy 2000). Low productivity translated into slow growth increasing their vulnerability to predation. Low upwelling brought warmer waters that welcomed subtropical species such as mackerel and whiting up the coast to compete for an already diminished food supply. These warm ocean conditions could last up to 30 years under the cycle of the Pacific decadal oscillation.

In the high seas past the 200 mile U.S. border there were times when the gills of salmon were caught in old floating nets, remnants of the gillnetting days or by recent illegal fishers. At other times migration patterns were altered due to strong patches of pollutants, perhaps the remains of chemicals leached from old barge-loads of inland garbage. Two years or more passed and many salmon, overwhelmed by homesickness, left the ocean-life to return to the small-time stream of their birth. Congregating once again into estuaries they waited for rain to bring ample waters opening the passage upstream. Concentrating their energies swimming upstream they ceased to eat.

Slowly working their way upstream salmon again faced the dangers they had evaded before (high water temperatures, disease, sedimentation, unsuitable habitat for resting, blocked or inhibited passage, predation and stress). With their keen sense of smell they passed familiar sites: the mouth of the Willamette and its heavy metals, the storm water outlet pouring in smells of oil and household pesticides, the golf course and more pesticides, the aluminum smelt factory and fluoride concentrations, the old mining site, they were all familiar. For those salmon that had been barged down the river a number of dams were unfamiliar. Working their way up fish ladders rather than the falls their ancestors had climbed, the salmon were being watched. Miniaturized "passive integrated tranponders," or PIT tags had been inserted into over one million juvenile

salmon and steelhead at dams, hatcheries, and streams that now emitted an electronic signal when passing scanners mounted in several fish ladders (Brinkman 2001).

Unbeknownst to salmon they had achieved movie star status, and many Pacific Northwesterners followed their every move through the media. Fan clubs organized themselves into varying degrees of support for the fish (Appendix A) under names like the National Marine Fisheries Service, Washington and Oregon Departments of Fish and Wildlife, Columbia River Inter-Tribal Fish Commission, Bonneville Power Administration, Salmon for All (Commercial fishers), Northwest Sport Fishing Industry Assn, and the Columbia River Alliance (Industry support group), For the Sake of Salmon (Conservation interests), International Pacific Salmon Fisheries Commission, and local watershed councils.

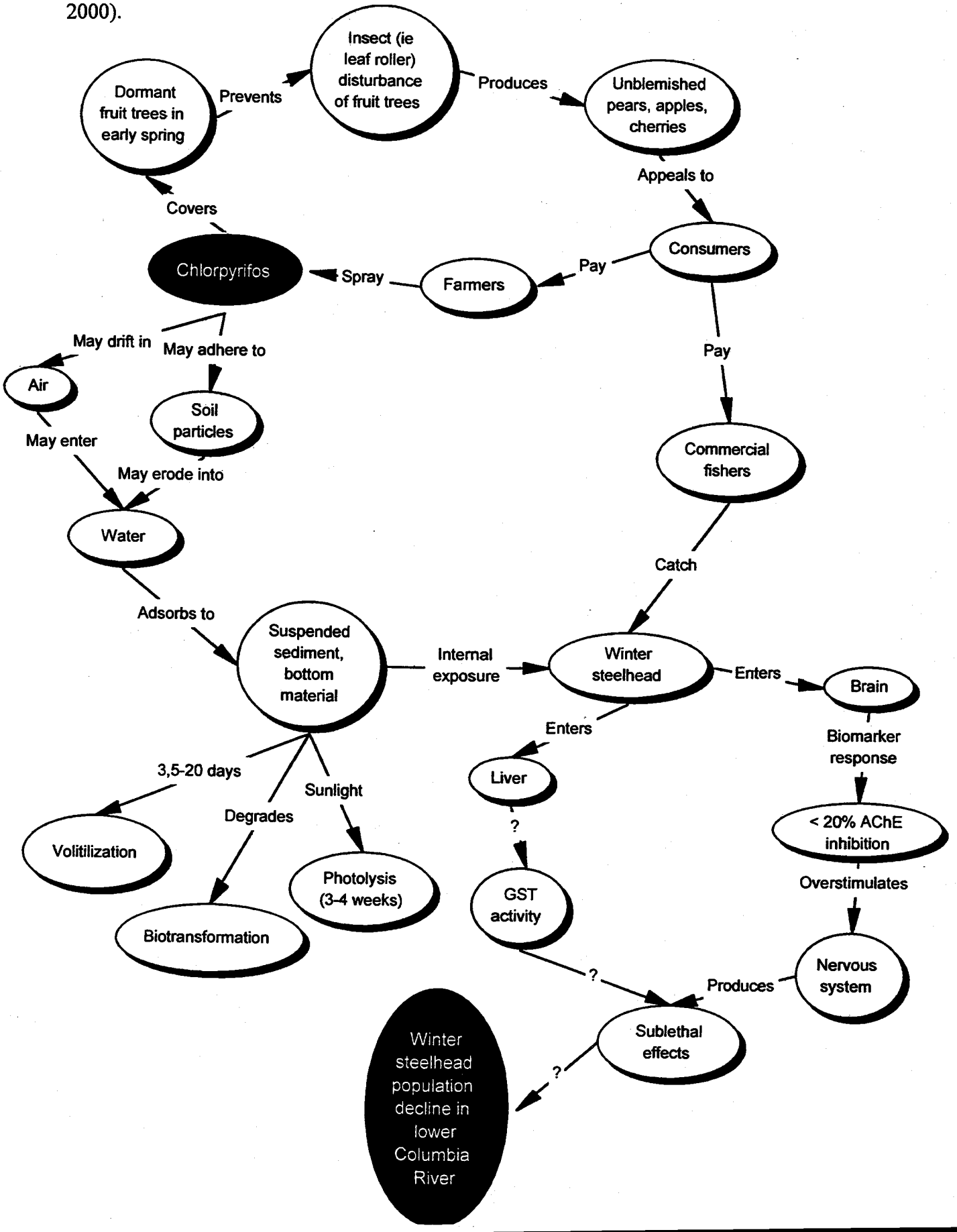
As the groups discussed ways to increase salmon populations, chinook chum, coho, sockeye, and steelhead all became listed as threatened in various reaches of the Columbia. Fewer and fewer wild salmon returned to the old gravel redds to transform the stream into a spawning ground once again. Depositing or fertilizing their eggs, the old salmon left their bodies in the water as nutrients for over 22 species (Duncan 1998), and if sedimentation didn't adhere to the chorion of eggs, or fill in the interstitial spaces reducing oxygen to the redd, fry would soon emerge (NMFS 1996).

On one particular day in early March, in a tributary to the Hood River in Oregon, a fry was wriggling around doing its business as was a fruit farmer upstream. The farmer was donning safety equipment in preparation to spray pear, cherry, and apple trees with an organophosphate insecticide called chlorpyrifos. The chemicals weighed on the farmers budget, but the cost/benefit ratio leaned favorably towards spraying due to an

increase in the quality of the produce. Consumers pay more for unblemished fruit. The farmer was whistling as this particular day was a fine one, but the opinion would change that evening while reading the Hood River Growers and Shippers Assoc. Newsletter. Apparently scientific studies had correlated adverse sublethal effects in salmon with internal exposure to organophosphates (OPs) (Zinkl et al. 1991, Grue et al. 1991), a class of insecticides to which chlorpyrifos belongs. The farmer feared satisfying consumer demand for unblemished fruit and a plentiful salmon supply would provoke an irreconcilable controversy between agricultural and fishing interests (Figure 3). Is chlorpyrifos a factor contributing to the Columbia River salmon decline? How can this question be answered?

Using the standard approach to investigate this question essentially entails disaggregating the effects of each of the elements in the situation (Figure 1), and thereby identifying the discrete contribution of each element or aggregation of similar units. The standard approach depends on the assumption that the isolation of each element does not affect how the elements interact as a whole (Clayton and Radcliffe 1996). Can adverse effects of chlorpyrifos in salmon be isolated and correlated with the population's decline amidst a plethora of factors for decline? A systems approach takes the standard approach a step further by placing as much emphasis on identifying and describing **connections** between objects and events, as on identifying and describing the events themselves (Clayton and Radcliffe 1996). Determining the effects of chlorpyrifos in Columbia River salmon at the population level will require research beyond the scope of this project; my objective is to identify one component of the whole system without making the assumption that isolating the component will not affect the component's behavior.

Figure 3. Cause and Effect Relationships between Chlorpyrifos, Agricultural and Salmon Fishing Interests. (Derived from Sandahl, 2001 and Extension Toxicology Network 2000).



Drawing relational diagrams (Figure 1), encourages the search for component interactions, causal flow, patterns, loops, and temporal and spatial consequences (William 2000) that may potentially affect the components behavior in the whole system.

Chlorpyrifos was detected by the Oregon Department of Environmental Quality (DEQ) in the Hood River and tributaries during March 1999 at levels exceeding state water quality standards (acute 0.083 µg/L, chronic 0.041 µg/L) in the range of 0.011-0.482 µg/L (Foster 1999). Chlorpyrifos is of particular concern due to the timing of its use, and its extreme toxicity to most freshwater fish and aquatic invertebrates (US EPA 1989). Chlorpyrifos is applied as a dormant spray in a concentrated effort in the first few weeks of early spring to control root-infesting and boring insects on pear, apple and cherry trees. This timing overlaps with the early life stage development of winter steelhead populations listed as threatened on the Endangered Species Act, and there is potential for exposure due to agricultural run-off following rainfall.

Organophosphate insecticides (OPs) inflict toxicity primarily by inhibiting acetylcholinesterase (AChE), an enzyme vital to normal nerve function that hydrolyzes the neurotransmitter acetylcholine (ACh) (Coppage and Matthew 1974). Chlorpyrifos is highly lipophilic and can accumulate at nerve synapses and neuromuscular junctions. Hyperexcitability occurs at these junctions producing isolated muscle twitches, tremors, convulsions, and occasionally paralysis (Chambers and Levi 1992). The 96 hour lethal concentration (LC 50) for chlorpyrifos in juvenile rainbow trout is 8 µg/L; lethality occurs at 60-70% AChE inhibition (Holcombe et al 1982). Chlorpyrifos levels in the Hood River are not acutely lethal to salmonids. However, studies show that at 20-50%

AChE inhibition organophosphates may produce sublethal, abnormal effects on growth, reproduction, maturation, swimming, feeding and cause hyperactivity (Zinkl 1991, Lockhart 1985, Jarvinen, 1983, Brewer 2001).

To link the presence of a toxicant to a sublethal effect the availability of chlorpyrifos from the environment into steelhead must be quantified. A biomarker measurement is defined by the National Academy of Sciences as a measurement of xenobiotically induced alterations in cellular or biochemical composition or processes, structures or functions in a biological system or sample (Klassen 1996). Through a dose-response relationship, biomarkers quantify biologically available pollutants and the susceptibility of organisms to these pollutants. There are two types of biomarkers, biomarkers of effect and exposure. A biomarker of exposure measures the presence of a xenobiotic substance or its metabolite, or the product of an interaction between a xenobiotic and a target molecule or cell (Klassen 1975). A biomarker of effect is any measurable biochemical, physiological, or other alteration within an organism that can be recognized as a health impairment or disease (Klassen 1993). Biomarkers of effect have an advantage over biomarkers of exposure as they measure an endpoint of the toxic stress incurred.

A disadvantage of biomarkers is that basal levels, as well as the effect on the biomarker of a given pollutant exposure, may be subject to potentially confounding morphological changes associated with infectious disease, season, sex, age, diet, natural variation, and/or environmental factors (Rees 1993). To account for such complicating factors, biomonitoring studies should include a suite of selected exposure and effect indicators at several levels of biological organization to the community level

(Adams et al.1990). The most commonly studied biomarker of exposure and effect for organophosphates is AChE inhibition.

The use of AChE as a biomarker of exposure and effect in rainbow trout is suitable up to a 20% inhibition; this generally occurs at a 1-2 μg/L chronic chlopyrifos exposure (Sandahl 2001). Sublethal effects in steelhead may be occurring below current levels of reliable detection. Detection of AChE activity at lower levels is currently under investigation (Sandahl 2001). The use of additional low level biomarkers may add credibility in lieu of potentially confounding factors. Other enzymes known to metabolize and detoxify organophosphate compounds are cytochrome P450, flavin-containing monoxygenase, glutathione s-transferase, and carboxylesterase(A and B-esterase) (Chambers and Levi 1992).

There are several reasons for selecting the liver of teleosts for the detection of biomarkers of exposure (Hinton and Lauren 1990). The first organ encountered by internalized toxicants is generally the liver. The liver is the major site of xenobiotic detoxification through the cytochrome P-450 and mixed function oxygenase systems, as well as the glutathione s-transferase (GST) family. Bile synthesized within hepatocytes then carries conjugated phase I and II metabolites of toxicants via the intestine to the gut lumen for excretion.

Glutathione is a tripeptide (L-y-glutamyl-L-cysteinylglycine) involved in many cellular functions, including certain cellular transport mechanisms, cellular protection, and the metabolism of numerous xenobiotics (Meister and Anderson, 1983). Although reduced glutathione can combine spontaneously with highly reactive chemicals,

conjugation with electrophilic compounds is usually promoted by a family of isoenzymes known as GSTs (Sultatos 1992). The response of GST to low levels of chlorpyrifos was measured in this study.

GST activity has been detected in various plants, insects, molluscs, and animals. GST concentrations are high in most tissues (liver, intestine, kidney, testis, adrenal and lung) where they are localized in the cytoplasm (>95%) and endoplasmic reticulum (<5%) (Klassen 1995). In the biotransformation of xenobiotics, GSTs are called phase II conjugating enzymes. Phase I reactions involve the hydrolysis, reduction, and oxidation of xenobiotics. Phase II reactions link metabolites to water soluble compounds resulting in an increase in xenobiotic hydrophilicity, and greatly increasing the rate of excretion. Phase II reactions generally, but not necessarily, occur after phase I reactions. GSTs increase the rate of glutathione conjugation by deprotonation of GSH. All glutathione substrates are hydrophobic, contain an electrophilic atom, and react non-enzymatically with glutathione at some measurable rate. Glutathione conjugates are thioethers, which form by nucleophilic attack of the glutathione thiolate anion (GS-) with an electrophilic carbon atom (or heteroatoms O, N, S) in the xenobiotic (Figure 4). GSH conjugates formed in the liver can be excreted intact in bile, or they can be converted to mercapturic acids in the kidney and excreted in urine (Figure 5).

GSTs have been grouped into four classes of isoenzymes alpha, mu, pi and theta based on similarities in amino acid sequences. It is postulated that the isoenzymes originate from a single ancestral gene (Mannervik et al. 1985). There is no known chemical substance that is suitable for measuring the catalytic activity of all

Figure 4. Conjugation of Chlorpyrifos with Glutathione (GSH) by Glutathione S-Transferase (GST) (Motoyama 1980).

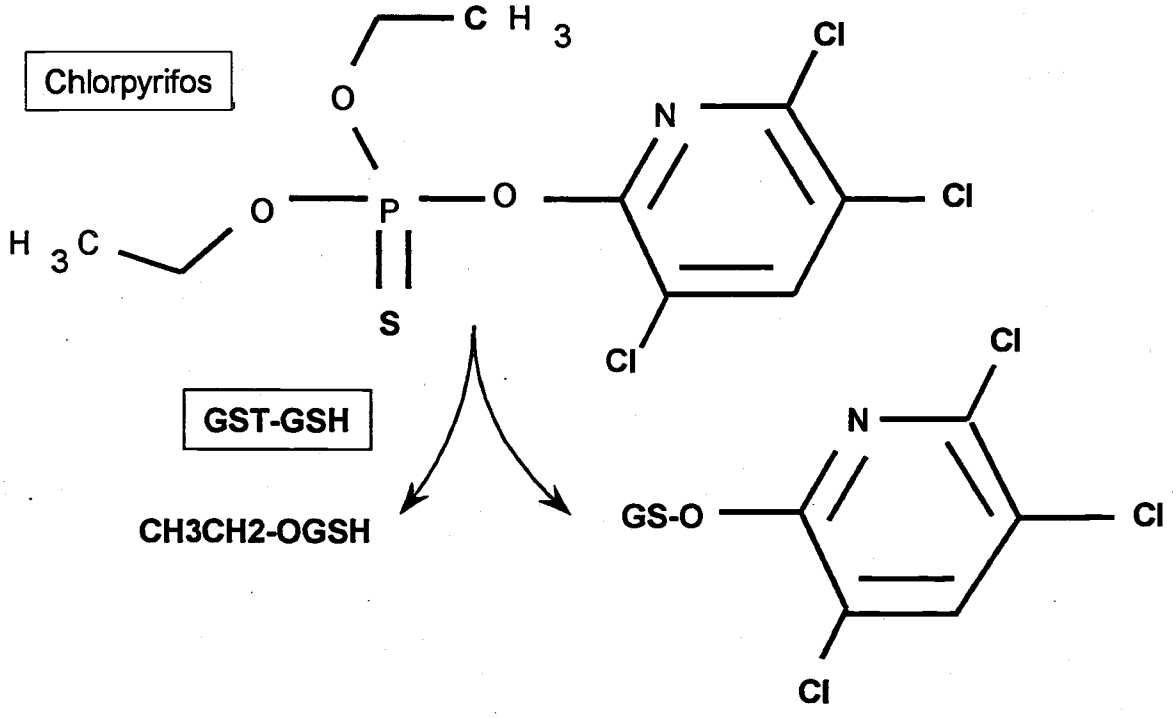
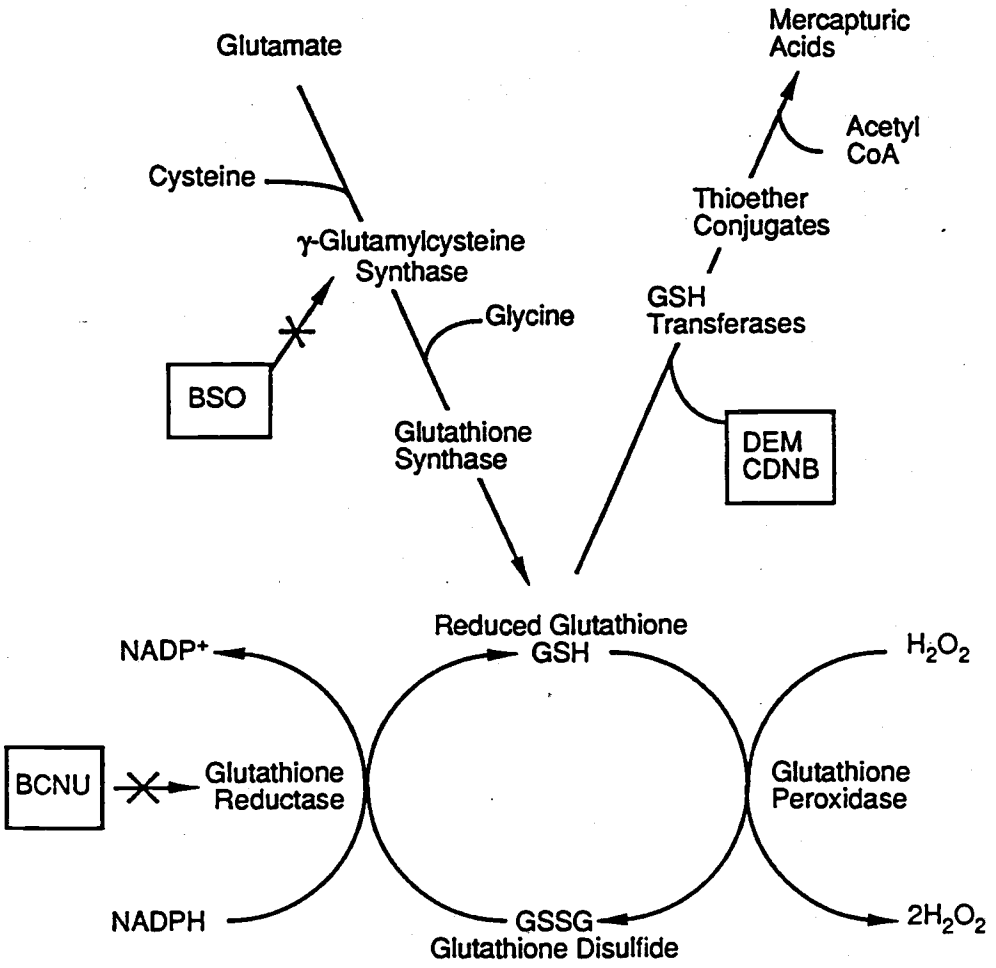


Figure 5. Hepatic Synthesis and Excretion of Glutathione S-Transferase (Connell 1999)



known GSTs. 1-Chloro-,4-dinitrobenzene (CDNB) is applicable to all isoenzymes except theta. CDNB is the most commonly used substrate, as the assay is simple to perform, sensitive, and approximates measurements of total GST. GST catalyzes the conjugation of CDNB to glutathione producing S-(2,4-dinitrophenyl) glutathione.

Although the use of CDNB to measure GST activity varies markedly across species (Stenersen et al. 1987), many fish species exhibit hepatic GST activities with CDNB in the range of those reported for mammals (George 1994), providing the potential for inter-species comparisons (Gallagher et al. 2000). Induction and/or inhibition of GST has been reported varying with the chemical exposed to, concentration and exposure duration, and the species (Ibrahim & Ottea 1995, Petrivalsky et al. 1997, Rao and McKinley 1969, Sheenan et al. 1991, Hodge et al. 2000). Nielsen et al. (1999) have evaluated GST induction as a biomarker in wolf spiders and GST has proved useful as a biomarker of pollutant exposure in molluscs (Baturo 1996, Fitzpatrick 1997). Intraperitoneal injections of rainbow trout with ß-napthoflavone (BNF) resulted in approximately a two-fold induction of hepatic GST activity (Andersson et al. 1985). In another study with the same species, hepatic GST activity increased by a factor of 2.5 as a result of BNF treatment (Goksoyr et al. 1987). A 10 day exposure of juvenile steelhead to the organophosphate, fenitrothion, in the range of 45 to 220 µg/L, significantly depressed GST activity (Davies et al.1994). In the same study aqueous exposure to chlorothaolnil (a fungicide) promoted higher levels of GST in juvenile steelhead.

To date, we are not aware of any published studies testing hepatic GST activity in *Oncorhynchus mykiss* as a biomarker of low level chlorpyrifos exposure. We hypothesize

that the induction or inhibition of cytosolic fractions of hepatic GST activity will serve as a biomarker of low level chlorpyrifos exposure in juvenile steelhead.

Materials and Methods

Test System

The test system was designed and performed by Jason Sandahl (Sandahl 2001) for AChE and GST measurements. Exposure tests were run in the latter half of 1999, and water characteristics determined by February of 2000. Technical grade chlorpyrifos (99.2%) was purchased from Chem Service Inc. (West Chester, PA). Test solutions were prepared in 5 ml acetone. Exposures were static and conducted in fiberglass tanks filled with 1500L spring water. Water temperature (±SD) was maintained at 12 (± 1)°C, pH at 7.9 (± 0.1), DO at 9.9 (± 0.3) mg/L, and hardness at 44.5 (± 0.5) mg/L as $CaCO_3$.

Tests were conducted with juvenile steelhead trout on-site at Oak Springs Hatchery (Maupin, OR); the source for the Hood River basin steelhead stock. Chlorpyrifos was introduced into tanks at 1.25, 2.5, 3.75, 5.00 μ g/L for 24 hr exposures, and 0.5,1.0, 1.5, 2.0, 2.5 μ g/L for 96 hr exposures. Water samples were immediately frozen at -20°C. Analyses were conducted using a Hewlett-Packard 6869 Gas Chromatograph (GC) coupled with a 5972A mass selective detector (MS), following a modified in-vial elution protocol (Runes 1999). The initial oven temperature was held for 1 min at 90°C and then increased at 15°C/min to a final temperature of 240°C and held for 3 min. The MS operated under selective ion monitoring (*m/z* 197,199, 314) at a temperature of 280°C. Results indicate that both field and laboratory blanks contained no

chlorpyrifos at a detection limit of 5 ng/L, and limit of quantitation at 10ng/L. Laboratory extraction recoveries ranged from 86-100%, and field recoveries 74-89% of nominal values.

Fish size (±SD) averaged 7.6 (±0.8) cm and 3.8 (±1.3) g. Feeding was suspended two days prior to tests. Chlorpyrifos solutions were mixed thoroughly in tanks before introducing fish. The 96 hr test consisted of five exposure levels replicated in triplicate, with 20 fish per tank (60 fish per exposure group, 300 fish total). The 24 hr test consisted of five exposure levels replicated in quadruplicate, with 20 fish per tank (80 fish per exposure group, 400 fish total). After a 96 hr exposure, fish were captured, anesthetized in MS-222 and immediately frozen at -20°C. Fish were partially thawed, livers excised and frozen at -80°C until GST analysis.

GST Analysis of Fish Liver Tissue

Measurement of GST with CDNB followed Bengt Mannervik and Per Jemth's protocol (Mannervik & Jemth 1999), with reagent concentration adjustments made for fish as suggested by Evan Gallagher (personal communication).

Four livers were pooled randomly by exposure group and homogenized at 20 mg/ml in Tris buffer 50 mM (pH 7.4), 250 mM sucrose, and 1mM disodium ethylenediamine tetraacetate. Homogenates were centrifuged at 1000g for 30 min and the supernatant separated to remove large particles. Exposure groups were processed in random order.

For each sample, 16μL of thoroughly mixed liver supernatant was transferred to a 1.5 ml eppendorf dilution tube, and combined with 934 μL Tris buffer 50mM (pH 7.4). Added to five triplicate plate wells was 190 μL of the liver supernatant/Tris buffer solution. Each well received 10 μL of the GST substrate, or CDNB (25 mM dissolved in 95% ethyl alcohol). Blanks for tissue and substrate were included for each sample. The reaction was initiated by addition of 50 μL reduced GSH (25 mM dissolved in distilled water), and immediately placed into a spectrophotometer (Spectra Max Plus: Molecular Devices Corp.) Change of absorbance (340 nm) was measured at 12 sec intervals for 10 min at 30°C. Final well concentrations were: liver tissue 0.6 mg/ml, CDNB 5 mM, and GSH 5 mM. All activities were normalized to wet tissue weight. Activities were calculated by Soft Max Pro 3.0 as μmol substrate hydrolyzed/min/mg wet tissue weight.

Statistical Analysis

The 3-5 min linear portion of slopes were kept within a 5% coefficient of variance. Nonenzymatic activity (blanks) were subtracted from enzymatic activity. Statistics were performed on GraphPad Prism 3.02 (2000); linear regressions determined significant dose-response relationships and ANOVA with Dunnetts Control Comparisons tested group similarities to controls.

Results

GST Activities

GST activity was inhibited (25% from highest to lowest dose) at 24 hr exposures ($p = 0.0022$). The equation for the slope was Activity = (-0.00433) X – 0.0796 with a 95% confidence interval of –0.00702 to –0.00164 (Figure 6a). The 95% confidence interval for the y-intercept was ± 0.00134. There was no significant dose-response relationship for GST activity after a 96 hr exposure (Figure 6b).

Variables Tested

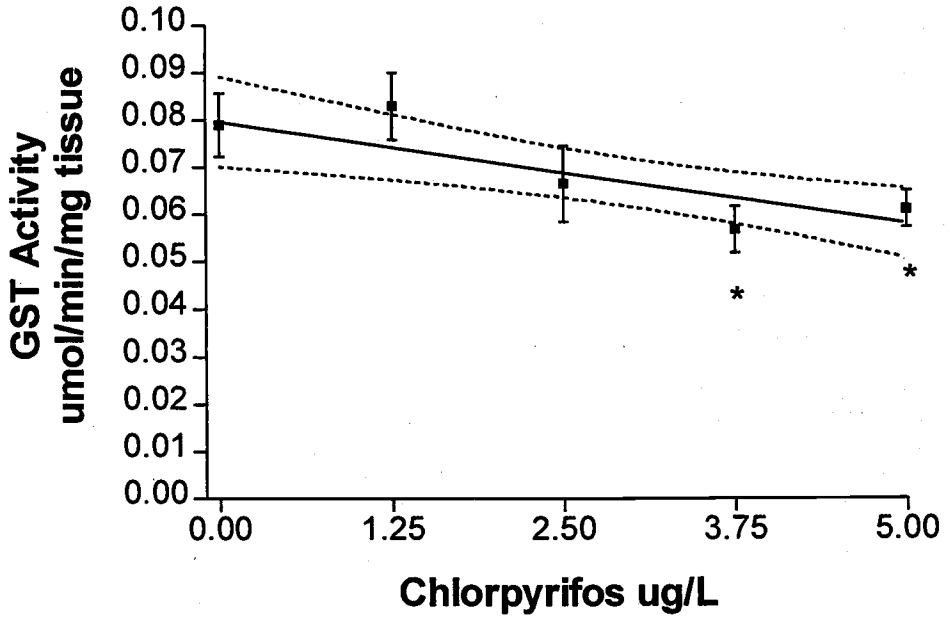
Steelhead weight does not appear to affect GST activity at 24 hr (Figure 7a) and 96 hr (Figure 7b) as there is no significant correlation ($p > 0.05$).

Nonenzymatic Conjugation of CDNB with Glutathione

CDNB reacted with endogenous substrate even without the catalytic action of the enzyme. Under the experimental conditions of the enzyme assay (without the cytosolic liver fraction) the average chemical reaction rate was 0.073 ± 0.005. Midway through the 24 hr experiments the average rate abruptly increased to 0.147 ± 0.044 (Figure 8).

Figure 6. The effect of (a) 24 hr and (b) 96 hr exposure to chlorpyrifos on cytosolic GST activity in juvenile steelhead livers (mean ± SE). Stars indicate significant P values as compared to the control by a Dunnetts one way ANOVA.

(a) $p < 0.05$, $n = 49$



(b) $p < 0.001$, $n = 51$

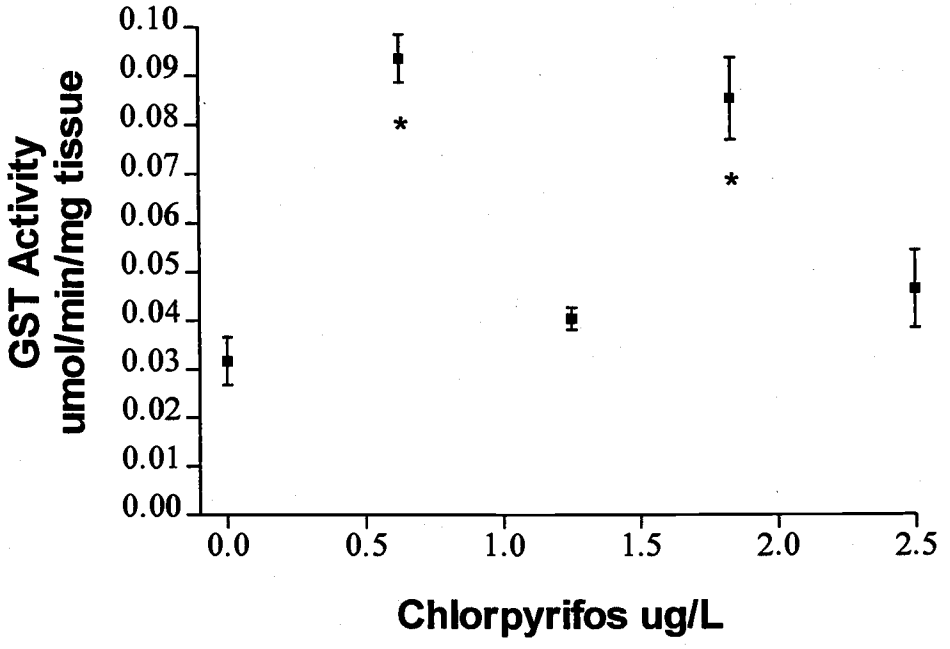
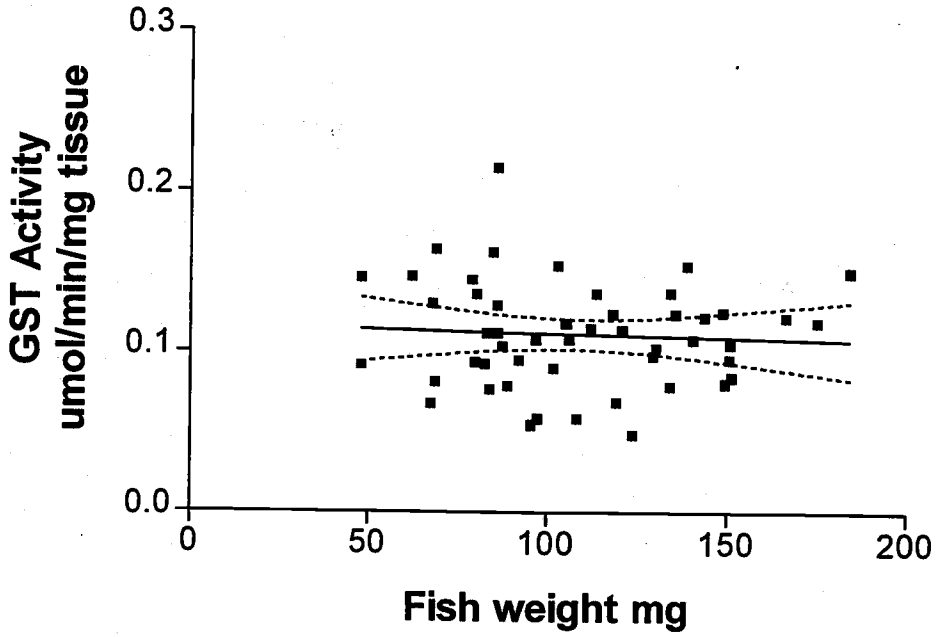


Figure 7. The correlation between weight and GST activity in winter steelhead exposed to chlorpyrifos in (a) 24 hr and (b) 96 hr exposures. Linear regressions show $p > 0.05$.

(a) 24 hr chlorpyrifos exposure



(b) 96 hr chlorpyrifos exposure

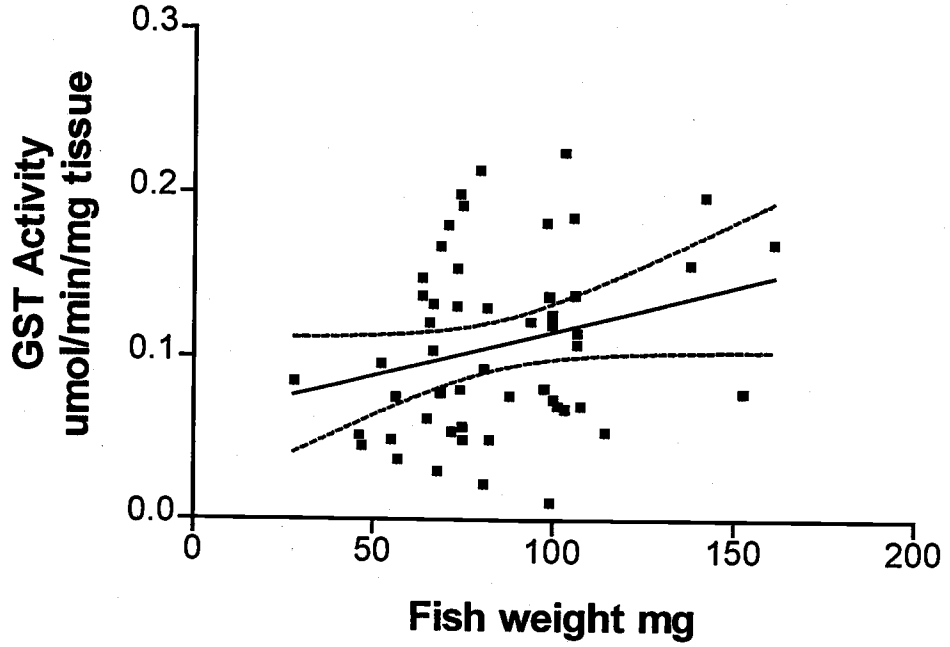


Figure 8. Nonenzymatic GST Activity through 24 hr and 96 hr experiments.

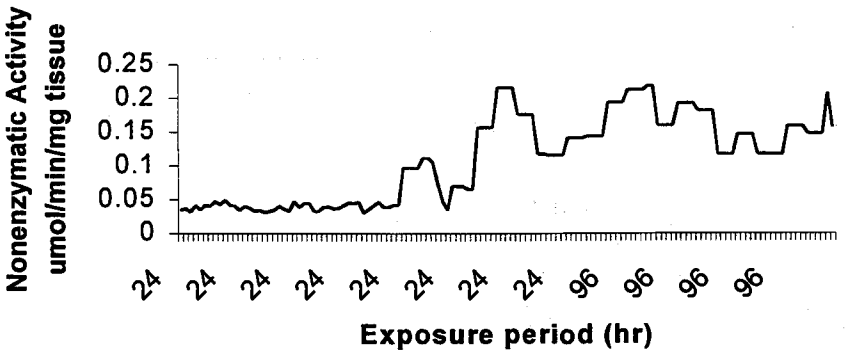
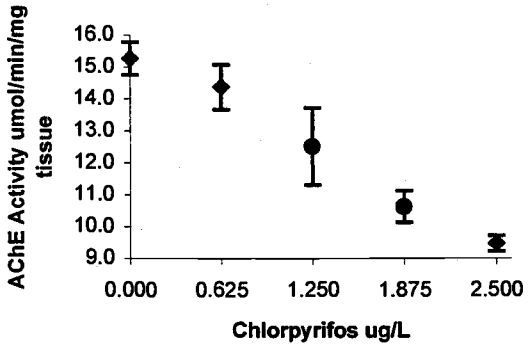
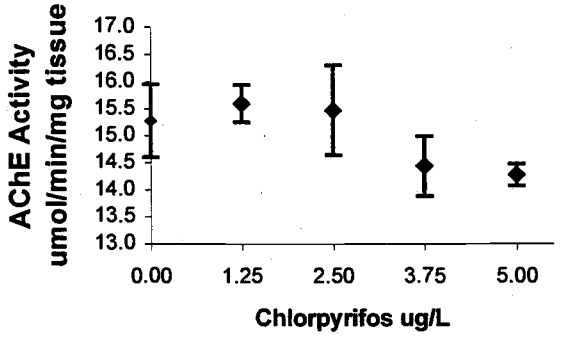


Figure 9. AChE Activity (means ± SE) in Juvenile Steelhead Brains Exposed to Chlorpyrifos for (a) 24 hrs and (b) 96 hrs

(a)



(b)



Discussion

GST Activity as a Biomarker of Exposure

GST inhibition in response to 24 hr chlorpyrifos exposure may serve as a biomarker of exposure. GST inhibition appears to be dependent on exposure time, as there was no dose-response relationship at 96 hours. The usefulness of a biomarker of 24 hr exposure in the field is questionable due to unknown exposure periods. Measurement of overall GST activity is probably not a sensitive biomarker of chlorpyrifos exposure in juvenile *Oncorhynchus mykiss* as there is no consistent correlation through time between pesticide exposure and GST response.

The use of CDNB to measure the response of cytosolic GST to environmental pollutants in fish has produced variable results (Tables 1 and 2). CDNB constitutes the activities of one or more isoenzymes, and an induction in one form may offset the decrease of another isoenzyme (Petrivalsky et al.1997). GST measurements with CDNB in steelhead may be useful only as a preliminary test of GST activity, thereby warranting further research into specific isoenzyme responses as biomarkers of pollutant exposure.

One dilemma associated with hepatic GST activity as a biomarker, is the high variability among different teleosts to the substrate CDNB. High variability makes interspecies and intraspecies comparisons among studies difficult. GST activities may vary with biological and environmental factors (sex, age, season, and natural variation) as well as with experimental design (acclimation, test duration, diet, water characteristics). GST activities in fish varied four-fold in some studies (Table 1). GST activity comparisons are further complicated by the absence of an established standard for

reporting GST activities; GST activity has been measured per mg protein (Table 1) and mg wet weight (Table 2). Confounding variables between tank and field experiments (i.e. pH, temperature, and diet) obligate further caution when comparing GST activities. The variability in GST activity exemplifies the importance of utilizing a suite of complementary biomarkers.

Variability of GST activity in this experiment was high, possibly due to an abrupt increase in nonenzymatic activity midway through the experiment (Figure 9). This may have been due to a substantial increase in the efficiency of the assay procedure as the experiment progressed resulting in incomplete dissolution of substrate in the well-plates. Furthermore, the amount of base necessary to maintain pH 7.4 increased midway through the experiment, possibly due to the summer's heat and/or variable pH calibration. The stability of GSH decreases with an increase in pH, primarily due to the ionization of the sulfhydryl group (pka 9) (Mannervik and Jemth 1999). Another contributing factor may have been the dissolution of CDNB into ethanol; ethanol's poor adherence to pipette tips due its low viscosity may have slightly affected precision. It has been proposed that salmonid GST is able to esterify ethanol with fatty acids (Dominey et al. 1991), therefore it is possible that ethanol may act as a substrate for the pi-class salmonid isoenzyme. However, the latter two speculations are less likely to account for an abrupt increase in nonenzymatic rates.

Table 1. Hepatic GST activity nmol/min/mg protein in control fish using CDNB as a substrate. Day = d, None Stated = NS

nmol/min/mg protein mean±SD or SE, (n)	Species	Tank Field	Acclimation	Test duration	Age (weight)	Feeding regime	Water temp	Reference
5,470 ± SD 1,150 (8) cytosolic	Rainbow trout	Tank	NS	2d	Juvenile (120-200g)	2x daily	13°C	Petrivalsky 1997
0.35± SE 0.04 (4) cytosolic	Rainbow trout	Tank	7d	7d	Juvenile	Unfed 2d prior to exposure	14-16°C	Donnarumma 1988
190± SE 11000 (18) cytosolic	Rainbow trout	Field	4d	21d	Juvenile (78.9-216.0g)	Fed 2x, after 1 &10d	10-14°C	Lindstrom-Seppa & Oikari 1990
430± SD 60 (5) cytosolic	Rainbow trout	Tank	NS	4d	Juvenile (100-200g)	NS	10°C	Andersson et al. 1985
1600± SD 580 (23) 1,830±2800(23) cytosolic	Steelhead trout	Tank	14d	9d 17d	Juvenile (110-210g)	Fed 1x a week	7°C	Hektoen et al. 1995
700± SE 20 (8) 600± 25(8)	Steelhead trout	Tank	NS	1d 3d 7d	Juvenile	Unfed	8-10°C	Celander et al. 1993
1,930± SD 620(23) 1.080±760(23) cytosolic	Cod	Tank	14d	9d 17d	Juvenile (110-170g)	Unfed	7°C	Hektoen et al. 1995
1,340± SE 120(8) 1570±210(10) microsomal	Nase	Field	NS	NS	7-8yr (771g)	NS	NS	Monod 1988
620± SE 100(6) 700±80(7) microsomal	Grayling	Field	NS	NS	3-4yr (356g)	NS	NS	Monod 1988
1,640± SE 280(9) microsomal	Roach	Field	NS	NS	4-5yr (207g)	NS	NS	Monod 1988
1,154± SE 124 (4) 843 ± 55 cytosolic	Large mouth bass	Field	NS	NS	2-5 yr	NS	NS	Gallagher 2000
1,969 ± SE 249 (4) 1,909 ± 180 cytosolic	Brown bullheads	Field	NS	NS	2-5 yr	NS	NS	Gallagher 2000

Table 2. Hepatic cytosolic GST activity (nmol/min/mg wet weight) in control fish using CDNB as a substrate. Day = d, None Stated = NS

nmol/min/mg tissue mean±SD or SE (n)	Species	Tank Field	Accli m-ation	Test duration	Age (weight)	Feeding regime	Water temp	Reference
6,140 ± SD 490 (10) 4,360 ± 0.960 6,540 ± 1,540 3,600 ± 910 cytosolic	Steelhead trout	Tank	5-10d	10d	Juvenile (1.1-30g)	1 x daily	12-15°C	Davies 1994
78.9± SE 21.1(49) 31.6 ±12.9(51) cytosolic	Steelhead trout	Tank	NS	1d 3d	Juvenile 3.8(1.3)g	Unfed 2d to exposure	12±1°C	Pecore Unpublished

Recommendations for future research

1) Petrivalsky et al. (1997) states that "Any practical use of fish hepatic glutathione-dependent enzymes as biomarkers should be preceded by detailed studies of their induction patterns, by chromatographic resolution of hepatic isoenzymes and determination of their substrate specificity, and by application of immunochemical methods." Deviations of steelhead GST activity from control rates cannot be classified as a dose-response until a normal activity range is established. Pi-class mediated substrates should be examined as the major salmonid hepatic isoenzyme appears to be pi (Dominey et al. 1991).

2) When measuring GST, ensure proper mixing of substrate and buffer solution, and use a solvent other than ethanol to dissolve the substrate.

3) Endogenous GST substrates should be further examined. Berhane and Mannervik (1990) have demonstrated that acrolein is one of the most active substrates known for the human pi-class GST. Acrolein is a toxic aldehyde produced during lipid peroxidation and by alcohol dehydrogenase, and may act as a substrate for the salmonid GST e*n vivo.*

4) AChE inhibition occurred in the brain at 24 hours (Figure 9) suggesting that the liver was not able to completely eliminate chlorpyrifos. Measurement of hepatic GST activity at lower levels (<1.25μg/L) may more closely reflect the initial reaction to chlorpyrifos perhaps decreasing the confidence interval in the 24 hr dose-response relationship.

5) A reliable GST isoenzyme exhibiting a dose-response relationship with chlorpyrifos should be tested in tank conditions mimicking the comparison site (Hood

River) as closely as possible. For example, to eliminate seasonal variation as a variable between lab and field GST activities, research should be conducted in early spring (when intense spraying occurs). Tanks acclimatizing juveniles to a mixture of other chemicals detected in the river, spring river temperatures, pH, and salinity should be included to compensate for additive effects. Prior testing of GST activity toward dietary compounds would also increase confidence. Caged fish studies eliminate many difficulties in field/tank comparisons, however identifying causes of GST variation is made more difficult.

6) Measure additional biotransformation enzymes such as the Cytochrome P450 system or UDP-glucuronosyltransferase (Stegeman 1990).

GST as a Component of the Whole System

The introductory story is a simplified version of the complex, adaptive system affecting steelhead population levels in the Columbia River watershed. Adaptive systems interact with their environment and change in response to environmental change (Clayton and Radcliffe1996). Complex, adaptive systems tend to withstand alterations within the system maintaining an equilibrium up to an undefined threshold; rapid or extensive changes may push the system over its threshold resulting in changes in the system as a whole (Bella 1997). Over the last century the extent of environmental changes within the Columbia River watershed (Appendix B) have surpassed the threshold and winter steelhead in the lower Columbia River have not been able to adapt. Environmental changes include obstructed passage, extensive alterations to habitat, genetic changes introduced by hatchery fish, and over-fishing

along with others identified in Figure 1. The changes exacted on steelhead habitat composition and complexity, as well as internal biochemistry, probably accumulated until population levels could no longer be sustained.

One might hypothesize that salmon population numbers could be increased to sustainable levels simply by eliminating key factors for decline as identified in Figure 1 (i.e. heavy metals, storm water, chronic turbidity, high water temperatures, blocked passage, predators, stress, diminished spawning grounds and food supplies). Identifying causes for decline is made difficult due to the nature of complex, adaptive systems. Systems, by definition, "have behavioral or other emergent properties that the components of the system do not, and which are not readily explicable with reference to the subcomponents" (Clayton and Radcliffe 1996). For example, a salmon is much different than its constituent parts: heart, brain, gills, liver, scales, and eyes. Emergent properties, such as a salmon, enable complexity to be described in terms of a hierarchy of levels of organization in which each level is described in terms of its emergent properties rather than constituent parts (Bawden and Packham 1991). In a complex, adaptive system (i.e. Sedimentation in Figure 1), the immediate cause of sedimentation is illustrated as erosion, which in turn may be caused by run-off from excessive rains flowing over logging roads, skid trails, or soil compacted by heavy farm equipment. If logging roads were built elsewhere, erosion might still occur in the system from skid trails or compacted soil. If all three causes of erosion were eliminated, erosion might still occur due to an iterating chain of causal events, or a feedback loop (Clayton and Radcliffe 1996), where erosion is caused by the very effect it produces, sedimentation. Complex, adaptive systems make identifying factors for

decline difficult, as a system is an interconnected set of elements interacting through time and space.

The various factors threatening winter steelhead survival (dams, disease, predators, pollution), not only exist in and of themselves but on interacting temporal and spatial scales. Chlopyrifos concentrations in the Hood River basin waters reach their highest potential in early spring. The potential for internal exposure of fish depends on flushing rates of the pesticide, or water levels and flow, and potentially the temperature (Schimmel et al. 1983). The inhibition of hepatic steelhead GST at 24 hours indicates that conditions in the tank experiment were favorable for internal exposure to chlorpyrifos. Sublethal effects have not been correlated with experimental chlorpyrifos concentrations; further investigation of a direct correlation between chlorpyrifos concentrations and sublethal effects may need to include research into the surrounding environmental conditions favoring sublethal effects. For example, sublethal effects were observed in juvenile steelhead internally exposed to low levels of organophosphates. Imagine if the commercial use of organophosphates were prohibited, yet sublethal effects were still observed. Perhaps sublethal effects arose from the interactions of organophosphates with DDT, low pH, and chronic turbidity after a spring storm. If the low pH, DDT exposure, and chronic turbidity were found to be caused by spring rains transporting acidic compounds from mine tailings, DDT historically used for weed control in second growth forests, and sediment in the stream. Could the adverse sublethal effect be minimized by allotting a buffer zone of bank stablilizing riparian vegetation reducing run-off? Recognizing the complex

behavior of systems may be a useful tool in addressing and prioritizing causes of desired effects.

The threat of extinction provides a deadline for salmon recovery. Economic and cultural value has been assigned to salmon resulting in the investment of billions of hours and dollars toward elevating population numbers. Again, recognizing the nature of complex, adaptive systems may be a useful tool in prioritizing recovery efforts, for example focusing research on leverage points. Leverage points are efforts that will reap the greatest number of desired effects in proportion to effort or cost (Senge et al 1994). Ensuring a zone of riparian vegetation could be considered one leverage point in the example above as planting or protecting a tree requires little effort and would provide soil stability for many years. However, in a complex adaptive system more than one leverage point is generally required, as the removal of one component often does not eliminate the emergent property (for example, sedimentation in Figure 1). Drawing relational diagrams (Figure 1) encourages a search for leverage points (William 2000). Leverage points and system components should be validated with empirical data. Planting a tree may not serve as a leverage point if the definition of riparian vegetation is not narrowed to include specific plant species able to intercept the pesticide, acidic compounds, and loose sediment. Possible actions providing leverage should be validated as being economically feasible, within reasonable time limits, and as having multiple and far-reaching effects through time.

There are several organizations concerned with declining salmon populations (Figure 10) currently researching leverage points (dam removal, fish catch quotas, riparian buffers, pollution credits, energy conservation, abundance and climate based

fisheries management, international Pacific Salmon Treaty). Leverage points can also be examined at the personal level at home (i.e. decreasing natural resource consumption), and in the workforce (i.e. scientists researching practical factors for salmon decline). In scientific pursuit of leverage points, the following two questions are asked: Would you fund continued research for a biomarker of low level chlorpyrifos exposure in juvenile winter steelhead? If chlorpyrifos were removed from the system how would the threatened status of winter steelhead in the lower Columbia River change?

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Appendix A. Organizations Involved in Water Resource Issues (Derived from unknown source, and International Fisheries Division Office of Sustainable Fisheries 2001)

- **Federal Agencies**

Dept. of Agriculture (USDA)
    Forest Service (USFS or USDA - FS)
    Natural Resources Conservation Service (USDA - NRCS i.e. SCS)
Dept. of Commerce
    National Oceanic & Atmospheric Administration (NOAA)
        National Marine Fisheries Service (NMFS or NOAA - NMFS)
        National Weather Service (NWS or NOAA -NWS i.e. USWB)
Dept. of Defense
    U.S. Army Corps of Engineers (USACE or USCE or USCOE)
Dept. of Energy (DOE or USDOE)
    Bonneville Power Administration (BPA)
    Federal Energy Regulatory commission (FERC)
Dept. of Health and Human Services
    Public Health Service (USPHS)
        Indian Health Services
Dept. of Interior (USDA)
    Bureau of Indian Affairs (BIA)
    Bureau of Land Management (BLM or USBLM)
    Bureau of Reclamation (USBR or BOR)
    Fish & Wildlife Service (USFWS or FWS)
    Geological Survey (USGS)
    National Park Service (NPS)
Dept. of Justice (DOJ)
Dept. of Transportation (USDOT or DOT)
    Coast Guard (USCG)
    Federal Highway Aministration (FHWA)
Pacific Salmon Commission (PSC)

- Independent / Other

Environmental Protection Agency (EPA or USEPA)
Federal Emergency Management Agency (FEMA)

- **State of Oregon Agencies and Boards**

OR Dept. of Agriculture (DOA or ODOA)
OR Dept. of Energy (ODOE)
OR Dept. of Environmental Quality (DEQ or ODEQ)
OR Dept. of Fish and Wildlife (ODFW)
OR Dept. of Forestry (ODF)
OR Dept. of Geology and Mineral Industries (DOGAMI)
OR Dept. of Land Conservation and Development (DLCD)
OR Dept. of Transportation (ODOT)
OR Division of State Lands (DSL)

OR State Health Division
OR State Marine Board (OSMB)
OR State Parks and Recreation Dept.
OR State Police; Office of Emergency Management (OEM)
OR Water Resources Dept. (OWRD)
Governor's Watershed Enhancement Board (GWEB) & Individual Watershed Councils (59 in 1995)
Public Utility Commission (PUC)

- **Regional Government - Type commissions, Interstate Compacts, and SimilarGroups with State Representation**

Columbia gorge commission
Columbia River Inter-Tribal Fish Commission (CRITFC)
Klamath River Compact commission
Northwest Power Planning Council (NPPC)
Pacific States Marine Fisheries Commission (PSMFC)
Western States Water Council

- **Regional and Oregon Non - Governmental Groups with a Water Focus**

American Rivers
Associated Oregon Industries
Audubon Society
Columbia River Alliance
Columbia River Fishermen's Protective Union
Defenders of Wildlife
For the Sake of Salmon
Izaak Walton league of America
League of Oregon Cities
League Women Voters
national Council of the Paper Industry for Air & Stream Improvement
nature conservancy, The
Northwest coalition for Alternatives to Pesticides
Northwest Environmental Advocates
Northwest Renewable Resources Center
Oregon Environmental Council (OEC)
Oregon Farm Bureau
Oregon forest Industries Council
Oregon Forest Resources Institute
Oregon Lands Coalition
Oregon Natural Resources Institute
Oregon Lands Coalition
Oregon natural Resources Council (ONRC)
Oregon Rural Electric Cooperative Association
Oregon Trout
Oregon Water Resources congress (OWRC)
Pacific Northwest Utilities Conference committee (PNUCC)

Pacific Rivers Council
River Network
Sierra Club
Trout Unlimited
Wilderness Society, The

- International and Regional Management Arrangements

Atlantic Ocean

Convention for the Conservation of Salmon in the North Atlantic Ocean (Basic instrument for the North Atlantic Salmon Conservation Organization - NAFO)

Pacific Ocean

Convention for the conservation of Anadromous Stocks in the North Pacific Ocean and Bering Sea (Basic Instrument for the North Pacific Anadromous Fish commission - NPAFC)

Great Lakes

Convention on Great Lakes Fisheries Between the US and Canada (Basic instrument for the Great Lakes Fishery Commission - GLFC)

Global

Convention on Biological Diversity (CBD)
Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)

- Bilateral Consultative Arrangements

North America

Agreement Between the Government of the USA and the Government of Canada on Fisheries Enforcement

Central America

US -Mexico Fisheries Cooperation Program

South America

US - Chile Fisheries Cooperation Program

Asia

US - Japan Consultative Committee on Fisheries

Europe

Agreement Between the Government of the USA and the Government of the Union of Soviet Socialist Republics on Mutual Fisheries Relations (Basic instrument for the US - Russia Intergovernmental consultative Committee - ICC)

- Scientific Organizations and Councils

Pacific Ocean

North Pacific Marine Science Organization (PICES)

Arctic Ocean

Program for the conservation of Arctic Flora and Fauna (CAFF

Global

Global Environment Facility (GEF)
International Council for the Exploration of the Sea (ICES)
Joint FAO/ WHO International Codex Alimentarius Food Standards Programme

Apendix B. A brief history of the complex, adaptive system of the Columbia River watershed. Derived from: (Cone J and Ridlington S, 1996), (Courtland S, 1979), (Dept. of the Interior Information Service, 1957), (Endangered Species International Agreements and Permits, 2001), (Pacific Salmon Treaty, 2001), (Oregon State University Extension Service, 1998), (US Dept of the Interior, 1961), (White R, 1995).

Formation of the Columbia River Basin:	
Miocene	Volcanic eruptions deposit basalt layers
Pleistocene	Ice dam at glacial lake, Missoula, collapses creating largest freshwater flood in history. Columbia channels were carved.
40 million years ago (Eocene)	Earliest fossil evidence for ancestors of Pacific Northwest salmon
6 million years ago	Earliest fossil evidence for present salmon species
9,000 years ago	Sea-levels stabilize in Pacific Northwest, more favorable conditions for salmon. There are 9 different salmonid species native to Oregon.
5-8,000 years ago	Native American become salmon fishers
1806	Small pox depletes Native American populations, salmon numbers increase
1830	First steamship powered up the Columbia River
1855	Treaty shifted Native American land ownership; guaranteed Native access to usual and accustomed fishing places.
1866	First cannery in operation at Eagle Cliff (50 miles from Columbia River mouth)
1871	Transplant of shad and striped bass from the East Coast to San Francisco (eat salmon, competed for habitat)
1876	Great flood
1877	Thirty-five hatcheries in operation
1880	Railroad built freeing transportation from water
1880	Pound nets replace wooden fish traps
1883	From May to August 1,700 gillnetters on Columbia
1894	Flood, greatest recorded flow in the Columbia's history
1800's	Trees frequently logged and floated down river to mill sites
1900	Early 1900's, U.S. Army Corp. builds jetties, dikes, dredges sand using it to fill in marshes
1900	Seine nets and 40 fish wheels in operation
1900	Salmon industry is Oregon's third largest
1903	Shad in the Columbia river recognized as a nuisance
1917	World War I
1918	Worst winter storm in half a century, coal railcars were stuck, mass production of electricity viewed as solution
1920	First dam built
1920	Federal Water Power Act

1920-30	Combination of popular ballot initiatives and legislative acts eliminate traps, fish wheels, and seines from river.
1880-1930	Columbia river salmon catch was 33.9 million pounds/year
1930's	Legal prohibition of fixed gear (fish wheels)
1932	Rock Island dam built
1933	Mid-depression - Public Works viewed as a remedy for unemployment
1937	Bonneville Project Act passed by Congress, created the federal agency BPA to transmit and wholesale electricity through a system linking dams along Columbia and tributaries
1938	1,192 gillnetters on Columbia (272 day fishing season)
1938	Bonneville dam built eliminating dip-net fishing at Cascade falls
1938	Pacific Northwest Regional Planning Commission (PNWPRC) formed
1938-1941	Energy use for residential purposes quadrupled
World War II	Dams powered shipyards, aluminum mills built by Defense Plant Corporation, airplane factories built, Hanford Nuclear Power Plant built as well as 2 other reactors
1941	Grand Coulee built (without a fish ladder). The addition of Grand Coulee closed an estimated 70% of original spawning ground. Eliminated dip-net fishing at Kettle Falls
1943	Aluminum companies consumed 60% of BPA megawatt hours
1946	Fish & Wildlife service declare that McNary Dam alone may eventually exterminate salmon.
1931-1948	Columbia river salmon catch was 23.8 million lbs/year
1949	Fisheries development program first funded
1947-1955	Cold War, 5 nuclear reactors built
1948	Large flood
1949	Green Run experiment from Hanford - radioiodine (I 131) released into air over Pacific Northwest. Distribution depended on weather, season, growth of forage, location of grazing cows, market distribution of milk…
1950's	Six dams built below Grand Coulee, 4 on Snake River
1952	BPA sold more electricity to public customers than investor owned utilities.
1953	International North Pacific Fisheries Commission is formed between Canada, Japan, and U.S.
1954	North Pacific Fisheries Act - Authorizes Secretary of the Interior to Control fishing by US nationals on the high seas
1956	Success of salmon net fishing on the high seas off the State of Washington cause concern among salmon industry
1957	Priest rapids dam built
1957	Acreage limits increased for irrigated farms in Eastern Oregon to 160 acres, 320 per couple.
1957	Revision of North Pacific Fisheries Act -Salmon fishing with nets prohibited to US nationals on the high seas throughout the North Pacific Ocean
1960's	Trollers emerge as the most productive commercial salmon fishers

1961	International North Pacific Fisheries Commission convenes in Japan to Review International salmon research - concerned with how to identify difference between Asian and N. American stocks
	• Japan abstains from fishing for salmon east of 175 degrees west longitude, resources east of line to Canada and the U.S.
1964	Nuclear reactors acconut for a 2-3°F rise in temperature between Priest Rapids and Richland during august and September. (riverwater used to cool reactors returned at 74°F.
1964	Columbia River Treaty - 3 new Canadian dams hold back Columbia's flow diminishing flood danger and releasing water as required for power by U.S.
1965	Oregon Fisheries Commission "A mounting volume of evidence has pointed to the failure of the downstream migration as the major cause of the vanishing runs."
1966	Hanford Nuclear Power Plant produces electricity
1969	Chlorpyrifos is applied for agricultural uses
1970	Salmon fishers consist of (10% commercial, 30-40% part-time, 50-60% sport)
1970	Columbia distributes electricity from 26 federal dams, 5 more under construction
1970	Courts upheld 1855 Northwest tribes treaty rights
1970's	Commercial fishing for spring and summer Chinook and sockeye virtually vanished
1970's	Scientists connect high mortality among juveniles migrating to sea with gas-bubble disease, caused when water spilling over dams is supersaturated with nitrogen. Cumulative fish mortality past 9 dams is 77-96% BPA begins trucking, then barging juveniles downstream
1973	Endangered Species Act requires recovery efforts to save species from extinction
1972-1973	Winter storms, insufficient snow pack, drought followed
1973	1,104 gillnetters on Columbia river (73 fishing days)
1949-1973	Columbia river salmon catch was 10.9 million lbs/year
1974	Judge Boldt rules Northwest tribes will share 50% of runs
1975	Alaska, British Columbia, and Washington had programs limiting salmon fisher numbers
1975	Calculated to have spent $84 million on salmon mitigation
1976	U.S. Fisheries Conservation and Management Act of 1976 (extended national jurisdiction to 200 miles driving foreign fishers off. Created new mechanisms for restricting catch).
1978	Snake River runs fall drastically
1979	Hatcheries account for ½ of Columbia River salmon runs
1980	Mt. St. Helens erupts destroying habitat in Toutle River, Washington
1980	Pacific Northwest Electric Power Plan and Conservation Act mandates Northwest power Planning Council to take measures to enhance fish and wildlife, and provide Northwest with adequate and efficient power supply.
1981-1991	Salmon protection costs and fishways reach $1.34 billion
1982-1983	El nino - poor upwelling

1985	Pacific Salmon Treaty (Canada and U.S.), Pacific Salmon Commission formed (16 person body with equal representation from US and Canada, representing the interests of commercial and recreational fisheries as well as federal, state, and tribal governments) provides regulatory advice to both countries.
1986	Drought year
1991	Snake River runs given protection under Endangered Species Act
1991	One female Sockeye returns up Snake River to Redfish Lake. Named "Sally" they stripped her eggs where they are held in captive breeding
1992	Convention for the Conservation of Anadromous Stocks in the North Pacific Ocean, member nations were Canada, US, Japan, and the Russian Federation
1993	Columbia river salmon catch is 1.4 million lbs/year
1993-1994	El nino - poor upwelling
1994	Kenneth Peterson, President of Col. aluminum sues gillnetters to protect salmon
1994	American and Canadian negotiators agree to pay British Columbia, Canada $5 billion for downstream power rights on the Columbia River
1996	Lower Columbia Winter Steelhead listed as threatened
1997-1998	El nino-poor upwelling
1998	Canada bans all direct Coho fisheries
1998	Canada argues that U.S. has been taking more than its fair share of West Coast salmon, violating the equity principle in Pacific Northwest Salmon Treaty
1998-2000	La nina-strong upwelling
2000	Household use of chlorpyrifos, or Lorsban, was banned by the EPA in the U.S.
1999	Canada and U.S. reach a comprehensive agreement under the Pacific Salmon Treaty.
	• $140 million dollars allocated to invest in habitat, stock enhancement, science and salmon management initiatives in US & Canada.
	• Ten-year arrangement governing northern boundary fisheries, transboundary rivers, based on abundance-based management.
	• Strengthened cooperation among Canadian and US scientists and fisheries managers, new bilateral Panel on Transboundary Rivers and addition of a Committee on Scientific Co-operation to advise the Pacific Salmon Commission