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An Ecological Risk Assessment of the Acute and Chronic Effects of the Herbicide Clopyralid to Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract Clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) is a pyridine herbicide frequently used to control invasive, noxious weeds in the northwestern United States. Clopyralid exhibits low acute toxicity to fish, including the rainbow trout (*Oncorhynchus mykiss*) and the threatened bull trout (*Salvelinus confluentus*). However, there are no published chronic toxicity data for clopyralid and fish that can be used in ecological risk assessments. We conducted 30-day chronic toxicity studies with juvenile rainbow trout exposed to the acid form of clopyralid. The 30-day maximum acceptable toxicant concentration (MATC) for growth, calculated as the geometric mean of the no observable effect concentration (68 mg/L) and the lowest observable effect concentration (136 mg/L), was 96 mg/L. No mortality was measured at the highest chronic concentration tested (273 mg/L). The acute:chronic ratio, calculated by dividing the previously published 96-h acutely lethal concentration (96-h ALC_{50}; 700 mg/L) by the MATC was 7.3. Toxicity values were compared to a four-tiered exposure assessment profile assuming an application rate of 1.12 kg/ha. The Tier 1 exposure estimation, based on direct overspray of a 2-m deep pond, was 0.055 mg/L. The Tier 2 maximum exposure estimate, based on the Generic Exposure Estimate Concentration model (GEENEC), was 0.057 mg/L. The Tier 3 maximum exposure estimate, based on previously published results of the Groundwater Loading Effects of Agricultural Management Systems model (GLEAMS), was 0.073 mg/L. The Tier 4 exposure estimate, based on published edge-of-field monitoring data, was estimated at 0.008 mg/L. Comparison of toxicity data to estimated environmental concentrations of clopyralid indicates that the safety factor for rainbow trout exposed to clopyralid at labeled use rates exceeds 1000. Therefore, the herbicide presents little to no risk to rainbow trout or other salmonids such as the threatened bull trout.

There are ~325 million kg of pesticides applied annually in the United States; herbicides account for about 60% of total pesticide use and are applied in a wide range of weed management activities, including agriculture, lawn care, home/garden, silvicuture, range management, and utility/roadway maintenance (Kiely et al. 2004). Herbicides are increasingly being used in control of non-native, invasive plants on federal lands that are managed by the US Department of the Interior and the US Forest Service (USFS). Non-native, invasive plants are considered one of the primary ecological threats to ecosystems of the United States and are estimated to cost over $120 billion per year in economic costs and loss of ecosystem services (Duncan et al. 2004; Pimentel et al. 2005).

Although total government use of herbicides is difficult to estimate, the USFS reported that it applied ~ 50,000 kg of herbicides to 85,000 ha of land in 2004 primarily for noxious weed control; glyphosate, 2,4-D, dicamba, triclopyr, picloram, and clopyralid accounted for the majority of

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herbicide use (Cota, 2005). The USFS uses only a minute fraction of total herbicides applied in the United States compared to agricultural, commercial, and private use. However, herbicides are frequently applied by the USFS in environments adjacent to streams and other waterways that may contain aquatic species of concern. Acute toxicity data, supplied by chemical manufacturers during the herbicide registration process under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), are the primary data used for risk assessments of these herbicides to fishes. In spite of the widespread application of herbicides to terrestrial environments, there is surprisingly little published chronic toxicity data available for ecological risk assessments. This information is needed by resource managers in consultations regarding the use of herbicides for invasive plant control in watersheds that may contain threatened and endangered fish species and other aquatic resources (Federal Register 2004, 2005).

This study is part of a recent series of studies evaluating the nontarget ecological risk of commonly applied broad-leaved herbicides to salmonids. Previous risk assessments were published for picloram acid (Fairchild et al. 2009b) and 2,4-D acid (Fairchild et al. 2009a). In this study we conducted an ecological risk assessment of clopyralid to rainbow trout. The relative risk of clopyralid to the threatened bull trout, based on previous acute data published by Fairchild et al. (2008), is also discussed.

Materials and Methods

Test Chemicals

Dow Agrosciences (Indianapolis, IN) donated a commercial formulation of clopyralid (Lontrel 100^{TM}; CAS# 1072-17-6; 9.5% a.i. free acid) for this study. Clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) is a pyridine herbicide that is the active ingredient of several commercial herbicide formulations, including Lontrel^{TM}, Transline^{TM}, Stinger^{TM}, and Reclaim^{TM}, which are widely applied to control star thistle, Canadian thistle, and other annual and perennial broad-leaved weeds in the western United States (Weed Science Society of America 2002).

Study Site, Fish Sources, and Acclimation of Fish

Studies were conducted at the Columbia Environmental Research Center (CERC), United States Geological Survey (USGS), Columbia, Missouri. Rainbow trout were obtained as eyed eggs from Aquatic Biosystems, Inc. (Fort Collins. CO). Eggs were shipped in chilled (5°C), oxygenated water. Once received at the CERC, rainbow trout were acclimated to ambient well water conditions (temperature, 16°C; alkalinity, 258 mg/L as $CaCO_3$; hardness, 286 mg/L as $CaCO_3$, pH 7.8) and were fed twice daily at swim-up with #1 Finfish Diet (55% protein, 15% fat; Ziegler Brothers, Gardner, PA, USA).

Chronic Toxicity Testing

Chronic toxicity testing was conducted using proportional flow-through diluters according to ASTM (2004). Each diluter contained 12 replicate tanks divided into 2 replicate test chambers (7.5 L each). Ten juvenile rainbow trout (mean weight, 0.68 ± 0.10 g; mean length, 43.40 ± 2.09 mm) were stocked into each test chamber within each replicate tank (total $n = 4$ aquaria per concentration).

The diluter delivered 0.48 L test solution to each chamber every 20 min for a total volume of 35 L per day (turnover of 4.7 volume exchanges per day). Therefore, the maximum loading rate for fish (6.8 g wet weight biomass for juvenile rainbow trout; 0.91 g/L chamber density; 0.19 g/L/day) was below the maximum allowable loading rate (5 g/L chamber density; 0.50 g/L/day) of ASTM (2004). Prior to testing, fish were acclimated to test temperature (8°C) in well water over a 7-day period (1°C decrease per day). This temperature is lower than the standard temperature for rainbow trout [12°C; ASTM (2004)], but was used for comparison to other data that has evaluated the relative sensitivity of rainbow trout and bull trout exposed to picloram (Fairchild et al. 2009b) and metals (Hansen et al. 2002). Light intensity was a natural diurnal cycle of dampened natural sunlight ($\sim$ 14 h light/10 h dark). We exposed rainbow trout to the following nominal herbicide concentrations (as free acid form): 0, 16, 32, 64, 128, and 256 mg/L clopyralid. Exposure concentrations were based on previous estimates of chronic mortality published by Fairchild et al. (2008).

Temperature, dissolved oxygen, alkalinity, hardness, pH, conductivity, and ammonia were measured twice weekly in all chambers as previously described (Fairchild et al. 2009a, 2009b). Survival was measured in each exposure chamber daily over the 30-day test duration. On days 15 and 30, we measured growth of fish within each of two test chambers ($n = 20$ fish per concentration) using length ($\pm$ 1 mm) and weight ($\pm$ 0.01 g) as measurement end points.

Herbicide Analyses

In the chronic study, four herbicide samples were collected weekly from a randomly selected aquaria from the control, low, medium, and high concentrations for a total of 16 independent samples. On one date, triplicate samples were taken from one tank of the high-exposure concentration to determine precision and accuracy. Samples were frozen in brown polypropylene bottles in the dark until analysis.

Herbicides were analytically confirmed using ion chromatography similar to methodology described in the work by Fairchild et al. (2009b). Measured concentrations were calculated from a regression of measured versus nominal concentrations from the chronic study ($n = 16$ samples) and are reported in the free-acid form. Method quantitation limits were 0.366 mg/L clopyralid. Average recovery of external certified standards was 106%. Analytical precision was 2.87% based on analysis of $n = 3$ discrete replicate samples of the high concentration of clopyralid. All reported concentrations are presented as measured concentrations corrected for recovery.

Exposure Assessment

Estimated exposure concentrations (EECs) of clopyralid were derived from the US Environmental Protection Agency's (USEPA) worst-case Tier 1 assessment based on a direct application to a pond of 2 m depth, a Tier 2 assessment based on modeled concentrations using the USEPA Generic Exposure Estimate Concentration model (GEENEC2; http://www.epa.gov/oppefed1/models/water/index.htm), a Tier 3 assessment based on the Groundwater Loading Effects of Agricultural Management Systems model (GLEAMS) as applied by Durkin and Follansbee (2004), and a Tier 4 assessment based data reported by the USGS's National Water Quality Assessment (NAWQA) program (Gilliom et al. 2006) as cited by Durkin and Follansbee (2004).

Statistical Analysis

Chronic length and weight data were tested for normality and homogeneity of variance using the Shapiro–Wilk's statistic and Bartlett's test, respectively. Length and weight data met the assumptions of normality and homogeneity of variance and were, therefore, analyzed without transformation. We analyzed growth data using a one-way analysis of variance (ANOVA). Significant differences among treatments were tested using the Duncan's mean separation test. The Maximum Acceptable Toxicant Concentration (MATC) was defined as the geometric mean of the No Observed Effect Level (NOEC) and the Lowest Observed Effect Level (LOEC) as determined using ANOVA. The acute:chronic ratio (ACR) for rainbow trout exposed to clopyralid was calculated by dividing the previously published 96-h acutely lethal concentration (ALC_{50}) value (700 mg/L; 95% confidence interval 630–780 mg/L; Fairchild et al. 2008) by the MATC for growth. All significance levels were maintained at $p \leq 0.05$.

Results and Discussion

Acute Toxicity of Clopyralid

The USEPA pesticide registration database indicates that clopyralid is acutely toxic to rainbow trout and the bluegill (*Lepomis macrochirus*) at 104 and 125 mg/L (96-h ALC_{50}), respectively, whereas the zooplankton *Daphnia magna* is less sensitive to clopyralid (range 225–1133 mg/L; 48-h ALC_{50}) (USEPA 2000). Fairchild et al. (2008) determined that clopyralid was acutely toxic to juvenile rainbow trout and bull trout at similar concentrations: juvenile rainbow trout, 700 mg/L (96-h ALC_{50}), 532 mg/L (96-h ALC_{25}), 476 mg/L (96-h ALC_{10}), and 448 mg/L (96-h ALC_{5}); bull trout, 802 mg/L (96-h ALC_{50}), 582 mg/L (96-h ALC_{25}), 496 mg/L (96-h ALC_{10}), and 458 mg/L (96-h ALC_{5}). Fairchild et al. (2008) used the acute-to-chronic estimation procedures of Ellersieck et al. (2003) to estimate 30-day chronic lethal concentrations of clopyralid predicted to result in 1% mortality (CLC1) of 35 mg/L for rainbow trout and 40 mg/L for bull trout, respectively.

Chronic Toxicity of Clopyralid

We observed no significant mortality of juvenile rainbow trout exposed to clopyralid over the course of the 30-day chronic toxicity study (highest concentration = 256 mg/L clopyralid). Water quality parameters were within the limits described for an acceptable toxicity test (ASTM 2004) and are presented in Table 1.

There were no significant effects of clopyralid on growth of juvenile rainbow trout at day 15 at the highest concentration tested (256 mg/L) (Table 2). However, we observed 30-day NOEC, LOEC, and MATC values of 68, 136, and 96 mg/L clopyralid, respectively, for both growth end points (length and weight) (Table 2). The 30-day LOEC represented a decrease in weight of 9% of rainbow

Table 1 Average water quality conditions measured during the 30-day chronic exposures of rainbow trout to clopyralid

Variable	Mean value
Dissolved oxygen (mg/L)	7.55 ± 1.07 ($n = 334$)
Temperature (°C)	8.48 ± 0.40 ($n = 334$)
pH	7.97 ± 0.17 ($n = 66$)
Total ammonia (mg/L as N)	0.93 ± 0.01 ($n = 66$)
Conductivity (μS)	660 ± 76 ($n = 8$)
Alkalinity (mg/L as $CaCO_3$)	245 ± 12 ($n = 8$)
Hardness (mg/L as $CaCO_3$)	273 ± 12 ($n = 8$)

Note: Values represent the mean ± 1 SD with sample number (n) in parenthesis. The continuous chronic water quality criterion for total ammonia at pH = 8 and temperature = 8°C is 2.43 mg/L total ammonia as N (USEPA 1999)

Table 2 Average lengths and weights of juvenile rainbow trout exposed to clopyralid acid for 30 days

Measured concentration (mg/L)	Day 15		Day 30	
	Length (mm)	Weight (g)	Length (mm)	Weight (g)
0	49.5 (0.8)[a]	1.18 (0.06)[a]	57.6 (0.99)[a]	1.80 (0.99)[a]
17	48.8 (0.9)[a]	1.15 (0.08)[a]	56.1 (0.22)[a]	1.67 (0.04)[abc]
34	48.6 (1.0)[a]	1.12 (0.08)[a]	56.8 (1.54)[ab]	1.76 (0.16)[ab]
68	48.7 (0.8)[a]	1.16 (0.05)[a]	56.5 (0.61)[ab]	1.74 (0.07)[ab]
136	48.3 (0.8)[a]	1.11 (0.07)[a]	55.1 (0.90)[bc]	1.63 (0.06)[bc]
273	47.7 (1.1)[a]	1.11 (0.09)[a]	53.9 (1.02)[c]	1.56 (0.06)[c]

Note: Data represents mean $\pm$ 1 SD of $n = 2$ chambers with 10 fish per chamber. Different letters indicates significant differences from other treatments based on Duncan's means separation test ($p < 0.05$)

trout compared to controls. There are no other published data regarding the chronic effects of clopyralid on the growth of fish. However, Stehr et al. (2009) determined that clopyralid had no effects on the early development of zebrafish (*Danio rerio*) using a 5-day phenotypic screening test at concentrations up to 10 mg/L.

Acute:Chronic Ratio of Clopyralid

Acute:chronic ratios (ACRs) are typically used to calculate the margin of safety between acute and chronic effects for fish exposed to chemicals. We determined the ACR of clopyralid (7.3) by dividing the 96-h ALC_{50} (700 mg/L) published by Fairchild et al. (2008) by the MATC for growth (96 mg/L) from this chronic study. The ACR for rainbow trout exposed to clopyralid (7.3) was similar to that previously reported for rainbow trout exposed to 2,4-D (ACR = 6.6; Fairchild et al. 2009a) but threefold lower than that for rainbow trout exposed to picloram (ACR = 22; Fairchild et al. 2009b).

Expected Environmental Exposures of Rainbow Trout to Clopyralid

The Tier 1 worst-case environmental exposure assumed a direct overspray of a 1-ha pond of 2 m depth at the upper limit of application of clopyralid (1.12 kg/ha). The results of this analysis resulted in an estimated exposure concentration of 0.054 mg/L clopyralid.

The Tier 2 GENEEC2 model assumed that exposure followed a rain event occurring 2 days after application of 1.12 kg/ha clopyalid to a 10-ha watershed draining into a 1-ha generic water body of 2 m depth based on the chemical properties listed in Table 3; we assumed that application would be aerial application of a fine to medium droplet formulation with no buffer zone and no "watering in." Results are provided in Table 4. These are basically the same results as the Tier 1 model due to the spatial-scale assumptions that assume a 10% runoff to the aquatic system and the fact that clopyralid under the 2-day rainfall window is not sorbed to soil or degraded based on the published physical properties of the chemical (Table 3).

Table 3 Chemical properties of clopyralid acid used in modeling of exposure concentrations in aquatic environments

Parameter	Value
Application rate	1.12 kg/ha
Water solubility	1000 mg/L
K_{oc}	13 mL/g
Aerobic aquatic dissipation half-life	162 days
Aerobic soil metabolism half-life	12–70 days
Foliar half-life	100 days
Aquatic photolysis half-life	Stable
Hydrolysis half-life	Stable

Note: Parameters published by Wauchope et al. (1992), Durkin and Follansbee (2004), Dow Agrosciences (2008), and references therein

Table 4 Results of the Tier 2 GENEEC2 model of predicted aqueous exposures of clopyralid applied at a rate of 1.12 kg/ha

Model end point	Exposure concentration (mg/L)
Peak	0.058
Max. 4-day average	0.058
Max. 21-day average	0.057
Max. 60-day average	0.054
Max. 90-day average	0.052

Note: Model input parameters included the following: aerial application of fine to medium droplet formulation not watered in; no buffer zone; K_{oc} = 13 mL/g; soil half-life = 70 days; foliar half-life = 100 days; aquatic half-life = 162 days; and water solubility = 1000 mg/L

Tier 3 exposure estimate was derived from the GLEAMS model as applied by Durkin and Follansbee (2004). Durkin and Follansbee (2004) calibrated the GLEAMS model using the half-lives of clopyralid in various environmental compartments (sediment, soil, water, and foliar surfaces), water solubility, and soil sorption coefficients for various soil types varying in amounts of organic carbon (e.g., clay, loam, and sand). These physical constants were quite similar to those provided in Table 3. Site parameters included a base flow of 4,420,000 L/day, velocity of 0.08 L/s, stream width of 2 m, depth of 0.3 m; clopyralid applied at 1.12 kg/ha to a field of 4.5 ha in size, a root zone of 1.5 m depth, and four different soil layers. The model was run for varying amounts of annual rainfall (13–635 cm total) and various soil conditions. The highest maximum predicted stream water concentration (0.062 mg/L clopyralid) was predicted in sandy soil under annual rainfall conditions of 635 cm/year. The maximum concentration of clopyralid (0.073 mg/L) estimated in a small pond adjacent to a field application at a rate of 1.12 kg/ha was similar to that for the stream scenario.

For a Tier 4 exposure estimate, we relied on published environmental data in stream water and groundwater. The NAWQA program of the USGS has evaluated the concentrations of herbicides in water and groundwater since 1991 in over 50 major river basins and aquifers distributed across agricultural and urban land use; clopyralid has never been observed above the detection limit of 0.21 µg/L (Gilliom et al. 2006). However, the NAWQA program does not measure concentrations of herbicides in small ephemeral to intermittent streams typical of forestry catchments. Durkin and Follansbee (2004) evaluated the only published study of clopyralid runoff in streams adjacent to a 54-ha watershed treated by aerial application of clopyralid at a rate of 2.21 kg/ha (two times higher than our assumed rate of 1.12 kg/ha) published by Leitch and Fagg (1985); peak concentrations (0.017 mg/L) occurred shortly after rain events over the monitoring period where 133 mm rainfall was recorded over a 19-day period. Therefore, if we adjust the data of Leitch and Fagg (1985) for our modeled application rate of 1.12 kg/ha, the field exposure value for edge-of-field conditions would be 0.008 mg/L clopyralid.

Risk Assessment of Rainbow Trout Exposed to Clopyralid

Our assessment of the risk of rainbow trout exposed to clopyralid consists of mathematical and graphical comparisons of laboratory toxicity data to modeled and observed concentrations of clopyralid in the environment. Modeled and measured exposure concentrations of clopyralid applied at 1.12 kg/ha ranged from 0.008 to

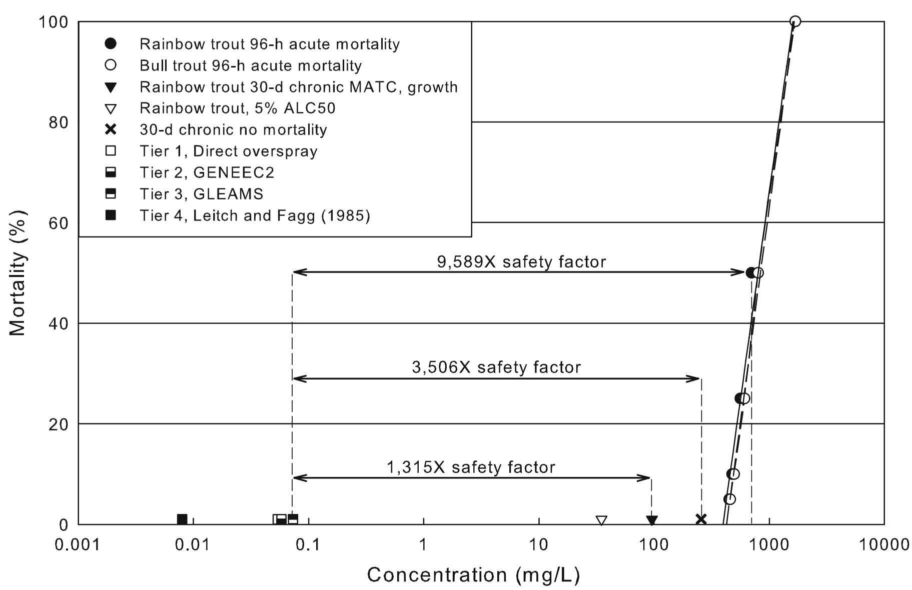

Fig. 1 Graphical presentation of modeled and measured environmental exposure concentrations (squares) compared to rainbow trout toxicity response concentrations (other symbols) for clopyralid acid. No mortality occurred at the highest chronic concentration of clopyralid tested (256 mg/L) in the 30-day chronic study. The Tier 3 GLEAMS model indicated that the highest anticipated exposure concentration of clopyralid acid is 0.073 mg/L. The 30-day MATC for growth (black triangle) of rainbow trout is 96 mg/L. The open triangle represents 5% of the 96-h ALC_{50} (700/20 = 35 mg/L) for rainbow trout from Fairchild et al. (2008), which has been used to estimate safe concentrations of herbicides for endangered and threatened salmonids (USEPA 2004). Circles represent various 96-h mortality rates (5%, 10%, 25%, 50%, and 100% acute mortality) modeled using probit analysis of acute data from Fairchild et al. (2008). Safety factors represent the ratio of the 30-day MATC for growth (96 mg/L) divided by the highest modeled exposure concentration (0.073 mg/L; 1315 × safety factor); the 30-day maximum concentration resulting in no mortality (256 mg/L) divided by the highest modeled exposure concentration (0.73 mg/L; 3506 × safety factor); and the 96-h ALC_{50} for rainbow trout (700 mg/L) divided by the highest modeled exposure concentration (0.073 mg/L; 9589 × safety factor)

Table 5 Measured acute toxicity, chronic toxicity, and acute:chronic ratio of rainbow trout exposed to clopyralid free acid

Chemical	96-h ALC_{50} (mg/L)	30-day CLC1 (mg/L)	30-day NOM (mg/L)	30-day MATC (mg/L)	5% ALC_{50} (mg/L)	Acute:chronic ratio
Clopyralid acid	700	477	256	96	35	7.3

Note: The 96-h ALC_{50} value was reported in Fairchild et al. (2008). The 30-day CLC1 (estimated 30-day exposure concentration resulting in 1% mortality) was calculated from acute data as reported in Fairchild et al. (2008) with rainbow trout. The maximum 30-day concentration of clopyralid resulting in no observed mortality (30-day NOM) was the highest concentration tested (this study). The MATC was determined as the geometric mean of the 30-day NOEC and LOEC affecting growth determined using ANOVA (this study). The 5% of the ALC_{50} (fractional acute value) was published in Fairchild et al. (2008) and is frequently used in ecological risk assessments for endangered and threatened species (USEPA 2004). The acute:chronic ratio was determined as the 96-h ALC_{50} from Fairchild et al. (2008) divided by the 30-day MATC for growth (this study)

0.073 mg/L clopyralid. These concentrations were plotted against various acute and chronic endvpoints for rainbow trout and bull trout in Fig. 1. Worst-case exposure results, published by Durkin and Follansbee (2004), revealed a margin of safety of 1315 for growth effects and a margin of safety of 3507 for mortality (e.g., no mortality observed at exposure to 256 mg/L clopyralid for 30 days). The margin of safety for acute effects (96-h ALC_{50}) is about 10,000 for both rainbow trout and bull trout. Collectively, the results of this risk assessment indicate an extremely low probability for adverse effects of clopyralid to rainbow trout and bull trout.

Actual chronic toxicity data are rarely available for risk assessment of the effects of herbicides or other chemicals on endangered species. Therefore, the USEPA proposed using 5% of the 96-h ALC_{50} as an estimate of a potential chronic effect concentration (e.g., 35 mg/L from this dataset; Table 5, Fig. 1). In this study we measured an MATC for growth of rainbow trout exposed to clopyralid at 96 mg/L; in addition, we applied the acute-to-chronic estimation procedures of Ellersieck et al. (2003) using the exposure:response profile from an acute toxicity dataset to calculate a 30-day CLC1 of 477 mg/L (Table 5). These results as well as those published for rainbow trout exposed to 2,4-D acid (Fairchild et al. 2008, 2009a) and picloram (Fairchild et al. 2008, 2009b) indicate that acute to chronic estimation procedures based on acute toxicity data (e.g. Ellersieck et al. 2003) provide statistically defensible approaches for the estimation of concentrations of herbicides protective of threatened or endangered species that are more accurate than the traditional use of safety factors applied to acute toxicity data.

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