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SUSAN RIVER TOXICITY TESTING PROJECT

FINAL REPORT
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Glossary of Terms and Acronyms

20% Evian	Evian® diluted with glass distilled water
^{o}C	degrees Celsius
µg/l	micrograms per liter
µm	micrometer
µmhos/cm	micromhos per centimeter
ASTM	American Society for Testing and Materials
$CaCO_3$	calcium carbonate
CV	coefficient of variation
DFG	Department of Fish and Game
DIEPAMH	de-ionized water amended to a hardness of 80 to 100 mg/L as CaCO3
DO	dissolved oxygen
EC	electrical conductivity
EC_{25}	effective concentration at which a toxicant causes an adverse affect on a quantal (all or nothing) response in 25% of the organisms (US EPA 2002)
EC_{50}	effective concentration at which a toxicant causes an adverse affect on a quantal (all or nothing) response in 50% of the organisms (US EPA 2002)
Eluate	methanol that is passed through a C8 column to remove any non-polar compounds adsorbed to it
g/l	grams per liter
IC_{25}	inhibition concentration at which a toxicant causes an adverse affect on a non-quantal response in 25% of the organisms (US EPA 2002)
LC_{50}	lethal concentration at which a toxicant causes death in 50% of the organisms (US EPA 2002)
LRWQCB	Lahontan Regional Water Quality Control Board

MeOH	methanol
mg	milligrams
mg/l	milligrams per liter
mg/surviving indiv	the weight in milligrams per surviving individual (fathead minnow)
Mini-TIE	a project-focused TIE procedure for duckweed that includes Chelex and solid phase extraction C8 manipulations
ml	milliliter
MS-222	tricaine methanesulfonate, fish anesthestic
NP	nonylphenol
NPE	nonylphenol ethoxylate
NTU	nephelometric turbidity unit
$p<0.05$	there is a 5% probability that a treatment will be flagged as being statistically different from the control even though the sample is nontoxic (false positive)
PRT	pathogen related toxicity
QAPP	Quality Assurance Project Plan
s.e.	standard error
SPE	solid phase extraction
SR-1	site sampled on Susan River at Hobo Camp trailhead to Bizz Johnson trail downstream of former USGS Gage
SR-2	site sampled on Susan River at McGowan Lane
SR-3	site sampled on Susan River at Leavitt Lane Bridge
SR-4	site sampled on Susan River upstream of Litchfield at Bridge 7-34 on Highway 395
SSEPAMH	Sierra Springs™ water amended to a hardness of 80 to 100 mg/L as CaCO3
SWRCB	State Water Resources Control Board
TIE	Toxicity Identification Evaluation

TIE trigger	50% or greater mortality and statistical differences from the control within 96 hours for *Ceriodaphnia dubia* and *Pimephales promelas* and a 50% or greater reduction in weight or frond number for *Lemna minor*
TMDL	total maximum daily load
UCD ATL	University of California, Davis Aquatic Toxicology Laboratory
US EPA	United States Environmental Protection Agency
USGS	United States Geological Survey
x	mean
YCT	*Ceriodaphnia dubia* food consisting of yeast, organic alfalfa, and trout chow

EXECUTIVE SUMMARY

During 1990, US EPA testing on the Susan River identified toxicity to larval fish and the aquatic plant, duckweed. The cause(s) of toxicity were not identified. Subsequently, the Susan River was placed on the federal Clean Water Act, Section 303(d) list of impaired waterbodies for unknown toxicity. The State Water Resources Control Board contracted with the University of California, Davis Aquatic Toxicology Laboratory to provide data to be used by the Lahontan Regional Water Quality Control Board in considerations of de-listing or in development of total maximum daily loads (TMDL). A primary objective of this project was to determine whether the pattern of toxicity observed by US EPA in 1990 continued to occur in 2003/04. An additional objective was to identify the cause(s) of any observed toxicity to test species.

Four sites on the Susan River were sampled 12 times (monthly, May through October 2003 and March through August 2004). These samples were tested with a larval fish (fathead minnow, *Pimephales promelas*), a cladoceran zooplankton species (*Ceriodaphnia dubia*), and a vascular aquatic plant (duckweed, *Lemna minor* a resident species of California).

Fifteen of forty-eight (31%) of the Susan River samples were toxic to at least one of the test species. Five Susan River samples were toxic to only larval fish, three samples were toxic to only *Ceriodaphnia dubia*, six samples were toxic to only duckweed, and one sample was toxic to both larval fish and *Ceriodaphnia dubia*. The magnitude of toxicity in all Susan River samples toxic to larval fish or to *Ceriodaphnia dubia* was insufficient to permit effective identification of contaminant cause(s). While toxicity in river samples is a violation of the Regional Board Basin Plan narrative water quality objective for toxicity, the ecological relevance of these findings are unknown.

A standard procedure for identification of chemical cause(s) of toxicity in duckweed testing has not been published. Nonetheless, experiments (beyond the scope of contract requirements) conducted at ATL strongly implicated additive/synergistic effects of the

herbicide Transline and surfactants (nonyphenol and nonyphenol ethoxylate) in Transline formulations as the causes of duckweed toxicity. The major use of Transline formulations in Lassen County is treatment of rights-of-way. Application to rights-of-way is mostly restricted to the June through September period, generally corresponding to the period when Susan River samples were toxic to duckweed.

While the toxicity observed in the Susan River samples is a violation of the Regional Water Quality Control Board narrative water quality objective for toxicity, potential impacts of the toxicity results (presented herein) on biological communities in the Susan River is incompletely known. Ecological relevance of waterway toxicity depends on magnitude, duration, frequency, and geographic extent of toxicity. Magnitude of toxicity in Susan River samples toxic to larval fish and *Ceriodaphnia dubia* was relatively low. Duration, frequency, and geographic extent of the observed toxicity is incompletely known, but data presented in this report indicate that geographic extent of toxicity to larval fish and *Ceriodaphnia dubia* was restricted and that duration and frequency of toxic events were low. Until more extensive monitoring (including a greater number of sites and more frequent sampling) potentially proves otherwise, the current set of data are consistent with low level or no impacts on aquatic life beneficial uses.

1. INTRODUCTION

1.1 Characteristics of the Study Area

The Susan River originates from Silver and Caribou Lakes, in southern Lassen County, and flows east through McCoy Flat Reservoir discharging into Honey Lake. The river supplies over thirty percent of the total surface water needs for Lassen County with precipitation and snow melt from the western portion of the watershed (Lahontan Regional Water Quality Control Board, 2004). The surrounding areas encompass an abandoned railroad and private mines. Fishing, cycling, hiking, horseback riding, and skiing are popular uses along the Susan River (Friends of the River, 2004). The Honey Lake Wildlife Area provides habitat for several threatened or endangered species, including the bald eagle, sandhill crane, bank swallow, and peregrine falcon (Department of Water Resources, 2004). The State Water Resources Control Board (SWRCB) and Regional Water Quality Control Boards have conducted investigations of California's inland surface waters over the past twelve years and identified toxicity to aquatic organisms in many waterways. Agriculture, mining, and storm water runoff were revealed to be the primary contributors to this toxicity (e.g., de Vlaming *et al*., 2000). In the early 1990's the United States Environmental Protection Agency (US EPA) reported toxicity in the lower section of the Susan River. US EPA testing on Susan River samples collected during 1990 revealed toxicity to larval fathead minnows and duckweed. The causes of the toxicity were not identified. Consequently, in 1996, the Susan River was placed on the Federal Clean Water Act Section 303(d) list of impaired waterbodies for unknown toxicity. The current investigation was initiated to assess whether the toxicity noted in 1990 could be confirmed in 2003-2004 and if so, determine the cause(s) of such toxicity. Data generated in this study are to be used by the Lahontan Regional Water Quality Control Board (LRWQCB) in consideration of delisting or in development of total maximum daily loads (TMDL).

1.2 Study Objectives

1. Investigate the validity of previous toxicity studies on the Susan River to aid the LRWQCB in ultimately confirming or denying the need for its placement on the Clean Water Act Section 303(d) list.
2. Identify specific cause(s) and source(s) of toxic contaminants to aid the LRWQCB in development of a TMDL for toxicity in the Susan River.

2. MATERIALS AND METHODS

Detailed descriptions of the materials and methods used for the Susan River Toxicity Testing Project can be found in the Quality Assurance Project Plan (QAPP) for Susan River TMDL Development (Fong *et al*., 2003). The QAPP defines procedures for sampling, testing and calibration, and criteria for data quality acceptability.

2.1 Sampling Sites

Site locations are based on historical (1990) toxicity data, land use practices, accessibility, and runoff patterns (Table 1). Staff of the LRWQCB and the SWRCB collected samples approximately monthly from May through October 2003 and March through July 2004. University of California, Davis Aquatic Toxicology Laboratory (UCD ATL) staff collected samples in August 2004. Samples were collected from the Susan River near the former United States Geological Survey (USGS) gage at the Hobo Camp trailhead, at McGowan Lane, Leavitt Lane Bridge, and upstream of Litchfield at Bridge 7-34 on Highway 395. Sample sites and rationale for choosing these sites are listed in Table 1. An overview of the sampling area can be found below (Figure 1) and sites are shown in Figure 2.

2.2 Sample Collection and Storage

Samples were collected as sub-surface grabs from mid-depth either off a bridge or along the bank in pre-cleaned, one gallon, amber glass bottles. One additional liter was collected in high density polyethylene containers for turbidity analysis.

To assess laboratory testing precision, a field duplicate sample was collected from one randomly selected site during four of the sampling events. The duplicate sample was collected using the same methods used for the primary test samples. A trip blank

(laboratory control water) was taken into the field during sample collection on 30 July 2003 and tested to assess possible contamination in the field.

Field measurements of pH, electrical conductivity (EC), dissolved oxygen (DO), and temperature were recorded for each site. Immediately after collection, samples were placed in ice chests with wet ice and transported to UCD ATL where temperatures were measured and samples were stored in the dark at 4 $\pm$ 2 degrees Celsius (ºC). Toxicity tests were initiated within 48 hours of sample collection.

Table 1. Summary of site selection criteria.

Site	**Map ID[1]**	**Rationale for Selection**
Susan River at Hobo Camp trailhead to Bizz Johnson trail downstream of former USGS Gage	SR-1	To duplicate 1990 US EPA toxicity testing site R-6-1, and represent water quality upstream of the City of Susanville.
Susan River at McGowan Lane	SR-2	To capture changes in water quality below confluence with Gold Run Creek, that may have geothermal discharges that could influence water quality. Also near 1990 US EPA site R-6-2.
Susan River at Leavitt Lane Bridge	SR-3	Best available access downstream of confluence with Jensen and Brockman Sloughs where Susanville Consolidated Sanitary District discharges and agricultural activity may influence water quality.
Susan River upstream of Litchfield at Bridge 7-34 on Highway 395	SR-4	To duplicate 1990 US EPA site R-6-3 downstream of confluence with Willow Creek.

1. Map IDs refer to sites on Figure 1.

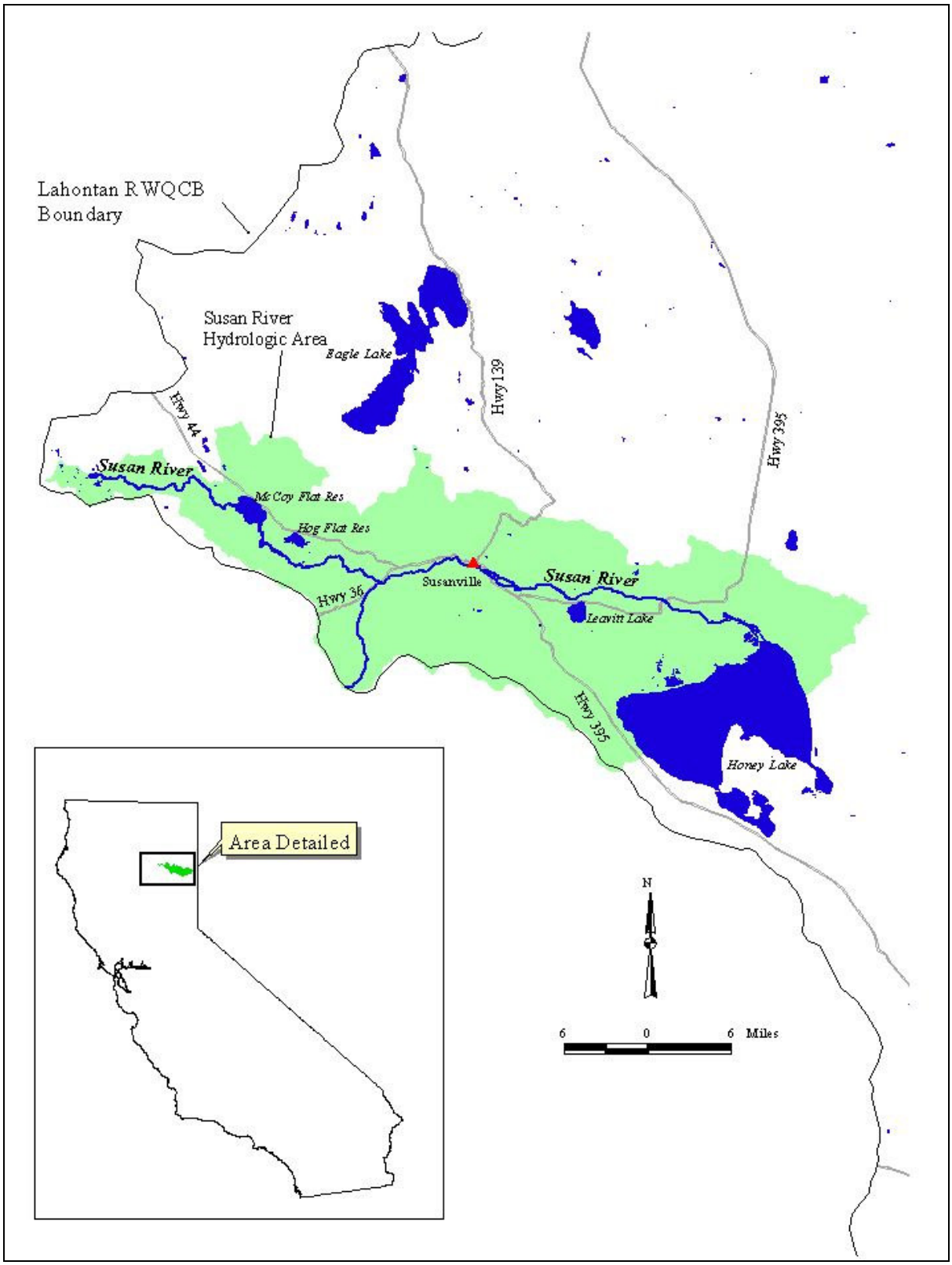

Figure 1. Overview of sampling area.

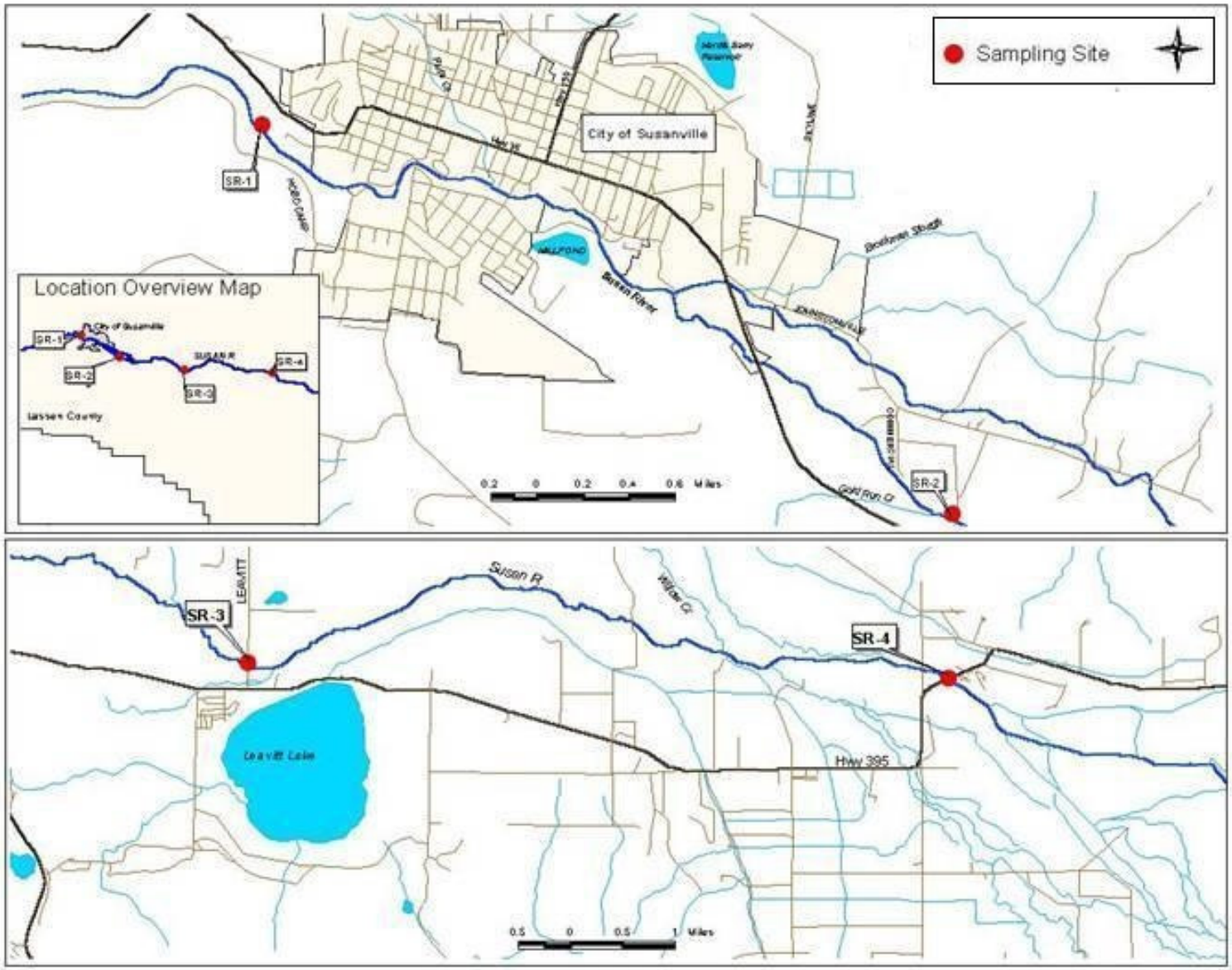

Figure 2. Susan River sampling sites (see Table 1 for descriptions).

2.3 Toxicity Testing

Ceriodaphnia dubia and larval *Pimephales promelas* (fathead minnow) toxicity testing procedures followed those outlined in Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (US EPA, 2002) with some exceptions. Aspects of these procedures that differ from the US EPA methods and the rationale for using them are outlined below. *Lemna minor* (duckweed) toxicity testing procedures followed those outlined in the American Society for Testing and Materials (ASTM) Standard Guide for Conducting Static Toxicity Tests with *Lemna gibba* G3 (1998). ASTM indicates within this method that it is applicable to *Lemna minor,* as well.

While US EPA methods do not specifically recommend aeration of the renewal water, UCD ATL protocols include aeration. This deviation is employed because the ambient samples tested at UCD ATL frequently require aeration to prevent oxygen super-saturation. Aeration time is limited until samples come to 102% saturation to minimize the loss of volatile toxicants.

2.3.1 Ceriodaphnia dubia

The *Ceriodaphnia* assay consisted of ten replicate glass vials. The US EPA recommends using plastic cups for the *Ceriodaphnia* toxicity test, but because plastic adsorbs organic compounds UCD ATL opts to use glass vials for determining the role of organic compounds in toxicity to *Ceriodaphnia* tests. Each vial contained 15 milliliters (ml) of sample and one *Ceriodaphnia* each. Less than 24-hour-old *Ceriodaphnia,* all born within a 16-hour period, were employed at test initiation. *Ceriodaphnia* were obtained from in-house cultures. *Ceriodaphnia* were transferred into a vial containing *Selenastrum*, YCT (a mixture of yeast, organic alfalfa, and trout chow) and 15 ml of fresh sample water daily. The test was incubated in a temperature-controlled room maintained at 25 ± 2 °C with a 16:8 hour light:dark photoperiod for six to eight days. Mortality and reproduction were measured daily and upon test termination.

2.3.2 Pimephales promelas

The *Pimephales* assay consisted of four replicate 600 ml beakers, each containing 250 ml of sample and 10 larval fathead minnows. The tests performed in May and August 2003 employed glass beakers and tests performed in other months employed Teflon® beakers. In

addition to testing in Teflon® beakers, "super clean" techniques were also used beginning in late July 2003. Super clean techniques require scraping the bottom of the test vessels with tubing while removing test sample and debris for the purpose of decreasing pathogen interference (see discussion on Pathogen Related Toxicity (PRT), section 3.2.2.1). Tests were initiated with less than 48-hour-old minnows obtained from Aquatox Inc, Hot Springs, Arkansas. Minnows were fed three times daily with the brine shrimp *Artemia* nauplii. Approximately 80% of the test solution was renewed daily. Dead fish, *Artemia*, and debris were removed from the test beakers daily. The test solution was incubated in a water bath at 25 ± 2 °C under ambient laboratory light with a 16:8 hour light:dark photoperiod for seven days. Mortality was measured daily upon test solution renewal. At test termination the surviving minnows were dosed with MS-222 (a fish anesthetic), dried to constant weight at 103-105 °C (approximately 16 hours), and weighed with a Mettler H54 AR balance. Mortality and biomass (growth) endpoints were measured upon test termination.

2.3.3 Lemna minor

Lemna minor is a resident species in California and was used in the tests conducted by US EPA on the Susan River in 1990. Prior to this project, duckweed testing had not been conducted at UCD ATL. The duckweed assay consisted of four replicate 250 ml beakers, each containing 100 ml sample and 4 colonies of three-frond plants. Duckweed plants were obtained from in-house cultures grown in Hoagland's E growth media. Duckweed plants were acclimated in 20xAAP media for approximately 24 hours prior to test initiation. Samples were filtered using a 0.22 micrometer (µm) nylon filter and inoculated with the standard ASTM media volume. The duckweed assay was a static non-renewal. Beakers were incubated at 25 ± 2 °C under a continuous light source at an intensity of 400 ± 40 ft-candles for 7 days. Beaker positions were randomized twice daily. At test termination duckweed was dried to constant weight at 103-105 °C (approximately 16 hours), and weighed with a Mettler H54 AR balance. Growth and frond count were measured upon test termination.

2.4 Quality Assurance

2.4.1 Laboratory Control Waters

Each toxicity test included a laboratory control. The laboratory control waters varied for each species, as specified in the project QAPP. For the *Ceriodaphnia* assay, the primary laboratory control was Sierra Springs™ water amended to a hardness of 80 to 100 milligrams per liter (mg/L) as calcium carbonate ($CaCO_3$) (SSEPAMH). As a precautionary measure, UCD ATL included a secondary control of Evian® diluted with glass distilled water (20% Evian) as well. De-ionized water amended to a hardness of 80 to 100 mg/L as $CaCO_3$ (DIEPAMH) was used for the *Pimephales* assay. For the duckweed assay, the laboratory control was Sierra Springs™ water.

2.4.2 Reference Toxicant Tests

Positive control reference-toxicant tests were conducted for each species using sodium chloride (NaCl) or Atrazine during the study period. These tests included the laboratory control and a dilution series of NaCl or Atrazine in laboratory control water. The purpose of these tests was to assess changes in organism sensitivity to a known toxicant. The LC/EC_{50} for each test was plotted to ensure that it fell within an acceptable range relative to previous results. If a test did not fall within acceptable ranges, then the results of toxicity tests performed in concurrent months were suspect.

The method UCD ATL uses to calculate the acceptable range of variation differs from that recommended by the US EPA. The US EPA recommends that acceptable data should fall within two standard deviations of the mean for the total data set. UCD ATL accepts data that falls within two standard deviations from the running mean. These standard deviations represent the standard deviation for the last data point and nineteen previous points. Corrective actions are only effective when the two-standard deviation range is calculated monthly, rather than delaying until the end of the survey period (as is the case with the EPA method).

As stated above, UCD ATL uses reference toxicant data to track changes in animal sensitivity over time. These changes may indicate problems with organism health, technician-handling techniques, and/or organism genetic variations. US EPA (2002) suggests that one outlying value may be expected to occur by chance when 20 or more data points are plotted. The UCD ATL

evaluates patterns of outlying values. When more than one outlying value occurs, corrective actions can be taken. For example, when two consecutive data points plot above the two standard deviation lines on an LC_{50} control chart, this may indicate that the test organisms are becoming less sensitive to reference toxicants. The appropriate corrective measure may be to introduce a new genetic line of organisms to increase sensitivity.

2.5 Water Quality

Water quality parameters of temperature, pH, (DO), and (EC) were measured on test samples upon initiation of the test. In the *Ceriodaphnia* and larval fathead minnow assays, temperature, pH and DO were measured on the 24-hour-old sample upon test sample renewal. The 24-hour-old sample is the water that the test organisms had been living in since test initiation. In the duckweed assay, temperature, pH and DO were measured upon test termination. Laboratory measurements were taken using a Check Temp™ digital thermometer, pH was measured with a Beckman 255 pH meter, DO was measured with a YSI model 58 oxygen meter with a 5700 series probe, and EC was determined with a YSI model 30 EC meter. All meters were calibrated daily according to the manufacturers' specifications. Ammonia was measured using a HACH DR/890 colorimeter within 24 hours of sample receipt. Hardness and alkalinity were measured on all samples utilizing titrimetric methods within ten days of sample receipt.

2.6 Toxicity Identification Evaluations

No Toxicity Identification Evaluations (TIEs) were performed during this investigation. The "trigger" for entering a toxic sample into the TIE process was "50% or greater mortality and statistical differences from the control within 96 hours" for *Ceriodaphnia* and larval fathead minnows (as defined in the project QAPP). Initially, the follow up procedures for duckweed toxicity did not include TIEs since procedures do not exist in the scientific literature. Several detections of toxicity to duckweed in 2003 emphasized the need for additional investigation. UCD ATL and the Contract Manager agreed on a project-focused TIE process ("mini-TIE") to aid in identification of potential cause(s) of toxicity to duckweed. In July 2004, the contract for this project was amended such that mini-TIE procedures would be performed on samples exhibiting $\leq$50% of the growth (frond number or weight) of the controls for duckweed. The TIE triggers were set as stated to ensure effective TIEs and prevent overuse of funds by investigating toxicity that may not be reproducible after extended holding times.

Two Susan River samples, SR-3 and SR-4, collected 30 September 2003 resulted in a greater than 50% reduction in duckweed weight and frond number compared to controls. However, TIEs were not conducted because the duckweed mini-TIE trigger was not in place at that time. During the entire course of this project, no other Susan River sample exceeded the *Ceriodaphnia*, larval fathead minnow, or duckweed TIE triggers.

2.6.1 C8 Solid Phase Extraction and Chemical Analysis

C8 solid phase extraction removes non-polar organic chemicals from water samples. Toxic samples collected on 2 July 2003 and 30 September 2003 were treated with C8 columns. 1800 ml of each sample were pumped through a C8 column at a rate of 10 ml/min. The columns were each eluted with 3 ml methanol on 10 March 2004. The eluate was delivered to the Department of Fish and Game (DFG) Nimbus Laboratory for organics analysis for pyrethroids, surfactants, herbicides, organophosphorous insecticides, triazines, organochlorines, and carbamates. Samples collected 30 June 2004, 28 July 2004, and 25 August 2004 caused low-grade toxicity (i.e., inhibition of growth or reproduction) and were preserved on C8 columns. The columns are being held at UCD ATL in case there is a need for future analysis.

In addition to analysis of the C8 eluates mentioned above, whole water samples collected March through August 2004 were delivered to DFG as soon as possible (generally within 24 hours of sample receipt at UCD ATL). They were analyzed for pyrethroids, surfactants, herbicides, organophosphorous insecticides, triazines, organochlorines, and carbamates. As of May 2004, copper, cadmium, lead, nickel, silver, and zinc were added to the chemical analyses.

2.7 Statistical Analysis

Toxicity was defined as a statistically significant difference ($p<0.05$) between a sample and the laboratory control water. Acute toxicity in the *Ceriodaphnia* and *Pimephales* assays was defined as a statistically significant increase in mortality in a test sample when compared to the laboratory control within 96 hours. Chronic toxicity was defined as a significant increase in mortality compared to the laboratory control in greater than 96 hours or a significant decrease in growth or reproduction compared to the laboratory control.

All *Ceriodaphnia* reproduction, *Pimephales* growth and mortality, and duckweed growth data were analyzed with Shapiro-Wilks Test for normality and Bartlett's Test for homogeneity of variance. When the data fit normal distributions and had homogeneous variances, they were analyzed using an Analysis of Variance and Dunnett's mean separation tests. When the data deviated significantly from normality or had heterogeneous variances, they were log transformed. When log transformation did not establish normality or homogeneity of variance, nonparametric Bonferroni corrected Wilcoxan tests were performed to compare each treatment to the control. *Ceriodaphnia* mortality data were analyzed with Fisher's Exact Test.

These statistical analyses differ from those outlined in US EPA (2002). US EPA (2002) protocols were designed for whole effluent toxicity testing in which all samples are tested in a dilution series, and the statistical analyses recommended by US EPA (2002) were designed to analyze data from a dilution series. The approach taken during this study was to assess the water quality at a particular site compared to laboratory control water, as well as to other sites. No dilution series were performed during the initial screening of the samples. As a result, the US EPA (2002) statistical protocols were not appropriate for the data obtained during this study. UCD ATL staff consulted the University statistician (Neil Willits, UCD) to determine the most appropriate statistical analyses for these data. The statistician recommended the analyses discussed above.

3. RESULTS

3.1 Species Performance / Test Acceptability Requirements

US EPA protocol (2002) specifies that the test performance of each species in laboratory control water meet criteria to be considered acceptable. For chronic *Ceriodaphnia* tests, US EPA specifies that in the control mortality is not to exceed 20%, and 60% of the surviving females must have three or more broods with a minimum average of 15 neonates. One test failed to meet test acceptability requirements in this project. On day one of the *Ceriodaphnia* test conducted with samples collected on 30 September 2003, 40% mortality was noted in the primary control (SSEPAMH). The test was re-setup on 3 October 2003 and it met test acceptability criteria. In all other *Ceriodaphnia* tests conducted during this project, either the primary or secondary control met test acceptability criteria. US EPA protocol requires control treatments in the

Pimephales assay to have a minimum average weight of 0.25 mg/fish and a maximum mortality of 20%. All tests conducted during this survey met these criteria. For duckweed, the ASTM protocol designates that the total number of fronds at test termination in the control treatment be five times that at initiation. The duckweed test conducted with samples collected 29 May 2003 failed to meet ASTM test acceptability criteria. Fungus was present in many replicates, compromising test results. Additional culturing and testing procedures were subsequently incorporated to prevent contamination. To compensate for the failed test, an additional sampling event occurred in June 2004. All other duckweed tests conducted during this project met test acceptability criteria. Deviations from the QAPP specifications are reported below in sections 3.2.1.1, 3.2.1.2, 3.2.2.1, and 3.2.3.1.

3.2 Toxicity Test Results

Results are reported by species in the order of *Ceriodaphnia*, larval *Pimephales,* and duckweed. The sample receipt temperature exceeded the data quality objective of 6 °C in five samples. Samples collected from SR-4 on 29 May 2003 and 26 August 2003 were 7.9 and 7.6 °C upon arrival at UCD ATL, respectively. The sample collected at site SR-2 on 26 August 2003 was received at 8.3 °C. Samples collected at sites SR–1 and SR-2 on 26 May 2004 were received at 7.9 and 7.4 °C, respectively. In our professional judgment, these temperature exceedances were not of a magnitude to affect toxicity test results. Toxicity test results are summarized in Table 2.

Fifteen of forty-eight (31%) of the Susan River samples were toxic to at least one of the test species. Five Susan River samples were toxic to only larval fish, three samples were toxic to only *Ceriodaphnia dubia*, six samples were toxic to only duckweed, and one sample was toxic to both larval fish and *Ceriodaphnia*.

3.2.1 Ceriodaphnia dubia

Summary data sheets for *Ceriodaphnia* testing appear in Appendix A. During the twelve months of testing, *Ceriodaphnia* exhibited reproductive toxicity in only four Susan River samples. Decreased reproduction compared to the control was observed in samples collected on 30 July 2003 at sites SR-1 and SR-2, and in the samples collected 25 August 2004 at sites SR-1 and SR-4. Because the magnitude of toxicity in these samples was below the Toxicity Identification

Evaluation (TIE) trigger, neither additional toxicity tests, nor TIEs were conducted. Chemical analysis performed on the 25 August 2004 samples revealed the presence of certain metals, but they were present at concentrations well below the LC_{50} level (Appendix G, Table 1). However, the sample was preserved on C8 columns for possible future analysis.

3.2.1.1 Exceedance of Sample Holding Time

The UCD ATL QAPP sample holding time of 48 hours was exceeded for samples collected on 30 September 2003. As mentioned above in Section 3.1, the initial test conducted with these samples was initiated on time, but 40% mortality occurred in the primary control on day one of the test. The test was taken down and another test was initiated on 3 October 2003, within 72 hours of sample collection, in an effort to gain useful information. US EPA methods allow for a maximum holding time of 72 hours with contractor approval, therefore it is our professional judgment that these data are reliable. Chemical analysis of these samples did not reveal contaminant concentrations of concern (Appendix G, Table 1).

3.2.1.2 Use of Primary and Secondary Control Waters

UCD ATL employs both SSEPAMH and 20% Evian in all *Ceriodaphnia* tests, but typically uses SSEPAMH as the primary control. Both are acceptable controls according to the US EPA method manual. In the test conducted on samples collected 2 July 2003, 20% Evian was used as the primary control because there was insufficient reproduction data for the *Ceriodaphnia* tested in SSEPAMH to meet test acceptability criteria. This is because all data collected on the seventh day of the test (for ambient samples and controls) were unreliable due to technician error and therefore could not be used. The test did provide useful and reliable data because the 20% Evian control met test acceptability criteria; ambient samples were compared to this control in statistical tests. No significant toxicity was detected in any of the samples. To further support our belief that no toxicity was missed the previous year, none of the June or July 2004 samples were toxic to *Ceriodaphnia*. These data contribute to our judgment that the use of 20% Evian as the control for the 2 July 2003 *Ceriodaphnia* test did not result in failure to detect significant toxicity in Susan River samples.

20% Evian was used as the primary control in the tests with samples collected on 26 August 2003 and 28 April 2004. Only 50% and 30%, respectively, of the *Ceriodaphnia* tested in

SSEPAMH produced a third brood of neonates by test termination. In the control, a minimum of 60% of surviving female C*eriodaphnia* must have a third brood to meet test acceptability criteria. Neonate production in all Susan River samples collected on 26 August 2003 and in three of four samples taken on 28 April 2004 was high compared to controls, indicating that these samples did not contain toxic concentrations of contaminants. In our judgment, it is unlikely that use of 20% Evian as the control for samples collected on these two dates resulted in failure to observe significant toxicity in the river samples.

To investigate the equivalence of control waters, UCD ATL performed two experiments in which *Ceriodaphnia* were tested in SSEPAMH and 20% Evian. Although the *Ceriodaphnia* tested in SSEPAMH tended to reproduce and survive better than those in 20% Evian, there were no statistical differences between the two control waters. During this project 20% Evian controls met *Ceriodaphnia* test acceptability criteria in all twelve tests, whereas SSEPAMH controls achieved test acceptability criteria in nine of twelve tests. In a concurrent project at UCD ATL the two controls were equivalent in meeting test acceptability criteria in 41 *Ceriodaphnia* tests. As indicated above, the use of 20% Evian as the primary control in three *Ceriodaphnia* tests unlikely affected the outcome of those tests (e.g., detection of toxicity was not compromised).

3.2.2 Pimephales promelas (Larval Fathead Minnow)

Summary data sheets for larval *Pimephales* testing are located in Appendix B. During the twelve months of testing, six samples were toxic to larval *Pimephales*. Increased mortality compared to the control was observed in the sample collected on 26 August 2003 at site SR-1. Because the mortality (30%) occurred on the fifth day of the test, the magnitude of mortality in this sample was below the TIE trigger and no follow up was conducted. Not only was this mortality insufficient to perform a TIE, but there was high variation among replicates (coefficient of variation (CV) = 38.7%). This high variability led us to suspect that the observed mortality was due to pathogen interference rather than chemical contaminant(s) effects (see section 3.2.2.1).

Larval *Pimephales* exhibited decreased biomass in five additional samples (30 March 2004 at SR-3, 30 June 2004 at SR-1, 28 July 2004 at SR-2, and 25 August 2004 at SR-1 and SR-2). For this project, biomass reduction did not trigger TIE follow-up. However, samples collected 30

June, 28 July, and 25 August 2004 were preserved on C8 columns for possible future analysis. The whole water chemical analyses conducted on these samples by DFG revealed that four of the samples contained certain metals, but at levels well below the LC_{50} level. No contaminants were detected in the sample collected at SR-3 on 30 March 2004 (Appendix G, Table 1).

3.2.2.1 Pathogen Interference

Intermittent lethality to larval fathead minnows has been observed in tests using standard US EPA toxicity test methods. This phenomenon, termed Pathogen Related Toxicity (PRT), has been reported around the United States in fathead minnow tests with ambient waters (Miller *et al.,* 2003). This toxicity was often associated with unusual characteristics including high among-replicate variability (CV >40%) in fathead survival (US EPA, 2002). Studies showed that incidences of the toxicity could be reduced or even prevented by sterilization procedures or intensive cleaning of test containers. These characteristics suggest that the observed anomalous fathead minnow mortality was caused by pathogen(s) rather than chemical toxicity.

In July 2003, the Contractor Manager requested that the remaining larval fathead minnow tests be conducted in Teflon® beakers. The samples tested 2 July 2003 continued to exhibit high variability among replicates, so super clean techniques were employed beginning in late July along with the Teflon® beakers to minimize possible pathogen effects. The use of Teflon® beakers and super clean techniques decreased variability among replicates with the exception of the sample collected at site SR-1 on 26 May 2004 that had a CV that exceeded 40%.

Due to technician error, the test initiated 27 August 2003 was conducted in glass beakers rather than the requested Teflon beakers. High variability among replicates was noted in the sample collected at SR-1, although the CV did not exceed 40%. An additional sampling event occurred in August 2004 to replace this test, and toxicity test results for samples from sites SR-1 and SR-2 indicated reduced larval growth.

3.2.3 Lemna minor (Duckweed)

Lemna minor summary data sheets are included in Appendix C. During the twelve months of testing, six samples were toxic to duckweed. Duckweed exhibited decreased frond number in samples collected 26 August and 29 October 2003 at site SR-4. Decreased frond number and

weight were noted in samples collected 2 July and 30 September 2003 at SR-3 and SR-4. There are currently no accepted TIE procedures developed for duckweed. C8 eluate from the toxic samples collected on 2 July and 30 September 2003 was submitted to DFG for organic chemical analysis. The analysis revealed that esfenvalerate, nonylphenol, nonylphenol ethoxylate and clopyralid (very low concentrations) were present at both sites in July and September 2003. The relationship between duckweed frond count, as well as weight, in Susan River samples from sites SR-3 and SR-4 is depicted in Figures 3 and 4. In samples collected on both July 2, 2003 and August 2003, the highest concentrations were nonylphenol at SR-4. During 2004 there were no Susan River samples toxic to duckweed. There were scans for the chemicals in the Transline formulation in the 2004 samples, but none were detected. These finding support our hypothesis that chemicals in the Transline formulation were the cause or contributed to the observed duckweed toxicity in samples from sites SR-3 and SR-4. Further research into these chemicals as potential causes of the toxicity is discussed in section 3.6. Results are summarized in Appendix G, Table 1.

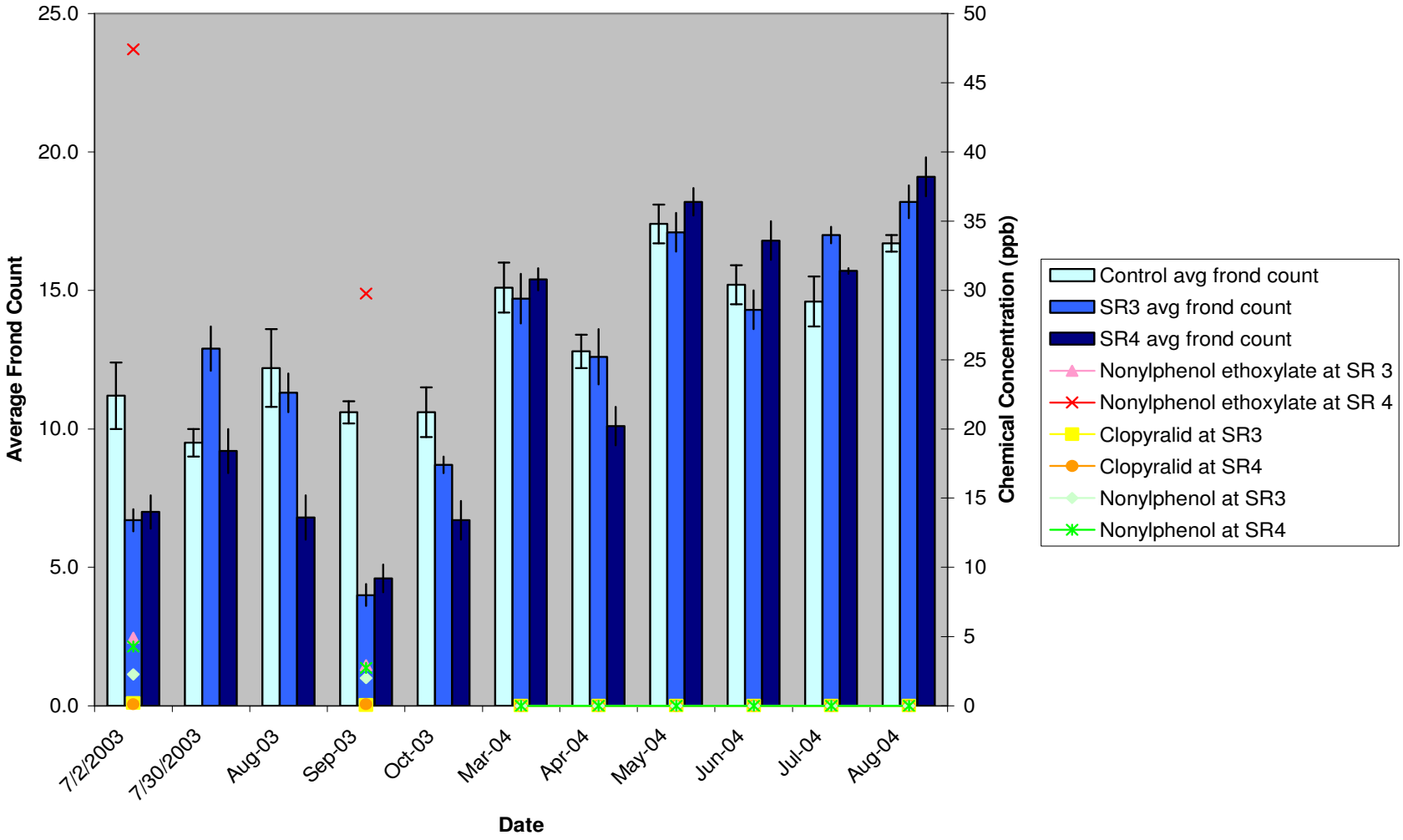

Figure 3. Duckweed frond count after exposure to Susan River water samples collected at sites SR-3 and SR-4 during the course of this project. Also illustrated are concentrations of nonylphenol, nonylphenol ethoxylate, and clopyralid in samples toxic to duckweed. Error bars represent one standard deviation from the mean.

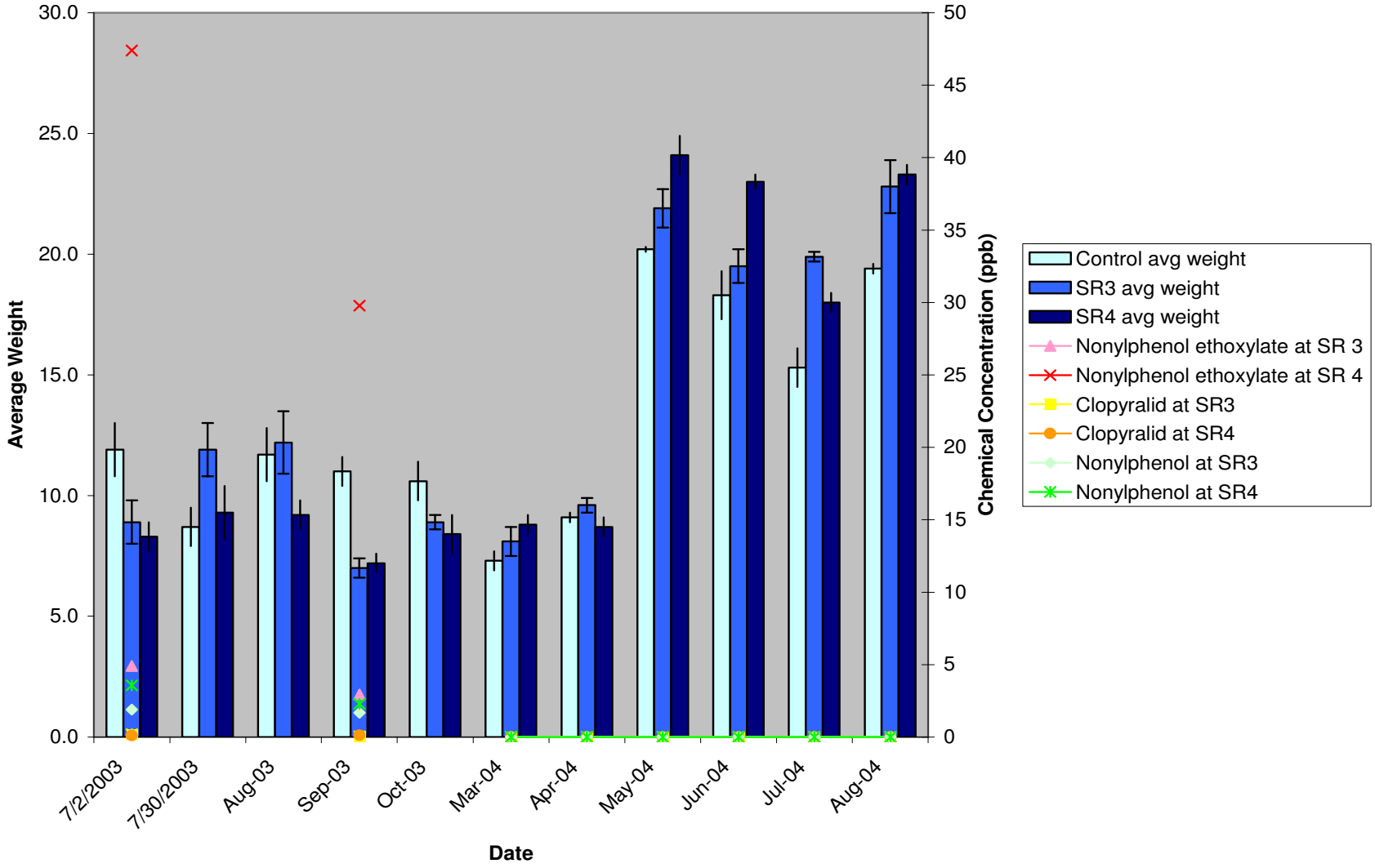

Figure 4. Duckweed weight after exposure to Susan River water samples collected at sites SR-3 and SR-4 during the course of this project. Also illustrated are concentrations of nonylphenol, nonylphenol ethoxylate, and clopyralid in samples toxic to duckweed. Error bars represent one standard deviation from the mean.

3.2.3.1 Duckweed Protocol Deviations

Due to technician error, duckweed tests initiated in March 2004 employed two three-frond colonies and tests initiated in April 2004 employed two six-frond colonies rather than four three-frond colonies as ASTM specifies. None of the samples tested in these two months exhibited toxicity compared to the control. Because of these errors, additional sampling events occurred in July and August 2004. While experiments should be conducted to confirm potential sensitivity differences, we believe that it is very unlikely that the six-frond duckweed colonies are *less sensitive* to contaminants than three frond colonies. Moreover, our perspective is that use of only two three-frond colonies and the use of six frond colonies did not result in an inability to detect significant toxicity in Susan River samples. Further, chemical analysis of these samples did not reveal contaminant concentrations of concern.

Table 2. Summary of toxicity testing results.

Sample date	SR-1	SR-2	SR-3	SR-4
5/29/03[1]	-	-	-	-
7/2/03	-	-	DW, DF	DW, DF
7/30/03	CR	CR	-	-
8/26/03	FM	-	-	DF
9/30/03	-	-	DW, DF	DW, DF
10/29/03	-	-	-	DF
3/30/04	-	-	FB	-
4/28/04	-	-	-	-
5/26/04	-	-	-	-
6/30/04	FB	-	-	-
7/28/04	-	FB	-	-
8/25/04	CR, FB	FB	-	CR

Acronyms and Symbols:
"-" indicates no toxicity detected.
DW = Statistically significant decrease in duckweed weight.
DF = Statistically significant decrease in duckweed frond count.
CR = Statistically significant decrease in *Ceriodaphnia* reproduction.
FM = Statistically significant increase in *Pimephales* mortality.
FB = Statistically significant decrease in *Pimephales* biomass.
Date-Specific Footnotes:
1. No toxicity testing data available for duckweed for this date.

3.3 Quality Assurance

3.3.1 Quality Assurance/Quality Control Samples

Four field duplicates, a trip blank and a laboratory blank were assessed during this project. In the duckweed test initiated 27 May 2004 a statistically significant difference in the mean frond count was noted between the control and the laboratory blank (17.4 and 15.0 fronds, respectively). The toxicity in the laboratory blank was almost certainly a false positive (Type I error in statistical testing—indication of toxicity when none actually exists). With the statistical alpha value (probability of a false positive) set at 0.05 (predetermined and the norm for most biological statistical testing), as it was in our statistical tests, a false positive is expected by chance alone in one out of every 20 samples tested (Sellers *et al.,* 1992). The very low magnitude of difference in mean frond count between control and laboratory blank (17.4 versus 15.0), as well as a lack of statistical difference in duckweed weight between the two groups, supports the concept of the laboratory blank being a false positive. False positives happen by random chance, so there is very little than any laboratory can do to prevent such events other than lower the alpha value in statistical analyses. However, lowering the alpha value in a statistical test results in an increased chance of false negatives (Type II statistical error—samples that are actually toxic being designated as nontoxic).

3.3.2 Reference Toxicant Testing

Reference toxicant testing was conducted with NaCl on *Ceriodaphnia* and larval fathead minnows. The herbicide, Atrazine, was used for reference toxicant tests on duckweed. US EPA (2002) recommends reference toxicant testing to ascertain changes in animal sensitivity. *Ceriodaphnia* sensitivity was assessed with 7-day LC_{50} mortality tests and 7-day EC_{25} reproduction tests. *Pimephales* sensitivity was assessed with 7-day LC_{50} mortality tests and 7-day EC_{25} biomass tests. *Lemna minor* responses were assessed with 7-day EC_{50} and IC_{25} tests for growth and frond increase. *Ceriodaphnia* NaCl LC_{50} values ranged from 2053 to 5363 µmhos/cm and NaCl EC_{25} values ranged from 352 to 2670 µmhos/cm. *Pimephales* NaCl LC_{50} values ranged from 2.4 to 6.6 g/L and NaCl EC_{25} values ranged from 1.8 to 5.6 g/L. *Lemna minor* Atrazine EC_{50} values ranged from 92.1 to 263.1 g/L and Atrazine IC_{25} 39.5 to 112.9 g/L.

Of particular interest is the detection of outlying values exceeding the upper or lower 95 percent control limits of the long-term mean. General trends indicating changes to species sensitivity are also assessed. US EPA (2002) states that "at the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone", therefore, those control charts with only a single outlying value will not be discussed. During this project no more than a single outlying value occurred in any of the reference toxicant control charts. Reference toxicant test control charts are located in Appendix E.

3.4 Sample Water Quality Measurements

Water quality measurements are summarized in Appendix F. All water quality parameters were within acceptable physiological limits for the test organisms. Ammonia values ranged from no detect to 0.4 mg/L. Turbidity ranged from 0.7-37.0 ntu. Turbidity and EC increased downstream throughout the project with one exception. It is unlikely that these water quality parameters affected toxicity results in any samples.

3.5 Chemical Analyses

DFG analytical chemistry data and a summary of chemical concentrations can be found in Appendix G. Based on LC_{50} values in the literature, concentrations of individual chemicals included in these analyses could not account for toxicity to test species. *Ceriodaphnia*, larval fathead, and duckweed LC_{50}s for esfenvalerate, nonylphenol, nonylphenol ethoxylate, copper and nickel are higher than observed in any sample analyzed.

3.6 Follow-up Research on Duckweed Toxicity

Because cause(s) of toxicity to duckweed was of particular concern, UCD ATL performed additional experiments to determine the LC_{50}s of organic chemicals detected by DFG. The nonylphenol (NP) LC_{50} for duckweed was 1,380 µg/L and for R-11 (commercial equivalent of the surfactant nonylphenol ethoxylate) was 7,500 µg/L (Appendix C, Tables 12 and 13). Multiple attempts were made to determine the clopyralid (commercial equivalent to the herbicide Transline) LC_{50} for duckweed, but UCD ATL was unable to observe a dose-response effect. Concentrations up to 6,250µg/L clopyralid had no effect on duckweed growth or frond count.

LC_{50}s of these potential toxicants were much higher than those detected by DFG. Clopyralid is typically applied with a surfactant (IPM UCD Online 2004), so the combinations of NP, nonylphenol ethoxylate (NPE) and Transline detected by DFG would be expected. UCD ATL determined the ratios in which NP, NPE and Transline were detected by DFG on four occasions. The average of these ratios was used to calculate concentrations of each chemical to be used in a "cocktail" that represented the average dosages in Susan River samples. The cocktail (0.11 µg/L clopyralid, 1.410 µg/L NP and 21.25 µg/L R-11) was added to glass distilled water and employed in a duckweed assay. Although these concentrations were much lower than those used in individual LC_{50} tests, duckweed tested in the cocktail had statistically decreased weight and frond number than those tested in the glass distilled control and methanol control (Appendix C, Table 14). These results suggest additivity and/or synergism of these chemicals. It is also possible that samples toxic to test species contained contaminants that were not included in chemical analyses.

4. DISCUSSION

A primary objective of this study was to determine whether the pattern of toxicity (observed by US EPA) in the Susan River during 1990 continued to occur in 2003/04. Toxicity testing data collected by US EPA in 1990 on duckweed correspond to UCD ATL findings, but test results with larval fathead minnows do not match in the two studies. US EPA did not observe any toxicity to *Ceriodaphnia.* During the current study, potential (see discussion below) *Ceriodaphnia* reproductive inhibition was noted in four Susan River samples. In the following subsections we discuss and summarize (1) *Ceriodaphnia* testing results, (2) larval fathead minnow testing results, (3) duckweed testing results, (4) probable cause(s), and (5) our best professional judgment on the reliability of the data gathered in this project.

4.1 *Ceriodaphnia* Results

None of the Susan River samples collected in this project caused statistically significant *Ceriodaphnia* mortality. Data collected in 1990 by US EPA indicated no acute or reproductive toxicity to this species. In the current study, *Ceriodaphnia* reproductive inhibition was only noted in four river samples. Samples collected during 30 July 2003 at sites SR-1 and SR-2 resulted in a 15 and 20% reduction in neonate production, respectively. These effects were statistically significant because of low variability within treatment replicates in the tests,

especially in controls. There is a possibility that these low-level apparent effects could have been due to a contaminant(s), but this is unknown because the samples were not analyzed for contaminants. In our judgment, however, these two statistically significant responses were false positives related to the low variability among replicates. Furthermore, the biological relevance of these results is questionable because of the low magnitude toxicity and uncertainty regarding duration and frequency of toxicity. In a review of the literature, Suter *et al*. (2000) suggest that a 20% or less reduction in biological or toxicological parameters is acceptable (i.e., unlikely to negatively impact biological communities). This threshold could be higher when endpoints are sublethal parameters. With low-level responses statistical false positives are also more likely (see section 4.2). Greater reproductive inhibition was observed in samples collected in August 2004 at sites SR-1 and SR-4. Neonate production was reduced 34 and 48%, respectively, in samples from the two sites. If these reproductive responses were indeed related to water quality, (contaminants in those two samples were not detected or were below the effect level) the duration, frequency, and geographic extent of the problem are unknown; thus, the actual instream biological impacts of these results are difficult to predict. Furthermore, the lack of EC50 data for the metals found in the Susan River samples makes it harder to predict their sublethal effects. Whether the reproductive inhibition observed in this study compared to the 1990 US EPA work is related to greater precision in test species response in the UCD ATL investigation or to contaminant problems that have developed since 1990 is unknown.

4.2 Larval Fathead Minnow Results

In the 1990 US EPA study larval fathead minnow mortality was 97 and 53% in samples collected during July at sites 1 and 2 (same as sites SR-1 and SR-2 in the current investigation), respectively. There was no discussion in the US EPA data sheets as to whether the mortality was related to pathogen interference. Statistically significant larval fathead mortality was not seen at any site during July 2003 or 2004 in the current study. Growth inhibition was noted in the site SR-2 July 2004 sample; this lower biomass was not pathogen related. The sample collected at site SR-1 in August of 1990 resulted in 80% larval minnow mortality. Again, it is unknown if the fathead mortality in the 1990 US EPA study was PRT related. In this study larval minnow mortality was 30% in a sample collected at site SR-1 during August 2003. This mortality was almost certainly related to PRT.

In the current study, a total of five Susan River samples resulted in larval fathead growth inhibition. That this growth inhibition was consequent to PRT is very unlikely, because there was low variability among replicates and technician observations did not discover indication of PRT. Furthermore, PRT has traditionally been associated with mortality, not with growth inhibition. PRT tends to be more common in samples with low ECs. EC tended to be lower at sites SR-1 and SR-2 than at the two lower river sites. Four of the five samples resulting in reduced biomass were collected at sites SR-1 or SR-2. However, there were a large number of samples collected at sites SR-1 and SR-2 with low ECs that were not toxic to larval fish. Because of the low EC at the upper river sites any future studies on the Susan River that include larval fathead testing should include all precautions to avoid PRT. While toxicity in river samples is a violation of the Regional Board Basin Plan narrative water quality objective for toxicity ("All waters shall be maintained free of toxic substances in concentrations that are toxic to, or that produce detrimental physiological responses in human, plant, animal, or aquatic life."), the instream biological impacts of these findings are unknown. Biomass reduction in the five toxic samples ranged from 12 to 19%; therefore, the magnitude of toxicity was not particularly striking in these samples. Duration, frequency, and geographic extent are incompletely known because of the infrequency of sampling and low number of sites. However, data presented herein indicate the geographic extent of toxicity was restricted (because toxicity was not consistently present at all sites) and that duration of toxicity was very low (not consistently present at sites through time).

Similarities do exist in larval fathead minnow data between the current investigation and the 1990 US EPA study. In both the US EPA 1990 study and the current study Susan River samples collected at the upper river sites during the June-August period were more likely to result in toxicity to larval fathead minnows. If there are to be future investigations aimed at identification of the cause(s) of larval fathead growth, sampling should focus on this June-August period. Cause(s) of small decreases in biomass compared to controls has proven difficult to determine with TIE procedures. To enhance the potential of cause(s) determination we would recommend a large number of replicates with each treatment.

4.3 Duckweed Results

Toxicity to duckweed was recorded in all Susan River samples collected during July and August of 1990. In the current study, all six samples toxic to duckweed were collected at sites SR-3 and SR-4 during the July to September 2003 period. Thus, there is correspondence with regards to the temporal pattern of toxicity to duckweed between the 1990 and current studies. However, in this study Susan River samples toxic to duckweed were from the lower river (sites SR-3 and SR-4) in 2003 only.

4.4 Cause(s) of Toxicity

An additional objective of this study was to identify the cause(s) of any observed toxicity to the test species. The toxicity detected in the samples tested with *Ceriodaphnia* or larval fathead minnows was not of sufficient magnitude to trigger (as defined in the project QAPP) a TIE. As indicated above, the instream biological significance of this study's results with daphnid and larval fish screening is unknown. On the other hand, the toxicity noted in Susan River samples is inconsistent with the narrative water quality objective in the Regional Board Basin Plan.

While toxicity to duckweed was documented in six Susan River samples during this study, TIE procedures had not been developed for this species to definitively identify cause(s) of toxicity. However, DFG chemical analyses and experiments at UCD ATL suggested that toxicity of four Susan River samples to duckweed might be due to additive/synergistic effects of Transline, nonylphenol, and nonylphenol ethoxylate found in four out of six of these samples. Other contaminants that were not included in chemical analyses may have contributed to the observed duckweed toxicity. One would not expect that these three chemicals, used for vegetation control, would be toxic to the other two test species based on the chemical analysis results.

Six Susan River samples collected at sites SR-3 and SR-4 inhibited duckweed growth. Toxicity detected in samples collected on 2 July 2003 and 30 September 2003 could be related to additive/synergistic effects of Transline, nonylphenol, and nonylphenol ethoxylate identified in these samples. Chemical analyses were not performed on the samples collected from sites SR-1 or SR-2 for these dates (which did not exhibit toxicity). Unfortunately, this data gap confounds efforts to correlate the lack of toxicity to the water quality (absence of herbicide formulation) of

these samples. Similarly, the cause(s) of duckweed growth inhibition in samples collected at site SR-4 during August and October 2003 is unclear, as these samples were not submitted for chemical analysis.

Transline is applied to control vascular plant growth, so it is not surprising to observe the duckweed response. The major use of Transline in Lassen County is treatment of rights-of-way (IPM UCD Online 2004). Both sites SR-3 and SR-4 are adjacent to roadways. Applications to rights-of-way are mostly restricted to the June through September period (IPM UCD Online 2004). This corresponds to the period when river samples were toxic to duckweed.

4.5 Reliability of Results

The number of technician errors in this project is inconsistent with concurrent projects and the history of successful projects at UCD ATL. Duckweed testing had not been performed at UCD ATL prior to this project. Communication with other laboratories indicated that achieving proficiency in this test can be elusive. While there were deviations from procedure specifications in two duckweed tests and a larval fathead minnow test, in our professional judgment, no river sample significant toxicity escaped notice.

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