UC Davis Aquatic Health Program Laboratory

Susan River Toxicity Project 2016

Final Report - Final

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Glossary of Terms and Acronyms

637SUS001	Susan River near Litchfield
637SUS003	Susan River above Confluence with Willard Creek
637SUS004	Susan River at Commercial Road
µg/L	Micrograms per liter
µm	Micrometer
ASTM	American Society for Testing and Materials
$CaCO_3$	Calcium carbonate
CV	Coefficient of variation
DO	Dissolved oxygen
EC	Electrical conductivity
EC_{25}	Effect concentration at which a toxicant causes an adverse effect on a quantal (all or nothing) response in 25% of the organisms (US EPA 2002)
EC_{50}	Effect concentration at which a toxicant causes an adverse effect on a quantal (all or nothing) response in 50% of the organisms (US EPA 2002)
g/L	Grams per liter
IC_{25}	Inhibition concentration at which a toxicant causes an adverse effect on a non-quantal response in 25% of the organisms (US EPA 2002)
IC_{50}	Inhibition concentration at which a toxicant causes an adverse effect on a non-quantal response in 50% of the organisms (US EPA 2002)
LC_{50}	Lethal concentration at which a toxicant causes death in 50% of the organisms (US EPA 2002)

L1650%	50% L16 media and water amended to a hardness of 80-100 mg/L as $CaCO_3$ used with *Ceriodaphnia dubia*
LRWQCB	Lahontan Regional Water Quality Control Board
mg	Milligrams
mg/L	Milligrams per liter
mg/surviving indiv.	The weight in milligrams per surviving individual (fathead minnow)
mL	Milliliter
MS-222	Tricaine methanesulfonate, fish anesthetic
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)
ROEPAMH	Reverse-Osmosis water amended to a hardness of 80-100 mg/L as $CaCO_3$ used with fathead minnow
ROEPAMHR	Reverse-Osmosis reconstituted water amended to a hardness of 80-100 as $CaCO_3$ used with *Hyalella azteca*
SE	Standard error
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*, *Pimephales promelas*, and *Hyalella azteca*, and a 50% or great reduction in cell growth for *Selenastrum capricornutum*
TMDL	Total maximum daily load
UCD AHPL	University of California Davis, Aquatic Health Program Laboratory
US EPA	United States Environmental Protection Agency
X	Mean
YCT	*Ceriodaphnia dubia* food consisting of yeast, organic alfalfa, and trout chow

Executive Summary

The Susan River is designated for both the Warm Freshwater Habitat and Cold Freshwater Habitat beneficial uses, and for the Spawning, Reproduction, Development, and Migration of Aquatic Organism uses. For the purposes of this report, three segments of the Susan River, Headwaters to Susanville, Susanville to Litchfield, and Litchfield to Honey Lake, are listed on the USEPA 303(d) list of impaired waters, as impaired due to "unknown toxicity". The Susan River was first listed by USEPA in 1990, and investigations conducted in 2003/2004 demonstrated that toxicity was still observed in this water body.

The primary objectives of this study were to determine whether the Susan River is exhibiting toxicity as has been historically observed in prior studies and determining the source(s). Monthly ambient samples were collected from the Susan River in April, May, and June, 2016. Samples were applied in toxicity tests with the water flea *Ceriodaphnia dubia*, fathead minnow *Pimephales promelas*, the green freshwater alga *Selenastrum capricornutum*, and the epibenthic amphipod *Hyalella azteca.* In addition, Continuous Low-Level Aquatic Monitoring (CLAM) apparatus were deployed at each site.

Three sites on the Susan River were sampled three times over the 2016 study period, for a total of nine samples. Two out of the nine samples (22.2%) were toxic to at least one of the test species.

In 2016, a statistically significant reduction in *H. azteca* survival was observed in the Susan River above the Confluence of Willard Creek site (637SUS003) collected April 6, 2016, and statistically significant reductions in *C. dubia* reproduction and *S. capricornutum* cell density were observed in the Susan River at Litchfield site (637SUS001) collected May 10, 2016. The toxicity observed during the current study was of low enough magnitude that TIE triggers ($\geq$50% reduction in an endpoint within 96 hours) were not met, and thus no TIEs were conducted to determine the cause of toxicity. Analytical chemistry on the CLAM passive samplers demonstrated the presence of the herbicide Hexazinone in every sample collected during the current study, although not all concentrations were able to be quantified as they were in between the Method Detection Limit and the Reporting Limit. Measured concentrations of Hexazinone fell well below those documented to cause acute toxicity and did not exceed the Office of Pesticide Programs Aquatic Life Benchmarks for freshwater organisms.

Across the three investigations into this water body, 63 samples were collected since 1990. Six samples were collected in 1990, 48 samples were collected in 2003/2004, and nine samples were collected in 2016. Of those 63 samples, 27 were toxic to at least one test species, leading to a toxicity frequency of 43%. Of these 27 toxic samples, three were determined to be false positives. With this in mind, frequency of observed toxicity is reduced to 38%.

Investigations in 1990 demonstrated nine instances of toxicity, observed in all three reaches of the Susan River. In 2003/2004, this increased to 12 instances of observed toxicity, again observed in all three reaches. During the current investigation, only two instances of toxicity were observed; once in the Headwaters to Susanville reach and once in the Litchfield to Honey Lake reach of the Susan River.

This frequency of toxicity exceeds the narrative water quality objective in the Basin Plan, *All waters shall be maintained free of toxic substances in concentrations that are lethal to or that produce other detrimental responses in aquatic organisms.* However, it would appear that the Susan River is on the mend.

1. Introduction

The State Water Board throughout the state of California has waters designated to protect beneficial uses, such as aquatic life, drinking water, and water quality standards. These beneficial uses serve as a basis for establishing water quality objectives. The State and Regional Water Quality Control Boards are charged with identifying and addressing these problems and maintaining water quality standards, which are accomplished through permitting and monitoring programs, TMDL implementation, and special studies.

Toxicity testing is a critical component of many monitoring programs and provides evidence of direct adverse effects of chemicals to species of interest or concern. Coupled with analytical chemistry, these analyses provide important information on the presence and effects of toxic contaminants in aquatic environments.

1.1 Characteristics of the Study Area

The Susan River is an internally drained river in eastern Lassen County with its headwaters near Lassen Volcanic National Park and its terminus in Honey Lake in the Great Basin. The Susan River is designated for both the Warm Freshwater Habitat and Cold Freshwater Habitat beneficial uses, and for the Spawning, Reproduction, Development, and Migration of Aquatic Organism uses (2010 State IR Report # 27172). For the purposes of this report, three segments of the Susan River, 1) Headwaters to Susanville, 2) Susanville to Litchfield, and 3) Litchfield to Honey Lake, are listed on the USEPA 303(d) list of impaired waters, as impaired due to “unknown toxicity”.

1.2 Study Objectives

The Lahontan Regional Water Quality Control Board (LRWQCB) has requested evaluation of the Susan River in order to determine whether or not these Susan River segments can be de-listed from the 303(d) list of impaired waters. Therefore, our study objects were three-fold: 1) Determine the toxicity of the Susan River with the application of USEPA toxicity tests, 2) Identify compound(s) causing toxicity when observed with Toxicity Identification Evaluations, and 3) Use the results of this study to determine whether the Susan River can be removed from the 303(d) list.

2. Materials and Methods

2.1 Sampling Sites

Monthly ambient samples were collected from the Susan River in April, May, and June, 2016. One sample from each reach of the Susan River was collected for toxicity testing, as outlined in Table 1. Samples were applied in toxicity tests with the water flea *Ceriodaphnia dubia*, fathead minnow *Pimephales promelas*, the green freshwater alga *Selenastrum capricornutum*, and the epibenthic amphipod *Hyalella azteca*. Site locations are outlined in Figure 1.

Table 1. Sample sites, names, and location

Sample ID	Site Name	Reach of Susan River	Location	
			Latitude	Longitude
637SUS001	Susan River near Litchfield	Litchfield to Honey Lake	40.37771	-120.39514
637SUS003	Susan River above Confluence with Willard Creek	Headwaters to Susanville	40.39603	-120.78140
637SUS004	Susan River at Commercial Road	Susanville to Litchfield	40.39705	-120.62122

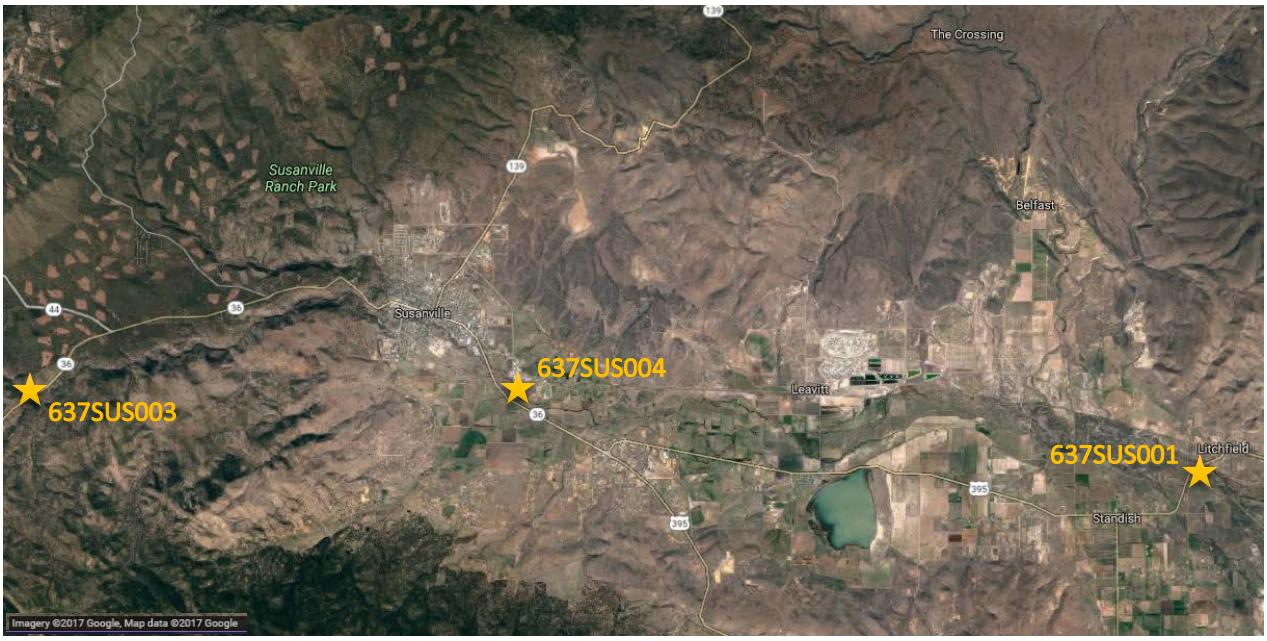

Figure 1. Satellite map of 2016 study site locations

2.2 Sample Collection and Storage

2.2.1 Toxicity testing

Staff from the LRWQCB collected mid-channel one-time grab samples from the Susan River. All samples for toxicity testing were collected in clean 4-L amber glass bottles. Water samples were transported, stored, and preserved following protocols outlined in the UCD AHPL Standard Operating Procedures (UCD AHPL, 2016). All containers used for water collections were labeled with the site ID, collection date and time, initials of the sampler, and then rinsed three times with ambient water prior to filling. Up to 40 L were collected from each site location on the Susan River for laboratory toxicity testing. All samples were placed on wet ice for transport to the UCD AHPL and kept between 0-6°C (USEPA 2002). Upon receipt, samples were stored in the dark in an environmental chamber maintained between 0-6°C. Laboratory toxicity test samples were used the day after collection. Copies of Chain of Custody forms are in Appendix A.

2.2.2 Analytical chemistry

Continuous Low-Level Aquatic Monitoring (CLAM) apparatus were deployed at each site for every sampling event. CLAM filters were deployed in duplicate at each site for approximately 12 hours. At the end of each 12 hour event, CLAM filters were collected by LRWQCB staff and shipped overnight to Department of Fish and Wildlife Water Pollution Control Laboratory (DFW WPCL) for sample extraction and analysis. CLAM filters were analyzed for the following compounds: prometon, simazine, imidacloprid, diazinon, chlorpyrifos, diuron, carbaryl, fipronil + fipronil degradates, oryzalin, oxyfluorfen, pendimethalin and prodiamine. Analytes and detection limits are outlined below in Table 2. Raw data of analytical chemistry is in Appendix C.

Table 2. DFW WPCL analytical chemistry control limits (µg/L)

Analyte Name	Analyte class	Method Detection Limit	Reporting Limit
Diuron	Carbamate	0.155	0.620
Carbaryl	Carbamate	0.014	0.056
Pendimethalin	Herbicide	0.065	0.256
Prodiamine	Herbicide	1.09	4.37
Oryzalin	Herbicide	52.2	250
Oxyfluorfen	Herbicide	0.500	1.00
Imidacloprid	Neonicotinoid	0.062	0.250
Chlorpyrifos	Organophosphate	0.019	0.074
Diazinon	Organophosphate	0.003	0.011
Simazine	Triazine	0.059	0.235
Prometon	Triazine	1.09	4.37
Hexazinone	Triazine	0.014	0.055
Fipronil	Fipronil	0.008	0.033
Fipronil sulfide	Fipronil degradate	0.010	0.040
Fipronil amide	Fipronil degradate	0.064	0.257
Fipronil desulfinyl	Fipronil degradate	0.012	0.047
Fipronil desulfinyl amide	Fipronil degradate	0.059	0.237
Firpnoil sulfone	Fipronil degradate	0.012	0.049

2.3 Toxicity Testing

UCD AHPL toxicity testing methods were based on protocols developed by USEPA (2000, 2002), SWAMP (SWAMP 2008), and UCD AHPL SOPs (Stillway 2016). Chronic toxicity testing for *Ceriodaphnia dubia*, *Pimephales promelas*, and *Selenastrum capricornutum*, followed protocols outlined in *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (USEPA 2002). Acute, 96-hour water column testing for *Hyalella azteca* were based on protocols outlined in *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates* (USEPA 2000), and protocols described in the Quality Assurance Management Plan for the State of California's Surface Water Ambient Monitoring Program (SWAMP), and UCD AHPL SOPs (2016). Summaries of toxicity tests and water quality are in Appendix B. Copies of raw bench sheets are in Appendix D.

2.3.1 Sample preparation

Before test initiation and water renewals, water samples were mixed thoroughly in their original sample containers and sub-samples were filtered through a 60-µm screen to remove debris and other organisms. Water quality measurements including EC, DO, temperature, and pH were recorded for all treatments at test initiation and termination. DO and pH were measured on fresh sample water prior to renewals; DO, pH and temperature were measured on 24-hr (48-hr for *H. azteca*) waste-water.

DO was measured using a YSI 20 meter, pH was measured using a Beckman 480 pH meter, and EC was measured using a YSI 30 meter. Meters were calibrated daily according to the manufacturer's specifications. Ammonia-nitrogen was measured within 24 hours of sample receipt using a HACH DR-890 portable colorimeter and a HACH Am-Ver Low-Range Ammonia Test n'Tube Reagent Set. Hardness and alkalinity were measured within 72 hours of sample receipt using titrimetric methods.

2.3.2 Testing organisms

2.3.2.1 *Ceriodaphnia dubia*

C. dubia were cultured in-house, following methods outlined in USEPA and in UCD AHPL SOPs. Cultures originally obtained from Aquatic Research Organisms (Hampton, NH) and AQUA-Science (Davis, CA), were kept in a temperature-controlled room maintained at 25 ± 2°C. Test organisms employed in toxicity testing were derived asexually. Prior to test initiation and renewals, waters were warmed to test temperature (25 ± 1°C) in 400 mL glass Mason jars using a water bath maintained at 25 ± 2°C, and aerated at a rate of 100 bubbles per minute until DO concentrations were 4.0-8.6 mg/L. Nutrient-rich Sierra Springs™ water amended with inorganic salts to USEPA moderately hard specifications (hardness: 80-100 mg/L $CaCO_3$, alkalinity: 57-64 mg/L $CaCO_3$, EC 250-300 µS/cm, pH 7.8-8.2; USEPA, 2002) was used as the control.

Toxicity tests were initiated using blocking by known parentage with less than 24-hr old *C. dubia*, born within an 8-hr period. Each of 10 replicate 20 mL glass vials contained 15 mL of sample water and one organism. *C. dubia* were transferred into a fresh vial of solution and fed YCT (mixture of yeast, organic alfalfa and trout chow) and *S. capricornutum* daily. Tests were conducted at 25 ± 1°C with a 16-hr light: 8-hr dark photoperiod under fluorescent light. Mortality and reproduction were assessed daily and at test termination.

2.3.2.2 *Pimephales promelas*

Fathead minnows were purchased from AquaTox, Inc. (Hot Springs, AR). Upon receipt, fish were fed and acclimated to laboratory conductions until their use in a test. Prior to test initiation and renewals, waters were warmed to test temperature (25 ± 1°C) in 1L glass beakers using a water bath maintained at 25 ± 2°C, and aerated at a rate of 100 bubbles per minute until DO concentrations were 4.0-8.6 mg/L. Reverse-osmosis water amended with inorganic salts to USEPA moderately hard specifications (hardness: 80-100 mg/L $CaCO_3$, alkalinity: 57-64 mg/L $CaCO_3$, EC 250-300 µS/cm, pH 7.8-8.2; USEPA, 2002) was used as the control.

Toxicity tests were initiated using fish less than 48-hours old. Each of the four 600-mL beakers contained 250 mL of sample water and 10 minnows. Eighty percent of the test solution was renewed daily, at which time debris and dead fish were removed from the test chambers. Fish were fed *Artemia* nauplii three times daily. Tests were conducted at 25 ± 1°C with a 16-hr light: 8-hr dark photoperiod under fluorescent and ambient light. Mortality was assessed daily. At test termination, surviving fish were euthanized and dried to a constant weight at 103-105°C, and weighed using a Mettler AE163 balance to determine dry biomass.

2.3.2.3 *Selenastrum capricornutum*

S. capricornutum were cultured and maintained in-house at UCD AHPL from cultures originally obtained from the Culture Collection of Algae, University of Texas (Austin, TX). Axenic algal cells were placed in media for 4-7 days prior to test initiation to ensure cells were in exponential growth.

S. capricornutum 96-hr chronic tests consisted of four replicate 250 mL glass flasks with 100 mL of sample and 1 mL of 1.0×10^6 cells/mL of *S. capricornutum*. A fifth replicate flask was inoculated and used for daily chemistry measurements. Tests were conducted with the addition of EDTA. Test chambers were incubated in a temperature-controlled environmental chamber maintained at 25 ± 2°C under constant cool white fluorescent light. Flasks were kept in random placement in a mechanical shaker in constant orbital motion at 100 cycles per minute and were randomized twice daily. Cell growth was measured at test termination with a Coulter Counter Z1 particle counter (Beckman Coulter, Pasadena CA).

2.3.2.4 *Hyalella azteca*

H. azteca were purchased from Aquatic Research Organisms (Hampton, NH). Upon receipt, organisms were moved to a 10-L aquarium, fed and acclimated to laboratory conditions for 48-hrs. Prior to test initiation and

renewals, waters were warmed to test temperature (23 ± 1°C) in 600 mL glass beakers using a water bath maintained at 23 ± 1°C, and aerated at a rate of 100 bubbles per minute until DO concentrations were 2.5-8.9 mg/L. Reverse-Osmosis water amended to USEPA moderately hard reconstituted water specifications (hardness: 90-100 mg/L as $CaCO_3$, alkalinity: 50-70 mg/L $CaCO_3$, EC: 330-360 µS/cm, pH: 7.8-8.2; USEPA 2000) was used as the control.

Tests were initiated with 9-14 day old *H. azteca*. Each of five replicate 250 mL glass beakers contained 100 mL of sample water, a small piece of Nitex screen (approx. 6 cm^2) for use as artificial substrate, and 10 organisms. Animals were fed YCT at test initiation and on Day 2 after the water renewal. Tests were conducted at 23 ± 1°C with a 16-hr light: 8-hr dark photoperiod under fluorescent and ambient light. Mortality was assessed daily and at termination.

2.4 Statistics

Each sample was characterized by descriptive statistics, including the mean response and variation among replicates. Toxicity is defined as a statistically significant reduction in test organism performance in an ambient sample compared to a laboratory control.

This project was designed to create data comparable with data contained in the SWAMP and CEDEN databases. To this end, organism performance (control v. ambient sample) was evaluated using SWAMP standard statistical protocols. The SWAMP protocol involves the examination of significant differences in test organism performance by a one-tailed heteroscedastic t-test ($\alpha = 0.05$) and a categorization of the performance of organisms exposed to the ambient sample as either greater to or less than 80% of the control performance (SWAMP, 2008). For the purposes of this report, samples were considered toxic only when both a significant t-test result and performance below the 80% threshold of the control was observed. All analyses were performed using custom Excel spreadsheets created by the SWAMP Data Management Team at Moss Landing Marine Laboratories (Office Excel 2007 (v.12), Microsoft Inc., USA) and UCD AHPL Data Management staff.

In *H. azteca* tests, survival comparisons were calculated as [# surviving / (# surviving + # dead bodies found)]. Animals missing from the test vessels may have died because of exposure to test waters, and then disappeared due to rapid decomposition, but it is also possible that animals have died due to desiccation when individuals resting on the water surface leave the water or are washed out of the water and adhere to the side of the test vessel. Thus, only animals whose remains are found submerged in the test vessels were included in the counts of animals that died in test replicates.

Toxicity tests may include conductivity controls when one or more ambient samples have a lower or higher specific conductance than the SWAMP's species specific thresholds. A low conductivity control is included in test batches when a sample's conductivity is below 100 µS/cm. This low conductivity control is first statistically compared to the standard test acceptability control to determine whether low conductivity has a negative impact on the test organism. In instances where the low conductivity control impairs a particular endpoint, the ambient sample with the lower conductivity is compared to the low conductivity control, rather than the standard test acceptability control, to determine whether the ambient sample is toxic. A low conductivity control was included with the April test batch to match the conductivity of Susan River at Commercial Road (637SUS004).

2.5 Quality Assurance

2.5.1 Test acceptability criteria

Test acceptability criteria (TAC) for toxicity tests included minimum control organism survival and sub-lethal fitness requirements. Tests where organisms did not meet these minimum requirements were repeated. All tests for this project met Test Acceptability Criteria.

- Chronic *C. dubia* toxicity tests require 80% or greater average control survival, with at least 60% of the surviving females having an average of 15 neonates and three broods.
- Chronic *P. promelas* toxicity tests require 80% or greater control survival and an average biomass of $\geq$ 0.25 mg/individual.
- Chronic *S. capricornutum* toxicity tests require an average cell growth of 1 x 10^6 cells/mL and a less than or equal to 20% coefficient of variation among control replicates.
- Acute *H. azteca* toxicity tests require 90% or greater average control survival.

2.5.2 Reference toxicant tests

Reference toxicant (RT) tests were included in this project to assess changes of organism sensitivity over time. These tests included the laboratory control and a dilution series of a chemical in laboratory control water. The LC_{50}/EC_{25} for each RT endpoint was plotted to determine whether it fell within the 95% confidence interval (CI) of the running mean. If an effect concentration, LC_{50} or EC_{25} was outside of the 95% CI, test organism sensitivity can be considered atypical and results of tests conducted during the month of an RT outlier could be considered suspect.

The method UCD AHPL uses to calculate the acceptable range of variation differs from that recommended by USEPA. USEPA recommends that acceptable data should fall within two standard deviations of the mean for the total project data set. UCD AHPL 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 a project.

Change in organism sensitivity may indicate problems with organism health, technician-handling techniques, and/or organism genetic variations. USEPA (2002) suggests that one outlying data value may be expected to occur by chance when 20 or more data points are plotted. UCD AHPL evaluates patterns of outlying values. When more than one outlier occurs, corrective actions will be taken. For instance, when two consecutive data points exceed the upper two-standard deviation line on an LC_{50} control chart, this may indicate that the test organisms are becoming less sensitive to reference toxicants. An appropriate corrective action measure in this case may include introducing a new genetic line of organisms to increase sensitivity.

RT tests with *P. promelas* and *H. azteca* were conducted concurrently with each test initiation. RT tests with *C. dubia* and *S. capricornutum* were conducted monthly. Sodium chloride was the toxicant used in *C. dubia*, *P. promelas*, and *H. azteca* species; zinc chloride was the toxicant used with *S. capricornutum*. There were no outliers for any species during this project.

2.5.3 Field duplicates

A field duplicate sample was collected once during the project (April 6, 2016) at Susan River at Commercial Road (637SUS004). Field duplicate samples are in agreement when the primary sample and its duplicate are both either statistically similar to or statistically different from the control. The primary sample and its duplicate were in agreement for all endpoints.

2.5.4 Precision

Precision is the degree to which the primary sample agrees with its duplicate. Precision is measured by calculating the Relative Percent Difference (RPD) between sample measurements. The RPD between a sample and its duplicate was calculated by using the following equation:

$$RPD = \left(\frac{\left[2 \left| Dup_1 - Dup_2 \right| \right]}{\left[Dup_1 + Dup_2 \right]} \right) \bullet 100\%$$

RPDs were calculated on water chemistry measurements of DO, pH, EC, hardness, alkalinity, and ammonia-nitrogen, as well as on toxicity testing endpoints such as survival, cell growth, reproduction, biomass, and weight. SWAMP Measurement Quality Objectives for precision require duplicate RPDs to be equal to or less than 20%. RPDs are discussed in more detail below in Section 3.3.1.

2.5.5 Toxicity identification evaluations

No Toxicity Identification Evaluations (TIEs) were performed during this project. The trigger for TIE follow-up was a 50% or greater reduction in a species' endpoint when compared to the control, within 96 hours of test initiation. None of the samples evaluated in this project met the TIE trigger.

2.5.6 Deviations from protocol

Two technician-error deviations occurred during this study. The LRWQCB requested that *H. azteca* testing be conducted only for Susan River near Litchfield (637SUS001), due to budgetary constraints. In the first sampling event on April 6, 2016, all Susan River samples were employed in an *H. azteca* toxicity test. This error was corrected in subsequent *H. azteca* toxicity tests in the May and June events. Additionally, dissolved oxygen at test termination (Day 4) was not recorded in the April 6 *H. azteca* test.

2.5.7 Completeness

UCD AHPL strives for a minimum of 90% completeness of work performed in accordance with SWAMP guidelines. All tests met TAC and therefore completeness for this project is 100%.

2.5.8 Analytical chemistry

Analytical chemistry was provided by the Department of Fish and Wildlife, Water Pollution Control Laboratory (Rancho Cordova, CA). CLAM filters were analyzed for the constituents outlined above in section 2.2.2, Table 2. Quality Assurance/Quality Control assessments were conducted by DFW WPCL following SWAMP protocols (SWAMP 2008) for each laboratory batch analyzed. The April (WPCL_L_155-16_W) and May (WPCL_L_230-16_W) analytical batches were considered Acceptable, with Minor Deviations. The deviations included a high continuing calibration verification (CCV) without bias, and low surrogate recovery. The June (WPCL_L_343-16_W) analytical batch was considered Acceptable. No laboratory data flags were applied for field surrogates in any analytical batch.

In general, field surrogate recoveries were low. As noted by DFW WPCL staff, low field surrogate recoveries have been observed with other projects in field deployed filters, due to the matrix that accumulates on the filter during deployment. Field surrogate recoveries ranged from 13.1-61.4% for the April analytical batch and between 26.0-71.4% for the May analytical batch. Field surrogate recoveries were improved for the June batch, ranging from 38.8-125%. As a result of these low field surrogate recoveries, field concentrations of herbicides may be underestimated. However, as these data were considered 'Acceptable' by the analyzing laboratory, we believe that the data are reliable.

3. Results

3.1 Species Performance / Test Acceptability Requirements

USEPA (2002) specifies that the test performance of each species in laboratory control water meet criteria to be considered acceptable, as described above in Section 2.5.1. All tests met acceptability criteria during this project.

3.2 Toxicity Test Results

Tabular summaries are provided in Appendix B, and hard copies of bench sheets are provided in Appendix D.

3.2.1 *Ceriodaphnia dubia*

Control performance for *C. dubia* was robust during the project, with survival ranging between 90-100%, and average reproduction of 25.8-35.0 neonates per surviving gravid. In the May 10, 2016 event, *C. dubia* in the Susan River at Litchfield site (637SUS001) exhibited a statistically significant reduction in the reproduction endpoint, with an average of 17.6 neonates per gravid when compared to the control, which had an average of 25.8 neonates per gravid. No other endpoint reductions were observed with this species.

3.2.2 *Pimephales promelas*

Control survival for the fathead minnow ranged from 97.5-100%, with an average biomass between 0.337-0.411 mg/individual. There were no statistically significant reductions observed with this species during the study.

3.2.3 *Selenastrum capricornutum*

Algal growth in the control ranged from 2.45-2.79 x 10^6 cells/mL. In the May 10, 2016 event, *S. capricornutum* in the Susan River at Litchfield site (637SUS001) exhibited a statistically significant reduction in cell growth, with 2.00 x 10^6 cells/mL, compared to the corresponding control, which had an average cell growth of 2.59 x 10^6 cells/mL. No other endpoint reductions were observed.

3.2.4 *Hyalella azteca*

H. azteca survival ranged from 98-100%. In the April 6, 2016 event, *H. azteca* in the Susan River above the confluence with Willard Creek site (637SUS003) exhibited a statistically significant reduction in survival. Survival in the Susan River site was 74%, compared to the corresponding control, which had an average of 98% survival. No other reductions were observed in this species for the remainder of the study.

3.3 Quality Assurance

3.3.1 Quality Assurance/Quality Control samples

One field duplicate sample was collected at the Susan River at Commercial Road site (637SUS004) on April 6, 2016. The primary sample and its duplicate were in agreement in all species endpoints. With one exception, all RPDs fell below the SWAMP MQO criterion of 20%. Individual RPDs for each species is outlined below in Table 3.

Table 3. Relative Percent Difference in field duplicate measurements (%)

Species	Survival/Growth	Repro/Biomass	EC	DO		pH	
C. dubia	0.00	4.39	2.27 1.51	0.63 2.05 2.01 0.28 5.34 0.74	0.63 1.00 0.97 1.01 0.80	0.00 0.11 0.05 0.05 0.02 0.05	0.02 0.01 0.03 0.08 0.06 0.05
P. promelas	0.00	3.58	1.42 26.94*	0.24 6.60 1.53 6.39 1.30 0.98 1.25	0.12 2.51 1.33 2.39 3.43 0.25	0.00 0.01 0.03 0.03 0.02 0.02 0.01	0.03 0.04 0.05 0.03 0.04 0.01 0.04
S. capricornutum	14.98	NA	11.07 3.40	0.35 18.91		0.01 0.28 0.02	0.05 0.01
H. azteca	9.30	NA	1.75 1.80	0.25 2.52	5.73 0.00	0.02 0.03	0.16 0.00

* EC measurements at test termination were 107.4 µS/cm for the primary sample and 81.9 µS/cm for its duplicate.

3.3.2 Reference toxicant testing

Reference Toxicant (RT) tests were conducted monthly within the project period. *C. dubia* sensitivity was assessed with 7d LC_{50} survival and 7d EC_{25} reproduction tests. *P. promelas* sensitivity was assessed using 7d survival and 7d EC_{25} biomass tests. *S. capricornutum* sensitivity was assessed with 4d IC_{50} growth tests, and *H. azteca* sensitivity was assed using 4d LC_{50} survival tests.

- *C. dubia* NaCl LC_{50} values ranged from 1.40 to 1.80 g/L, and EC_{25} values ranged from 0.350 to 0.904 g/L.
- *P. promelas* NaCl LC_{50} values ranged from 3.02 to 3.80 g/L, and EC_{25} values ranged from 1.56 to 1.96 g/L.
- *S. capricornutum* $ZnCl_2$ IC_{50} values ranged from 158.9 to 238.6 mg/L.
- *H. azteca* NaCl LC_{50} values ranged from 7538 to 8494 µS/cm.

There were no outliers during this project period. RT control charts for the AHPL are presented below in Figures 2-7. In March, 2016, the AHPL changed the way *C. dubia* RT tests were conducted, moving away from conductivity-based RT test concentrations (µS/cm), and towards measured, g/L-based concentrations, as is done with *P. promelas*. Therefore, the *C. dubia* charts have a limited number of data points associated with them. Project months of April, May, and June, 2016, are depicted as the last three data points in the referenced figures.

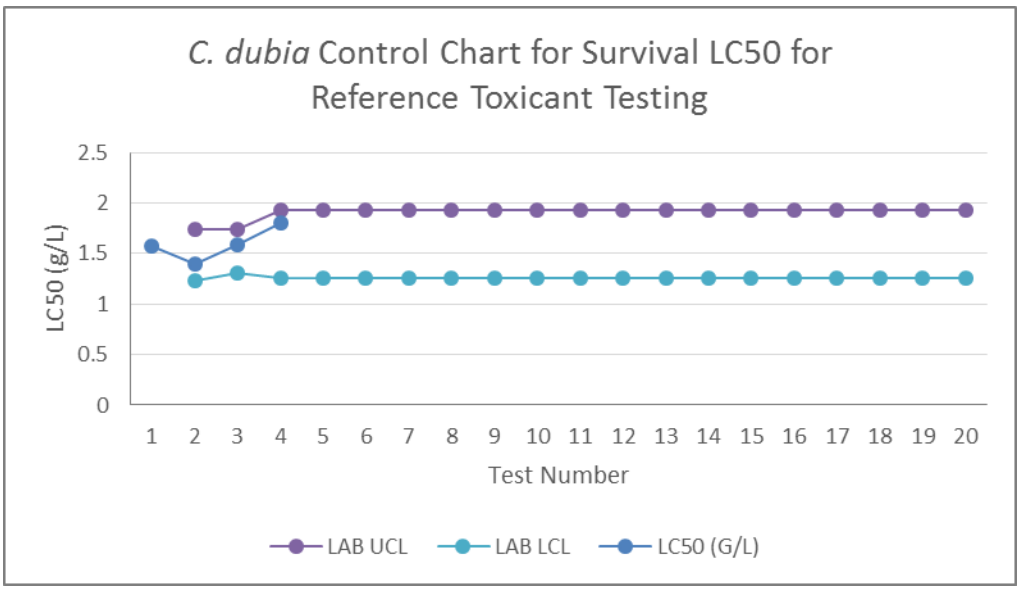

Figure 2. *C. dubia* control chart for survival LC_{50}

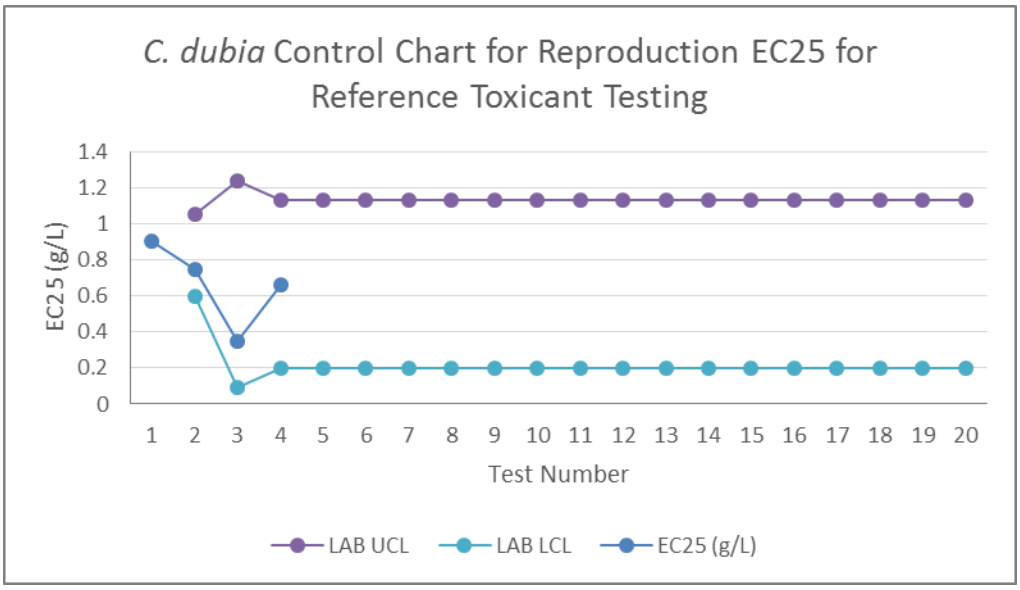

Figure 3. *C. dubia* control chart for reproduction EC_{25}

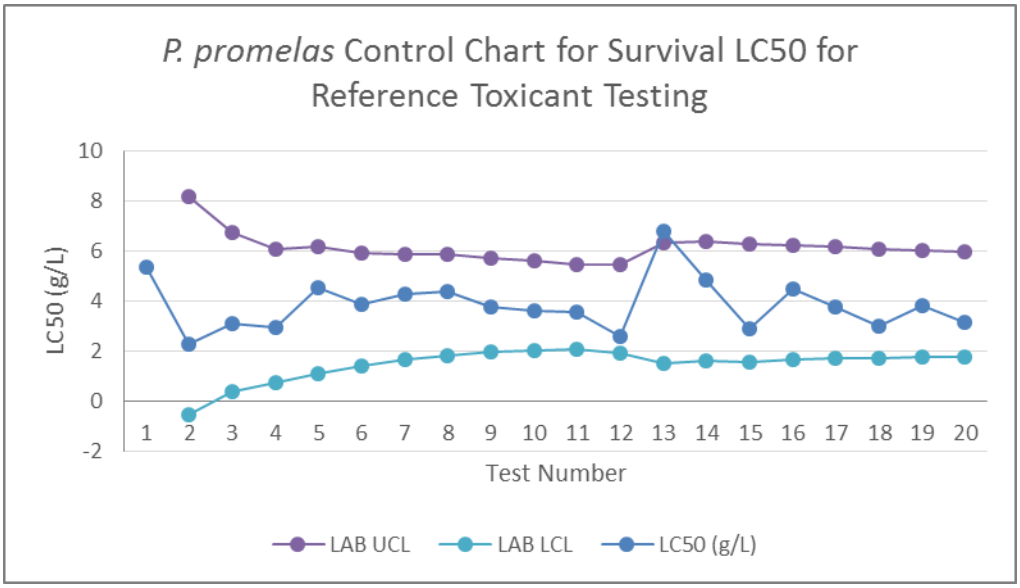

Figure 4. *P. promelas* control chart for survival LC_{50}

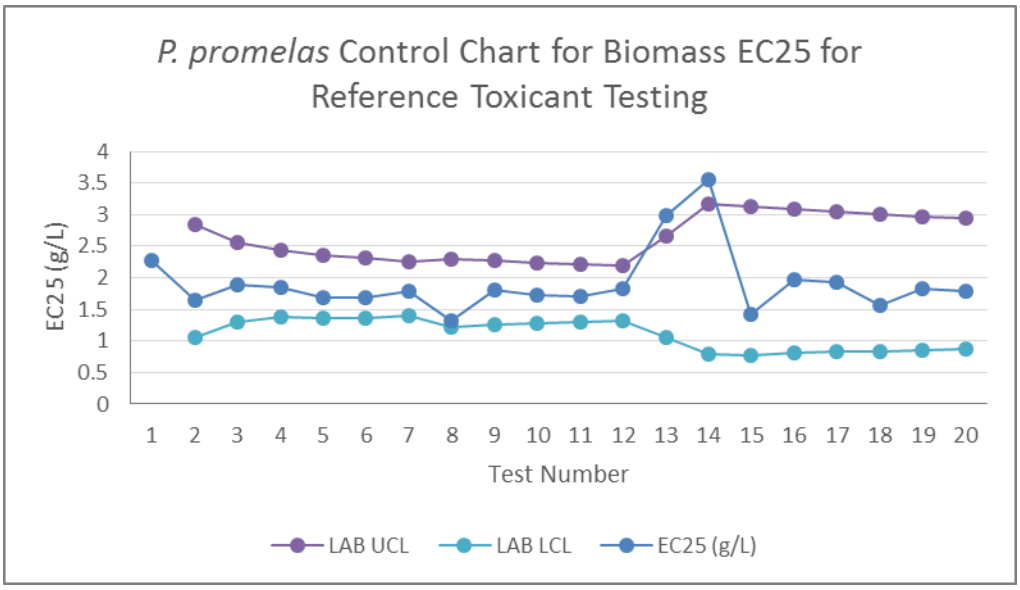

Figure 5. *P. promelas* control chart for biomass EC_{25}

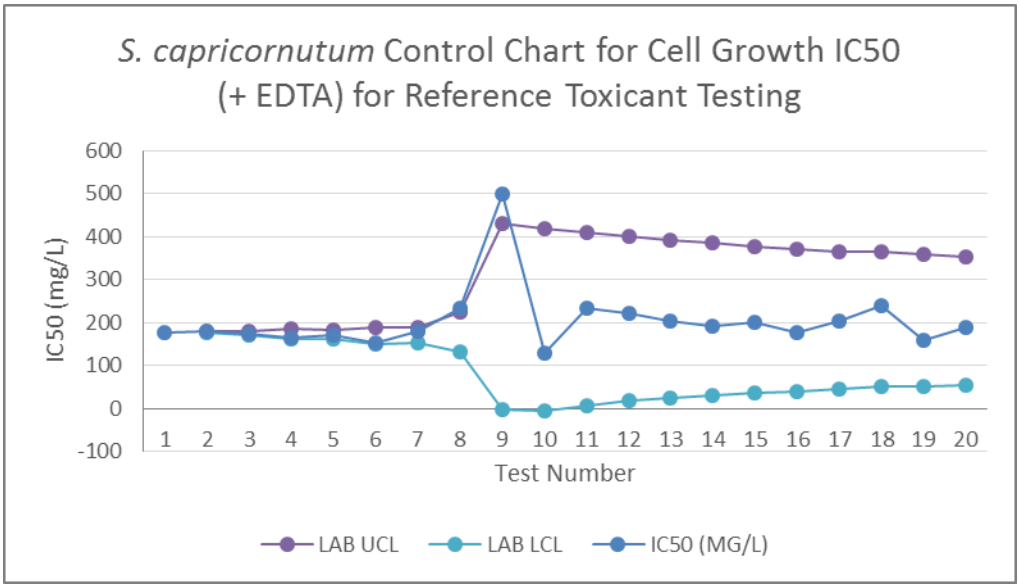

Figure 6. *S. capricornutum* control chart for growth IC_{50}

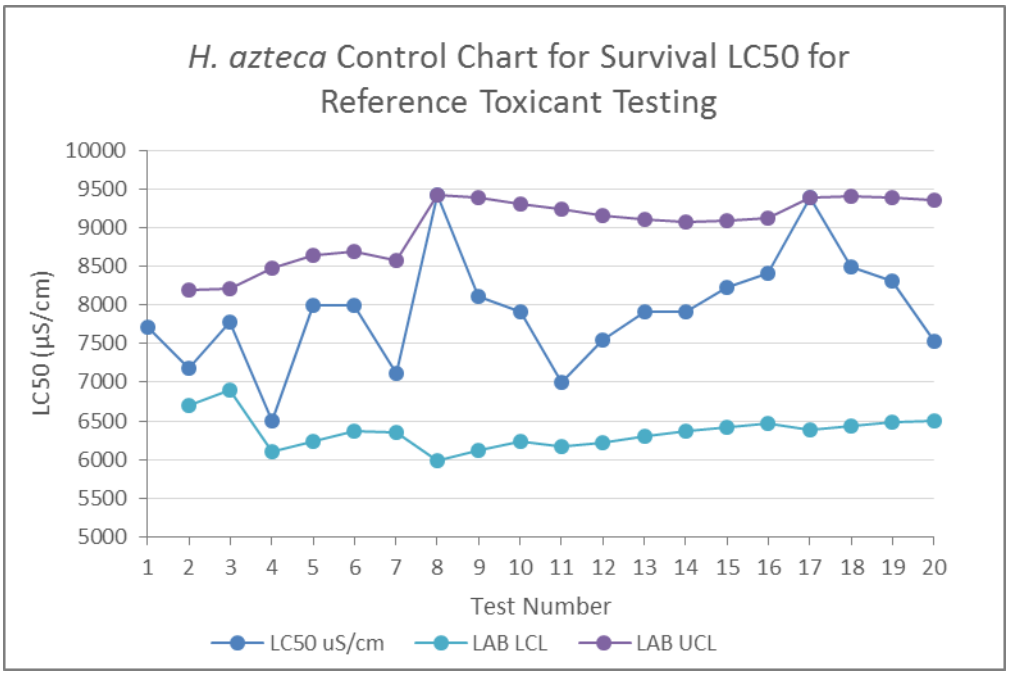

Figure 7. *H. azteca* control chart for survival LC_{50}

3.4 Sample Water Quality Measurements

Summary of water quality measurements are outlined in the Appendix B. All water quality fell within the prescribed ranges of USEPA for the test organisms. A Low Conductivity Control was applied in the April 2016 tests to match the conductivity of Susan River at Litchfield site (637SUS001). No adverse effects were observed due to low conductivity.

3.5 Analytical Chemistry

CLAM filters deployed by LRWQCB staff and analyzed by DFW WPCL showed the presence of the herbicide Hexazinone in every sample at each collection site, although many of these detected concentrations fell between the MDL and the RL for the analyte. No other analytes were detected. Results for all analytes are located in Appendix C. Concentrations of Hexazinone are presented below in Table 4.

Table 4. Analytical chemistry results for Hexazinone

Collection Date	Site	Replicate	Concentration (µg/L)	Notes
April 5-6, 2016	637SUS001	1	0.073	
		2	0.074	
	637SUS003	1	0.026	These values fell in between the MDL and RL; these values are estimated
		2	0.027	
	637SUS004	1	0.022	
		2	0.031	
May 9-10, 2016	637SUS001	1	0.030	
		2	0.031	
	637SUS003	1	0.023	
		2	0.023	
	637SUS004	1	0.018	
		2	0.015	
June 13-14, 2016	637SUS001	1	0.020	
		2	0.027	
	637SUS003	1	0.119	
		2	0.108	
	637SUS004	1	0.062	
		2	0.088	

4. Discussion

The primary objective of this study was to determine whether the Susan River is exhibiting toxicity as has been historically observed in prior studies. The Susan River was first listed by USEPA in 1990, and investigations conducted in 2003/2004 (Fong et al., 2004) demonstrated that toxicity was still observed in this water body. Three sites on the Susan River were examined for toxicity in 2016: Susan River at Litchfield (637SUS001), Susan River above the Confluence with Willard Creek (637SUS003), and Susan River at Commercial Road (637SUS004).

In this section, toxicity comparisons will be made among sample sites, species tested, and project years. Site code names, locations, and rationale for selection are outlined in Table 5 below. Figure 8 outlines the sample site names per study year. Site codes associated with the year of investigation will be used when making comparisons across years.

Table 5. Site codes, locations, and rationale for selection

Project Year	Site Code	Site Location	Site Rationale
2003/2004	SR-1	Susan River at Hobo Camp Trailhead to Bizz Johnson trail downstream of former USGS Gage	To represent water quality upstream of the City of Susanville; This site is comparable to 1990 site R-6-1
2003/2004	SR-2	Susan River at McGowan Lane	To capture changes in water quality downstream of confluence with Gold Run Creek that may have geothermal discharges that could influence water quality; This site is comparable to 1990 site R-6-2
2016	637SUS004	Susan River at Commercial Road, upstream of confluence of Gold Run Creek	Represent downstream influences of Susanville; This site is comparable to SR-2.
2003/2004	SR-3	Susan River at Leavitt Lane Bridge	Represent agricultural influences
2003/2004	SR-4	Susan River upstream of Litchfield at Bridge 7-34 on Highway 395	Represent downstream of confluence of Willow Creek; This site is comparable to 1990 site R-6-3
2016	637SUS001	Susan River near Litchfield	Capture private grazing and agricultural influences; This site is comparable to SR-4
2016	637SUS003	Susan River upstream of confluence of Willard Creek	Chosen as representative of reference conditions, downstream of USFS property

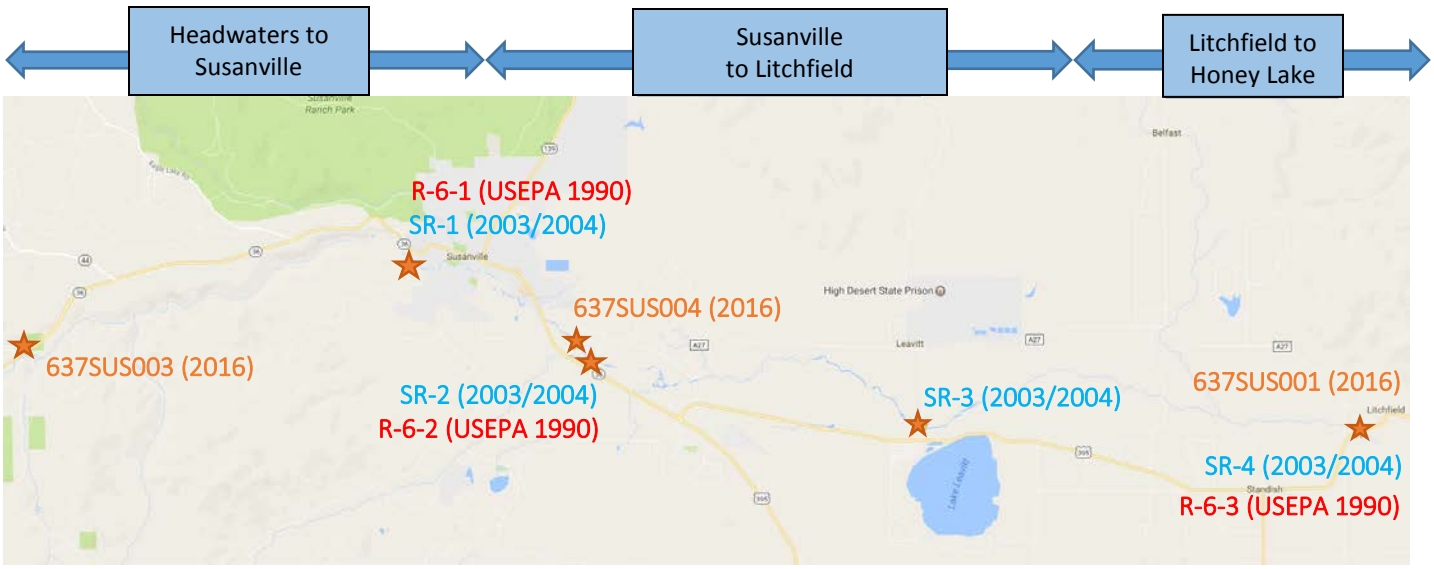

Figure 8. Map of study area with current and historical site codes. River reaches are indicated above the map in blue boxes. Site codes used in 2016 are delineated in orange font, blue font for 2003/2004 site codes, and red font for site codes used in 1990.

4.1 Statistically Significant Observations

Three sites on the Susan River were sampled three times over the 2016 study period. Two out of the nine samples (22.2%) were toxic to at least one of the test species.

A statistically significant reduction in *H. azteca* survival was observed in the Susan River above the Confluence of Willard Creek site (637SUS003) collected April 6, 2016, and statistically significant reductions in *C. dubia* reproduction and *S. capricornutum* cell density were observed in the Susan River at Litchfield site (637SUS001) collected May 10, 2016.

4.2 *C. dubia*

In the 1990 data collected by USEPA, no toxicity was observed with either *C. dubia* endpoint. No mortality was observed during the 2003/2004 study, however there was questionable reproductive impairment in two of the four Susan River samples which were toxic. 2003/2004 sites SR-1 and SR-2 collected July 30, 2003, resulted in a 15 and 20% reduction in neonates, respectively. Fong et al. noted that the reproductive impairment observed in these sites was most likely false positives related to the low variability among replicates.

Currently, the SWAMP statistical protocol involves the examination of significant differences in test organism performance by a one-tailed heteroscedastic t-test ($\alpha = 0.05$) and a categorization of the performance of organisms exposed to the ambient sample as either greater to or less than 80% of the control performance. Considering that samples are considered toxic only when both a significant t-test result and performance below the 80% threshold of the control is observed, the reproductive impairment observed in the July 2003 SR-1 and SR-2 sites do not meet this toxicity criteria. Therefore we agree with Fong et al. assessment that the July 2003 reproductive impairments were false positives.

Reproductive impairment was observed again in August, 2004 in sites SR-1 and SR-4, with neonate production reduced by 34 and 48%, respectively. Cause of toxicity at that time could not be determined.

In the current 2016 data set, reproductive impairment was observed once during the study period, in site 637SUS001 (Susan River at Litchfield) collected May 10, 2016; neonate production was reduced by 32%. As the Susan River at Litchfield site (637SUS001) is comparable to the SR-4 site collected in 2003/2004, which is comparable to the 1990 site of R-6-3, this reproductive impairment aligns with the toxicity historically observed in the Litchfield to Honey Lake reach of the Susan River.

4.3 *P. promelas*

Significant fathead minnow toxicity was observed with the 1990 USEPA data set, with 97 and 53% mortality in sites R-6-1 and R-6-2 collected in July 1990. R-6-1 collected in August 1990 resulted in 80% *P. promelas* mortality.

In the 2003/2004 data set, *P. promelas* did not exhibit statistically significant mortality at any site. A reduction in *P. promelas* survival (30%) was observed in site SR-1 collected August 2003, and was attributed to Pathogen-Related Toxicity (PRT). Significant reductions in biomass were observed in five samples, and four of those five samples were collected at sites SR-1 or SR-2.

There were no statistical significant reductions in either endpoint in *P. promelas* tested in the current 2016 data set, nor was any PRT observed.

There are similarities between the 1990 and 2003/2004 data sets regarding *P. promelas* toxicity, in that Susan River samples collected in the Headwaters to Susanville, and Susanville to Litchfield reaches during the June to August period were more likely to result in reduced organism performance. There was no toxicity observed with the current 2016 investigation, therefore it is possible that the causes of toxicity to the fathead minnow historically observed in the Susan River have since been ameliorated.

4.4 *S. capricornutum*

Investigations in 1990 and 2003/2004 utilized the vascular plant *Lemna minor* (duckweed) in order to determine toxicity. Toxicity to duckweed was observed in all Susan River samples collected in July and August, 1990. In the 2003/2004 investigation, all six samples collected from SR-3 and SR-4 during July – September 2003, were toxic. These specific instances in toxicity were attributed to the additive or synergistic effects of the herbicide Transline, and adjuvants nonylphenol and nonylphenol ethoxylate, which were identified in chemical analyses of these water samples. As noted in Fong et al.,Transline is applied to control vascular plant growth and was used in right-of-way applications in Lassen Co.

Toxicity was observed in all sites/reaches of the Susan River in the 1990 investigation. In the 2003-2004 study, duckweed toxicity was observed in the Susan River to Litchfield and Litchfield to Honey Lake reaches.

In the current study, the green freshwater alga *S. capricornutum* was used in lieu of *L. minor*. A statistically significant reduction in cell density was observed in 637SUS001 (Susan River at Litchfield) collected May 10, 2016. Cell density was reduced by approximately 22% compared to the control. This sample just barely met the two-fold toxicity criteria set forth by SWAMP, thus it is possible that this toxicity could be due to low variability among replicates. A secondary analysis using the Test of Significant Toxicity (TST) was applied to this sample, and resulted in a "Pass", indicating that the sample was not toxic. Therefore we deem this reduction of cell density a false positive due to low variability among replicates.

4.5 *H. azteca*

Inclusion of the amphipod *H. azteca* for evaluating the Susan River was first applied in the 2016 investigation, therefore there are no historical toxicity results to which we can compare organism responses. A significant reduction in *H. azteca* survival was observed in 637SUS003 (Susan River at the Confluence of Willard Creek),

with an approximate 25% reduction in survival compared to the control. Survival in this site was 74%. Given the slight reduction in survival, a follow up TST analysis was conducted on this result. However, results of the TST was "False", confirming the toxicity of this sample to *H. azteca*.

As this site was far upstream of all previous sites and was selected as a reference site, this toxic result is somewhat surprising.

4.6 Cause(s) of Toxicity

The toxicity observed during the current study was of low enough magnitude that TIE triggers (≥50% reduction in an endpoint within 96 hours) were not met, and thus no TIEs were conducted to determine the cause of toxicity.

Analytical chemistry on the CLAM passive samplers demonstrated the presence of the herbicide Hexazinone in every sample collected during the current study, although not all concentrations were able to be quantified as they were in between the Method Detection Limit and the Reporting Limit.

Toxicity was observed in 2016 in the Susan River near the Confluence of Willard Creek (637SUS003) in the April event for the *H. azteca* survival endpoint. Corresponding CLAM Hexazinone concentration at this site for this sample collection date was 0.027 µg/L (estimated), which fell between the MDL and the RL and is therefore unlikely to have caused the observed reduction in survival. It is possible that *H. azteca* were exposed to other contaminant(s) that may have been present in the water sample collected from this site; *H. azteca* are particularly sensitive to Pyrethroid pesticides. However without chemical analysis confirmation we are unable to identify the cause of this observed toxicity.

Toxicity was observed in the 2016 May event in both *C. dubia* and *S. capricornutum*, at the Susan River at Litchfield site (637SUS001). Corresponding CLAM Hexazinone concentrations at this site for this collection date was 0.031 µg/L (estimated), which fell between the MDL and the RL.

Although the frequency of detection for this herbicide was high (100% if estimated values are included; 33% frequency only those above the RL), measured chemical concentrations were consistently low, ranging between 0.015 and 0.119 µg/L. Because many of these Hexazinone concentrations are estimated (i.e., cannot be quantified), and given that field surrogate recovery was consistently low throughout the current project period, it is likely that CLAM concentrations of Hexazinone are underestimated and that in-stream concentrations of this herbicide may be higher than recorded.

Measured concentrations of Hexazinone fell well below those documented to cause acute toxicity and did not exceed the OPP Aquatic Life Benchmarks for freshwater organisms (USEPA, 2017). Acute and chronic benchmarks for freshwater invertebrates is 75,800 and 20,000 µg/L, respectively, and for non-vascular plants, the acute Aquatic Life Benchmark is 7 µg/L. Thus, it is unlikely that the toxicity observed in *C. dubia* and *H. azteca* was due solely to Hexazinone. Research by Tatum et al. (2010) confirms this assessment, as concentrations of formulated Hexazinone (Velpar L) of up to 550 µg/L active ingredient (a.i.) did not cause any noteworthy acute toxicity to *C. dubia* in 48-hour static non-renewal tests. Tatum (2004) noted that Hexazinone is classified by USEPA as slightly toxic to fish and aquatic invertebrates, with LC_{50} values ranging from 10-100 ppm (mg/L). These concentrations are orders of magnitude above those detected in the current study.

While our data suggest that the *S. capricornutum* impairment was a false positive due to low variability among replicates, and the secondary TST analysis confirms this assessment, there is evidence in the literature of the sensitivity of *S. capricornutum* to Hexazinone. Peterson et al. (1997) has documented 24-h NOEC concentrations ranging from 1-40 µg/L, and a 24h IC_{50} of 10 µg/L. In that study, *S. capricornutum* was

inhibited by 10% at the lowest test concentration of 1 µg/L. St. Laurent and Blaise (1992) has documented a 96-hr EC_{50} value of 24.5 µg/L (95% CI 14.5-33.1) of Hexazinone to *S. capricornutum*, and Montague (2000) has observed *S. capricornutum* exhibit a 120h EC_{50} of 7 µg/L. The concentrations detected in the current study are far below those documented in the literature to cause impairment, which supports our findings that it is unlikely that Hexazinone was the primary cause of toxicity during this investigation.

Interestingly, the highest Hexazinone concentrations were observed during the June 2016 event where there was no observed toxicity in any test species, although measured concentrations were still below those noted to cause organism impairment in Susan River at the Confluence of Willard Creek (637SUS003; average 0.114 µg/L) and Susan River at Commercial Road (637SUS004; average 0.075 µg/L). This leads us to believe that there were factors other than Hexazinone contributing to the toxicity observed in the 2016 dataset. As chemical mixtures are ubiquitous in the aquatic environment, it is likely that a combination of the Hexazinone observed during this project period and any number of unknown contaminants, can be attributed to the observed toxicity.

The frequency of detection of Hexazinone in the Susan River is not entirely surprising, given the land use in the surrounding areas and counties. The Department of Pesticide Regulation (DPR) Pesticide Use Report (PUR; 2015) lists Forest/Timberland and Alfalfa the second- and third-top sites in Lassen County, with Hexazinone as the third most-used pesticide in Forest and Timberland control. With Forest/Timberland and Alfalfa uses combined, approximately 2,150 pounds of Hexazinone (a.i.) was used in 34 applications over 1,977 acres in 2014, the most current year available in the PUR (DPR, 2015). Moreover, Susan River above the Confluence of Willard Creek (637SUS003) is located downstream of US Forest Service property, and Susan River at Litchfield (637SUS001) is located downstream of private farming and grazing operations; these locations align with the land uses associated with Lassen County, and it's possible that Hexazinone was used in these areas.

4.7 Summary of Toxicity

Samples were collected from the Susan River 63 times since 1990. Six samples were collected in 1990, 48 samples were collected in 2003/2004, and nine samples were collected in 2016. Of those 63 samples, 27 were toxic to at least one test species (Table 6), leading to a toxicity frequency of 43%. Of these 27 toxic samples, three were determined to be false positives. With this in mind, frequency of observed toxicity is reduced to 38%.

Investigations in 1990 demonstrated nine instances of toxicity, observed in all three reaches of the Susan River. In 2003/2004, this increased to 12 instances of observed toxicity, again observed in all three reaches. During the current investigation, only two instances of toxicity were observed; once in the Headwaters to Susanville reach and once in the Litchfield to Honey Lake reach of the Susan River.

This frequency of toxicity exceeds the narrative water quality objective in the Basin Plan, *All waters shall be maintained free of toxic substances in concentrations that are lethal to or that produce other detrimental responses in aquatic organisms.* However, it would appear that the Susan River is on the mend.

Table 6. Summary of observed toxicity – number of instances

Reach	Headwaters to Susanville			Susanville to Litchfield				Litchfield to Honey Lake		
Site Code	**R-6-1**	**SR-1**	**637SUS003**	**R-6-2**	**SR-2**	**637SUS004**	**SR-3**	**R-6-3**	**SR-4**	**637SUS001**
Project Year	**1990**	**2003/4**	**2016**	**1990**	**2003/4**	**2016**	**2003/4**	**1990**	**2003/4**	**2016**
Ceriodaphnia	0	1/1*	0	0	1*	0	0	0	0	1
Fathead Minnow	2	2	0	1	2	0	1	0	0	0
Algae	-	-	0	-	-	0	-	-	-	1*
Duckweed	2	0	-	2	2	-	2	2	2	-
Hyalella	-	-	1	-	-	0	-	-	-	0
Total per site:	**4**	**3**	**1**	**3**	**4**	**0**	**3**	**2**	**2**	**1**
Total per reach:	**8**			**10**				**5**		

*: These instances of observed toxicity are false positives due to low variability among replicates. These data points should be interpreted with caution and are not included in the total numbers.
- : Not tested

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