2. Project Description

The main hypothesis of the proposed research project is that feminization of fish observed in the Delta and its tributaries is due to the presence of chemical contaminants originating in agricultural, urban, and suburban areas in the upstream watershed. To test this hypothesis and to realize the project goals, the research will be conducted by an interdisciplinary team of environmental chemists and ecotoxicologists (see attached CVs for details). The project will be conducted in two Tasks, which are described below. The descriptions of tasks are followed by details of the experimental methods and project organization.

2.1 Task I: Quantification of (xeno)estrogens

In the first Task of the research project we will quantify the concentrations of (xeno)estrogens in the Delta and its tributaries. We also will develop and test protocols for *in vivo* bioassays of Chinook salmon exposed to waterborne contaminants.

Task 1.1: Quantifying the occurrence of (xeno)estrogens using chemical analysis

Although a variety of different chemicals that are capable of causing feminization of male fish are used in California, few measurements are available on contaminant concentrations in locations where early life-stage salmon exist. To obtain a better understanding of the occurrence and concentrations of (xeno)estrogens, samples will be collected from locations throughout the Sacramento River, San Joaquin River and freshwater Delta. Likely sampling sites are depicted in Figure 4.

The final selection of sites will depend upon accessibility of sampling sites and detailed evaluations of land use that could affect contaminant sources. Evaluation of land use classifications, which will be accomplished through analysis of census and planning data, will help us to identify surface waters where discharges are predominantly associated with undeveloped land, agricultural and urban/suburban land uses. Because different types of agricultural practices may result in significant variation in the nature and timing of contaminants releases, we will conduct a detailed evaluation of pesticide use in agricultural areas. As part of this analysis, we will use a GIS-based mapping tool that plots annual use patterns of specific types of pesticides at a one mile spatial resolution. The developer of the model, Dr. Susan Kegley, has generously agreed to help us implement the model as part of this project.

During Task I, the sample collection frequency will be biased towards winter wet weather periods. Monthly base-flow samples will be analyzed from each site during the period from December through April. Samples also will be collected immediately after two storm events to catch runoff-related pulses of contaminants. To assess dry-weather flows, samples will be collected during June, August and October.

Water samples will be analyzed for a suite of (xeno)estrogens hypothesized to be responsible for feminization of salmon in California's Central Valley and Delta, including steroid hormones, alkylphenols and selected pesticides. Analytical methods to be used for quantification of these compounds are described in section 2.3.2.

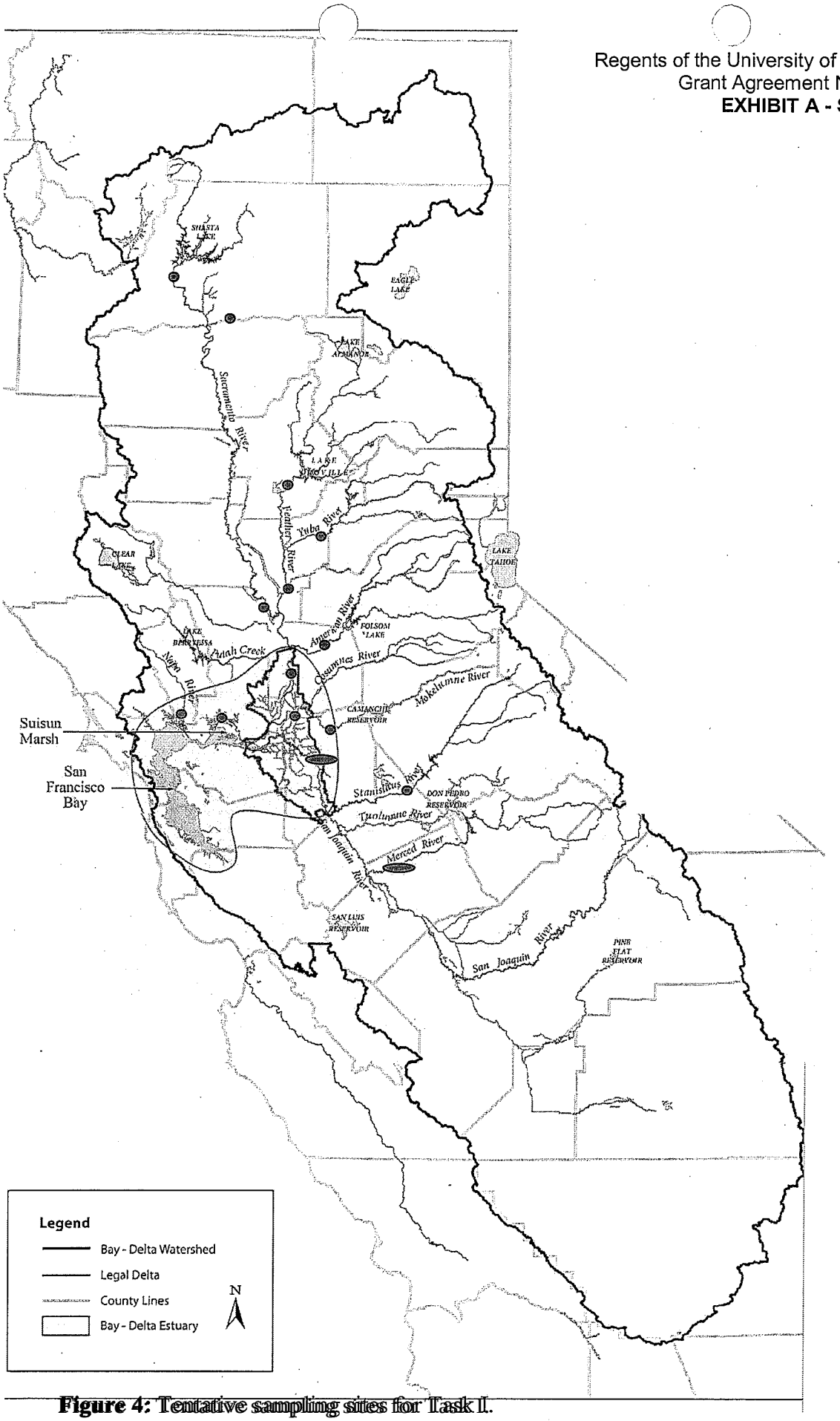

Figure 4: Tentative sampling sites for Task I.

Task 1.2: Quantifying estrogen agonists in sample extracts with an in vitro bioassay

The chemical analyses described in Task 1.1 may not identify all of the chemicals that could be responsible for feminization of salmon. To quantify the total concentration of estrogen' agonists (i.e., compounds capable of binding to the estrogen receptor), we will use the *in vitro* YES assay to quantify compounds that are capable of binding to the estrogen receptor in the extracts from water samples being analyzed for chemical contaminants. Comparison of results of the chemical analyses and the YES assay will be used to identify water sources in which unknown (xeno)estrogens are present.

Task 1.3: Development of in vivo assays for feminization in salmonids

To assess the potential for water and sediment samples to cause feminization, early life-stage Chinook salmon will be exposed to selected water samples. Feminization will be determined by measurement of vitellogenin in male fish during Task II of the project. Prior to conducting exposure studies with environmental samples, the salmon bioassay will be developed and validated as part of Task 1.3. In the first stage of these development activities, we will conduct dose/response studies with waterborne exposures to three known (xeno)estrogens (i.e., estrone, 4-nonylphenol and 3-phenoxybenzyl alcohol). These studies will identify threshold values for feminization of early life stage salmon attributable to exposure to these compounds. In addition, we will attempt to adapt the *in vivo* salmon bioassay to assess contaminated sediments by using river sediments from a reference site spiked with different concentrations of DDE. Although these data are being collected mainly to support research in Task II, they will provide information that will be of great interest to scientists concerned with feminization of salmon because it will provide a means for assessing the relative potency of waterborne exposures of (xeno)estrogens in rainbow trout and Chinook salmon.

2.2 Task II: Identification of Causative Agents

Data obtained in the Task I will provide us with an understanding of the temporal and spatial distributions of (xeno)estrogens. During Task II, we will further characterize spatial and temporal variations in concentrations of (xeno)estrogens and will expand the analytical methods to identify (xeno)estrogens that were not analyzed in Task I. We will use the *in vivo* Chinook salmon bioassay developed in the Task I to verify the relationships between chemical analyses and *in vitro* bioassays and their potential to cause feminization of male fish. We also will expand our analyses to include an assessment of the impacts of (xeno)estrogens in other important fish species, including Delta smelt and tule perch. Specific activities associated with each task are described below.

Task 2.1: Targeted sampling at selected locations

After completing Task I, sampling locations will be identified at which elevated concentrations of (xeno)estrogens and/or estrogen-binding activity (as evidenced by the YES assay) are present. To obtain further insight into the identities, sources and temporal distributions of these contaminants, additional sampling will be conducted at and near these sites during the second Task of the project. For example, if elevated concentrations of nonylphenol are detected during wet weather in one of the streams that receives considerable inputs of agricultural runoff, a concentrated sampling program will be employed in that watershed to assess potential upstream contaminant sources. Such an effort would concentrate on the

relationship between agricultural practices and concentrations of the contaminants through the application of pesticide application databases from the California Department of Pesticide Regulation. Likewise, if elevated concentrations of (xeno)estrogens are detected in surface waters downstream of livestock operations, we will evaluate the role of different management activities (e.g., confined animal feeding operations versus grazing) on (xeno)estrogen concentrations.

As part of this task, we anticipate collecting samples from approximately 25 locations in selected watersheds during three or four concentrated field sampling efforts. In conjunction with the targeted sampling, samples will continue to be collected from the locations sampled during Task I, but at a lower frequency, as determined by analysis of results from the first Task of the study.

Task 2.2: Toxicity/(xeno)estrogen identification evaluation (TIE)

Results from the *in vitro* bioassay and the chemical analysis conducted during the first Task of the study are likely to identify locations at which the concentrations of the measured compounds are unable to explain the total signal obtained with the *in vivo* bioassay. To identify the (xeno)estrogens responsible for the signal, we will subject extracts from samples in which the discrepancy was observed to chemical fractionation prior to application of the bioassays. In this Task of the research, we will employ the YES assay and an *in vivo* assay with rainbow trout *(Oncorhynchus mykiss).* Trout will be used instead of juvenile Chinook salmon in this part of the research because salmon eggs will not always be available and.the Chinook salmon assays will be more labor intensive than the trout assay, which is already running in our laboratories. This assay has been recently used to identify (xeno)estrogens from wastewater of New York City (Sapozhnikova *et al.,* 2005), and sediments near wastewater outfalls of Los Angeles and Orange County (Schlenk *et al.,* manuscript in review). The fractions showing YES or *in vivo* activity will first be separated by selective elution from solid-phase extraction columns. The fractions in which activity is observed then will be subjected to further separation by high performance liquid chromatography (HPLC). Once the active fractions have been identified with the *in vivo* and *in vitro* bioassays, the extracts will be subjected to gas chromatography/tandem mass spectrometry (GC/MS/MS) and HPLC/MS/MS to identify the causative agent(s). Previous studies have indicated multiple compounds with estrogenic activity in wastewater (Sapozhnikova *et al.,* 2005). If multiple compounds are identified from the samples in the proposed study, relative contributions toward biological activity will be assessed by reconstituting fractions or water with measured concentrations of identified compounds. In this way, causation of identified compounds will be verified.

Task 2.3 Conduct salmonid bioassays on selected samples

To test the hypothesis that feminization of salmon is attributable to chemical contaminants, early life-stage Chinook salmon will be exposed to water samples collected from selected locations in the Sacramento River, San Joaquin River and Freshwater Delta. Samples will be selected on the basis of chemical analyses and bioassays (i.e., tasks 2.1 and 2.2) to represent locations where high concentrations of (xeno)estrogens are present. Background sites and locations of which low concentrations of (xeno)estrogens are present also will be included. Sediment samples, from one or two locations in which elevated concentrations of sediment-associated contaminants, such as DDE or hydroxylated PCBs are present, also will be analyzed if

the *in vivo* bioassay can be adapted for use with sediments. Assessment of feminization will be made using expression of whole body vitellogenin.

Task 2.4: Analyze extent of feminization with other fish species in the watershed

In addition to salmon, other fish species may be affected by (xeno)estrogens. In particular, fish that spend a larger fraction of their lives in the Bay/Delta system may be exposed to higher concentrations of (xeno)estrogens than salmon. Most of the proposed research is focused on salmon because they are known to be sensitive to (xeno)estrogens and preliminary data indicate a relatively high frequency of feminized male salmon in the Sacramento and San Joaquin watersheds. However, other fish species may be impacted by (xeno)estrogens and at present no information is available on the extent of feminization of other key species of the Bay/Delta system.

To address the extent that xenoestrogens may be affecting fish that spend a great deal of their lifecycle in the watershed, particularly the estuarine portions that are not covered well by the other tasks, we will sample male fish of several species during their reproductive period. Blood plasma of male fish collected from the Delta will be assayed for egg proteins (i.e., choriogenins). Since males do not make these proteins except when exposed to (xeno)estrogens, their presence is a highly specific biomarker for (xeno)estrogens. The information provided by this task will provide a means for assessing whether or not (xeno)estrogens are acting as a stressor for other fish species that are relevant to the CALFED program.

2.3 Experimental Methods Descriptions

The research described in the previous section will be conducted using the experimental methods described in the following sections. We are already using many of these methods as part of ongoing research projects. In cases where new methods will be developed, we will use experimental methods developed by other researchers to guide method development activities.

2.3.1 Sample collection

As part of the activities described in the previous section, water samples will be collected from locations throughout the Sacramento River, San Joaquin River and Delta. Water samples will be collected by Applied Marine Sciences (AMS) using techniques developed as part of the San Francisco Bay Regional Monitoring Program (see www.sfei.org/rmp/index.html for details). The staff of AMS has over a decade of experience collecting water, sediment and biota samples in the Bay/Delta system and is well qualified to coordinate the field sampling elements of this program.

The water samples will be collected using a peristaltic pump fitted with Teflon tubing and a 0.2-pm in-line cap Midge filter. Whenever necessary, samples will be collected by boat from the center of the river. However, during storms and at locations where boat access is difficult, samples will be collected from bridges or from shore using a sample collection boom. As part of sample collection activities, standard quality assurance/quality control procedures will be followed. The QA/QC plan includes the collection of duplicate samples and field blanks at a frequency of at least 5% and maintenance of sample chain of custody to maintain continuity in sample handling.

Samples will be collected in glass containers and will be refrigerated during transit to UC Berkeley and UC Riverside. For chemical analysis and the *in vitro* bioassays, at least 8-L of

sample will be collected from each site in 4-L glass containers, with separate 1-L split samples for analysis of water quality parameters, such as total suspended solids, conductivity, pH and total organic carbon. For the *in vivo* bioassays, to be conducted during the second Task of the project, 100-L samples will be collected from a limited number of sites in glass carboys or other containers. Field spikes will be included in each set of samples to verify the stability of analytes during sample collection, handling and storage.

2.3.2 Chemical Analyses

In the first Task of the project, (xeno)estrogens will be analyzed using established methods. In particular, we will target those compounds hypothesized to be important to feminization of salmon (e.g., Table 2). However, we also will include other (xeno)estrogens that can be analyzed readily with the selected analytical techniques. The final selection of compounds to be analyzed will depend upon the method validation studies that will occur during the first three months of the project in which matrix spike recoveries and detection limits will be quantified using the collection methods, handling procedures and sample volumes to be used for the field study.

Solid-phase extraction (SPE) is the first step in the analysis of chemical contaminants and the preparation of samples for the *in vitro* bioassays. To concentrate (xeno)estrogens, we will use pressurized solid-phase extraction on C-18 discs followed by elution with methanol (Kolodziej *et al.* 2003). All of the known (xeno)estrogens listed in Table 2 are extracted efficiently on this material. However, to assure that polar (xeno)estrogens that are not extracted by C-18 are not responsible for the activity, 100-mL aliquots collected before and after C-18 extraction will be subject to SPE with more polar resins (e.g., cation exchangers) or will be subject to lyophilization prior to analysis with the YES assay.

The concentrated C-18 SPE extracts will be analyzed by GC/MS/MS after derivitization to enhance the volatility of the polar compounds. All of the compounds to be analyzed during the first Task of the project can be analyzed by GC/MS/MS (Kolodziej et al. 2003, Gross *et al.* 2004, Hoai *et al.* 2003, Schettgen *et al.,* 2002) after the appropriate derivitization techniques are applied. Although most of these compounds also can be analyzed by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS), we will use GC/MS/MS because it is sensitive and relatively free from interference attributable to organic matter that is present in the SPE extracts.

During the second Task of the project, we will conduct a TIE study to assess the sources of YES and *in vivo* vitellogenin activity using an approach similar to that used by Desbrow *et al.* (1998) and Sapozhikova *et al.* (2005) to identify sources of (xeno)estrogens in municipal wastewater effluent. The initial separation of (xeno)estrogens will be accomplished by selective elution of the C-18 discs with 50-mL aliquots of methanol/water mixtures of decreasing polarity (e.g., 10% methanol, 20% methanol, etc.) followed by measurement of YES and *in vivo* activity in each fraction. Fractions exhibiting activity will be subjected to finer separation by HPLC followed by fraction collection, as described in Huang and Sedlak (2001) and Sapozhikova *et al.* (2005) followed the YES and *in vivo* vitellogenin assays and GC/MS/MS analysis. For extracts in which *in vitro* or *in vivo* activity is greater than that predicted by the GC/MS/MS analyses, extracts will be subjected to analysis by HPLC/MS/MS by Dr. Shane Snyder of the Southern Nevada Water Authority. As part of this activity, we will identify unknown (xeno)estrogens by

matching the mass spectra of compounds detected in the extracts with putative compounds that elute at the HPLC retention time associated with each active fraction.

To assure the quality of results from the chemical analyses, appropriate quality assurance/quality control measures will be taken. These steps, which are routine parts of all trace organic analyses conducted in our laboratory, will include analysis of blanks, duplicates and matrix spike recovery samples at a frequency of at least 10%. In addition, each method will include surrogate and internal standards to monitor losses in individual steps in the analysis. After collection of the data, samples that do not meet QA/QC criteria will be eliminated from the database or will be analyzed again if sufficient sample extract remains.

2.3.3 YES Assay

To quantify the concentration of estrogen agonists, we will use the YES *in vitro* assay on sub-samples of the extracts obtained for the chemical analysis. The YES assay, which was developed by Routledge and Sumpter (1996), quantifies all of the compounds in a sample that are capable of binding to an estrogen receptor. The response of the bioassay is compared to a standard curve obtained with 17(3-estradiol and the results are expressed as estradiol equivalents (EEQ). The YES assay can be used with an extract from a water sample, provided that the solvent does not interfere with the receptor. A sample volume of only 100 µL of extract is needed to detect an EEQ of 1 ng/L in a water sample if a pre-concentration factor of 4,000 is applied. Therefore, the YES assay will require less than 10% of the sample extract from the chemical analysis.

2.3.4 In vivo Bioassays

The rainbow trout model will also be used to test *in vivo* estrogenic activity of samples. Juvenile animals of approximately 3 weeks of age will be obtained from the Thomas Fish Company (Anderson CA). The fish will be maintained under flow-through conditions at 10°C if injection exposure is utilized. If static exposure is utilized, 1 mL of extract will be added to 4L of filtered water containing 1 fish. The water will be renewed daily for 7 days. After exposure, animals will be euthanized in MS-222 and bled from the caudal vein using a heparinized syringe. Plasma/serum will be separated by centrifugation and evaluated by commercially available ELISA kits (Biosense, Bergen Norway). Inter-individual variability indicates an N of approximately 10 animals is necessary per exposure.

Data will be evaluated for homogeneity of variance to dictate whether parametric or nonparametric comparisons should be made between fractions or extracts. Appropriate post-hoc tests will be used to determine significance values.

Exposure route significantly affects uptake of xenobiotics into aquatic organisms. One caveat of the research is that the *in vivo* assays will evaluate extracts using aqueous and intraperitoneal injection exposure routes. The later route attempts to mimic oral exposure as the intraperitoneal cavity is drained by the hepatic-portal system allowing uptake to the liver which is the major organ for xenobiotic biotransfolination. Unfortunately, this exposure route omits any biotransformation at the gut/intestine that may also contribute to the overall metabolism of any given compound. In addition, environmental bioavailability is also not considered with the injections, as uptake across the gut or gill is bypassed. Overall, the vitellogenin response is a somewhat acute response (7 days) and would not likely identify xenoestrogens that require magnification to levels that may lead to ER activation or indirect estrogenic activity (i.e., anti-

androgenic activity). Thus, certain compounds may be excluded using this exposure regime. Pilot studies can be carried out using extended exposure regimes (i.e., 21-30 d) to determine whether differences exist within extracts for activity.

2.3.5 Choriogenin Assay

The choriogenin assay will be conducted on fish caught during the monthly trawls conducted by California Department of Fish and Game for the Interagency Ecological Program (IEP). Blood will be drawn from the caudal vein or gills of male fish caught in the trawl. Blood will be spun down and the plasma removed and frozen. Several species will be targeted (e.g., Delta smelt, tule perch), but sampling must necessarily remain adaptive, as the abundance of species in the trawls is not predictable. Plasma samples will be analyzed using a choriogenin polyclonal antibody in an ELISA. This antibody strongly cross reacts with choriogenins in a variety of fish species. One of the reasons that the choriogenin assay is preferred over assays for vitllogenin is that the protein structure of this egg component is more highly conserved and thus more widely cross-reactive with antibodies developed in other species. It is expected that both widely ranging species, such as Delta smelt, which will integrate biochemical responses to (xeno)estrogens over a broad area of the watershed, and species that range little within the watershed, such as tule perch will be sampled. Both of these species have been in decline so the data from this task will not only help identify the potential influence of (xeno)estrogens on salmonids in this portion of the estuary, but will be of broader interest to the CALFED program.

2.4 Interactions with CALFED Stakeholders and Scientists

One of the major goals of CALFED's science program is to assure that sponsored scientific research has an impact on water management practices in the Bay/Delta system. To assure that the results of this project achieve this goal, we will interact with scientists, managers and stakeholders throughout the project by employing the approach described below.

2.4.1 External Advisory Panel

To obtain input on the experimental design and interpretation of results, we will hold regular meetings with a panel of technical advisors with expertise in issues related to salmon and chemical contaminants in the Bay/Delta system (Table 3). The advisors will represent the perspectives of regulators, scientists and managers striving to protect and restore salmon in California's inland waters. Robert Holmes has helped to lead the Central Valley RWQCB's efforts to respond to threats posed by (xeno)estrogens. Dr. Bruce Macfarlane has conducted research on sediment-associated contaminants in the Delta and their effects on salmon. Dr. Thomas Harter is an expert on agricultural wastes and their effects on surface and groundwater systems. Additionally, to foster collaborative interactions with the Bernie May CALFED project, Kevin Williamson, who is managing the May CALFED project, will be included as an advisory panel member and will attend all advisory board meetings. All of the advisors listed in Table 3 have expressed a willingness to participate in the project.

Interactions with the external advisory panel will be facilitated through face-to-face meetings and occasional telephone conferences and e-mail correspondence. An initial meeting will be held with the advisory panel during the first month of the project to discuss details of the experimental plan. A second meeting will be held near the completion of Task I to discuss experimental design for the second Task of the project. A final meeting will be held two months

prior to completion of the project to discuss the implications of the research and mechanisms of communicating the results to stakeholders and scientists.

Table 3: External advisory panel.

Name	Affiliation
Robert Holmes	Central Valley Regional Water Quality Control Board
Dr. Bruce Macfarlane	NOAA
Dr. Thomas Harter	UC Davis

2.4.2 **Interactions with other CALFED Projects**

Several ongoing CALFED projects are addressing issues that may be important to the experimental design and interpretation of results from this project. For example, Dr. Donald Weston, of UC Berkeley, is leading a CALFED project on the fate of pyrethroid pesticides in the Central Valley and the preliminary findings of his research will be useful to our assessment of the potential for pyrethroids and their degradation products to cause feminization. The current call for proposals emphasizes research on salmonids and we hope to establish strong linkages with other CALFED projects focused on salmonids. Relevant CALFED-funded projects will be identified through discussions with CALFED staff as well as other scientists and stakeholders after initiation of the project.

To assure that results from those ongoing studies are considered in this project, we will make special efforts to facilitate interactions with other researchers through informal contacts between researchers and through formal meetings. For example, one mechanism for facilitating these interactions will involve inviting principal investigators and lead scientists on other CALFED projects to visit UC Berkeley or UC Riverside and present their recent results at departmental seminars. This approach also will give other CALFED-funded projects an opportunity to receive feedback from outside scientists. After presenting their latest findings, the invited scientists will spend the remainder of day with project personnel exchanging ideas and providing feedback on the experimental design.

2.4.3 **Dissemination of Results**

Results of our research will be disseminated to the research community through a variety of mechanisms. During the interim stages of the research, results will be presented at regional conferences, such as the biannual CALFED science conference and the California Regional meeting of the Society for Environmental Toxicology and Chemistry (SETAC). We also will develop and maintain a web site on the project on the server for UC Berkeley's Department of Civil and Environmental Engineering. The web site will be linked to home pages for each of the participating investigators and their research groups.

After completion of the different stages of the research, we will publish our results in peer-reviewed scientific journals, such as *Environmental Science and Technology*, *Environmental Toxicology and Chemistry* and *San Francisco Estuary and Watershed Science* (the newly established, open access journal focused on issues of relevance to CALFED). Results also will be presented at national and international scientific meetings. In addition, the lead investigators of this project are often invited to give keynote presentations at highly visible scientific forums and these should provide additional venues to communicate results of the research.

2.4.4 **Management Plan and Feasibility**

The proposed research is a collaborative effort involving environmental chemists and environmental toxicologists as part of an integrated project focused on the same goal: identifying the causes of feminization of salmon. As a result, all of the researchers will employ the same conceptual model. The boundaries of the research are clearly delineated (i.e., Professor Sedlak's research group will address the chemistry while Professor Schlenk's group will be responsible for the toxicology) despite the fact that the research is fully integrated. Because chemical analyses will be performed on the same water samples in which *in vitro* and *in vivo* bioassays will be conducted, the interpretation of data is interdependent. This integrated experimental design is particularly powerful because it offers independent lines of evidence for findings from the two approaches.

Although analyses will be conducted on the same water samples, the schedule of the individual groups will not be hindered by minor setbacks or delays experienced by one of the groups because samples will be collected by a third party (i.e., AMS). Furthermore, samples undergoing chemical analysis and *in vitro* bioassays will be extracted and split immediately after collection, thereby preventing any delays associated with analyses.

Professors Sedlak and Schlenk have successfully collaborated on other research projects. They are currently collaborating on a research project funded by the University of California's Coastal Marine Science Program, investigating the causes of feminization of flatfish in coastal waters. In that project, Professor Schlenk was responsible for bioassays while Professor Sedlak and Dr. Kolodziej, who was a doctoral student during the project, were responsible for chemical analyses of sediment samples. In addition, Professor Schlenk has served as an informal advisor to two of Professor Sedlak's doctoral students, who studied the fate of steroid hormones in an engineered wetland in Southern California. As a result if these previous interactions, the lines of communication between the two research groups are well established.

Throughout the research project, effective communication between the two groups will be reinforced through several different mechanisms. The main mechanism to ensure effective communication is through face-to-face contact. Full-day meetings of the lead investigators and key personnel will occur in Berkeley or Riverside at the start of the first and second Tasks of the project. In addition, meetings will take place among staff involved in sample collection and analysis at the beginning of the period of intensive field sampling (i.e., late fall). Furthermore, progress report meetings will be held by conference call every two months to discuss progress, scheduling and any impediments in the research program.

In addition to the group meetings and conference calls, the project manager, Dr. Edward Kolodziej, will dedicate approximately 20% of his time to project oversight and management. Dr. Kolodziej will be responsible for coordinating meetings, evaluating project schedules and timelines and facilitating communication between researchers. To accomplish these objectives, Dr. Kolodziej will visit UC Riverside once every three months to meet with Dr. Schlenk's research team. In addition, Dr. Kolodziej, will accompany AMS during the first rounds of sampling during the first and second Tasks of the research project. Dr. Kolodziej is particularly well suited for this task (see CV for details) because he is already familiar with many of the proposed sampling locations has a strong background in environmental chemistry and toxicology and already has worked closely with Professors Sedlak and Schlenk.

Permits:

With the exception of Task 2.4, no permits are required for any sampling or work done in this project, as we are not proposing to directly or indirectly alter the physical, chemical, or biological composition of any waterbody or site involved in the project. Task 2.4 will involve the collection of wild fish for choriogenin analysis, and Dr. Robert Spies of Applied Marine Sciences will undertake this task. Bob has determined that we will need only one permit for this task. A standard Scientific Collector's Permit issued by the California Department of Fish and Game would cover all collection, and AMS has routinely received these permits, with no problems, in the past for similar work. Also, AMS field personnel each have their own permit issued by CDF&G and the terms and conditions of our permits allows sampling all the requisite fish species with no other authorizations required. This task is not scheduled to begin until later in the project, so we do not have a current permit in hand at this time, but if you need a valid copy of the permit before the contract can be finished, please let us know so we can begin the process to get one.

3. Justification

Previous research in our laboratories and by scientists working in the Sacramento and San Joaquin watersheds and other locations suggests that feminization of Chinook salmon is occurring and that the most likely cause is exposure to (xeno)estrogens. Feminization is believed to have important impacts on the growth, survival and reproduction of many fish species. In species like Chinook salmon, that are sensitive to chemical stresses, feminization may be an important but overlooked stressor that contributes to the population declines that have been observed among salmonids in the Sacramento and San Joaquin ecosystems. Presently, very little is known about the causes of feminization of salmonids. The research described in this proposal will determine the chemical causes of feminization in Chinook salmon, will determine the sources of (xeno)estrogens in the Sacramento and San Joaquin watersheds, and will develop toxicological models for the effects of (xeno)estrogens on Chinook salmon. The research also will provide much needed data on the frequency of feminization of other key species that are resident to the Delta. Finally, the research also aims to identify cost-effective methods to control or alleviate the impacts of (xeno)estrogens on sensitive fish populations in these watersheds and the greater Bay-Delta.

This research project addresses several issues of importance to the CALFED program. In particular, the research is relevant to efforts to protect and restore Chinook salmon and other critical species because it provides much needed information about a family of chemical stressors that have not received much attention from the CALFED program. Many of the contaminants that are hypothesized to be responsible for feminization of fish are released from sources that exist in the Bay/Delta system. However, scientific information is not available to compare the relative importance of (xeno)estrogens and other fish stressors. If (xeno)estrogens prove to be important stressors, our research will help CALFED to design future monitoring programs and control measures for these stressors.

If feminization of salmon is caused by chemical contaminants present in water flowing through the Delta, efforts to enhance salmonids and other sensitive fish species by alteration of water flows and reallocations of water resources could be compromised because the concentrations of (xeno)estrogens or the timing of these discharges may be inadvertently altered by changing the relative flows from different sources of water. Furthermore, water diversions or

changes in agricultural practices that alter the retention times of (xeno)estrogens in environments where they might be removed through natural attenuation could result in exposure of fish to higher concentrations of contaminants. These issues are particularly important in the Delta where planned alterations in flows could result in changes in the composition and timing of water discharges.

4. Task Table: task summary, schedule and list of deliverables

task ID	task name	start month	end month	personnel involved	description	deliverables
1.1	Quantifying the occurrence of i(xeno)estrogens using chemical analysis	1	18	Sedlak, David L., Ph.D. Kolodziej, Edward P., PhD. *Post-Doctoral researcher-UCB Subcontractor for Sampling Services	Verify analytical methods (months 1-2). Collect and analyze water samples. Extract water samples and analyze extracts for (xeno)estrogens	1) Measurements of (xeno) estrogen concentrations in over 200 water samples from the Sacramento and San Joaquin watersheds. 2) Analysis of data correlating spatial occurrence of these compounds with pesticide application rates and hydrologic conditions.
1.2	Quantifying estrogen agonists in water samples with an in vitro bioassay		18	Schlenk, Daniel, Ph.D. *Post-Doctoral Researcher-UCR	Analyze estrogen agonists in sample extracts from task 1.1 using an in vitro bioassay	Quantification of total estrogenicity in over 200 water samples 2) Comparison of total estrogenicity with concentrations of (xeno)estrogens (Task 1.1) to identify locations where unknown (xeno)estrogens are present.
1.3	Development				Test	1) Developed

	of in vivo bioassays for feminization of early life-stage salmon	1	18	Schlenk, Daniel, Ph.D. *Post-Doctoral Researcher-UCR	dose/response relationship for early-life stage salmon by exposing fish to different concentrations that have been shown to cause feminization of other, fish species.	and validated Chinook salmon bioassay, including dose-response relationships for in water and sediments.
2.1	Targeted sampling at selected locations	18	36	Sedlak, David M., Ph.D. Kolodziej, Edward P., PhD. Post-Doctoral researcher-UCB *Subcontractor for Sampling Services	Collect samples from selected locations in task 1.1 where elevated concentrations of (xeno)estogens were detected as well as additional nearby locations. Extract samples and analyze for, chemical contaminants, YES activity and in vivo activity.	Quantification over 50 water samples from locations identified in Task 1.1. 2) Quantification of (xeno)estrogens in approximately 100 water samples collected from watersheds with, elevated (xeno)estrogen concentrations. 3) Identification of specific (xeno) estrogen sources in selected locations in the Sacramento and San Joaquin watersheds. 4) Description of specific strategies to control, reduce, and Alleviate stressors related to xenVestrogens

2.2	Toxicity identification evaluation	21	36	Sedlak, David L., Ph.D. Schlenk, Daniel, Ph.D. Kolodziej, Edward P., PhD. *Post-Doctoral Researcher-UCR *Post-Doctoral researcher-UCB Snyder, Shane A., Ph.D.	Perform chemical fractionation studies coupled to bioassays on samples from Task 2.1 in which (xeno)estrogens are detected. Analyze fractions by chemical methods, in vitro and in vivo bioassays.	1) Identification of unknown (xeno)estrogens, including likely sources and control strategies.
2.3	Conduct in vivo bioassays on selected samples	18	36	Schlenk, Daniel, Ph.D. *Post-Doctoral Researcher UCR	Perform in vivo bioassays with early life-stage Chinook salmon on samples from task 2.1 and 2.2 in which activity is detected.	1) Verification that (xeno)estrogens are responsible for observations of: feminized Chinook salmon in the Sacramento and San Joaquin watersheds.
2.4	Analyze extent of feminization with other fish species	18	36	Spies, Robert! B Subcontractor for Sampling Services	Collect fish plasma samples and analyze for choriogenins.	1) Evaluation of the extent to which other fish species in the watershed are feminized.

General deliverables involving all tasks:

- Presentations at conferences, regional and national, to occur prior to the 36 month project end date. One of the presentation deliverables will be a joint presentation at the CALFED Science Conference that integrates and analyzes the results of this study with the results of the May study (UC Davis).
- Development of a web site on the project on the server for UC Berkeley's Department of Civil and Environmental Engineering linking to home pages for each of the participating investigators and their research groups;
- Semi-annual reports will be submitted every 6 months following the project start date;
- Final report will be submitted 36 months from the project start date.