Quality Assurance Project Plan

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Investigation of the Cause of Eared Grebe Mortality at the Salton Sea: Algal Blooms and Biotoxins

Principal Investigator: Wayne W. Carmichael
Institution: Wright State University, Dayton, Ohio 45435

Section A: PROJECT MANAGEMENT

A1 Project Title and Approval Sheet

Investigation of the Cause of Eared Grebe Mortality at the Salton Sea: Algal Blooms and Biotoxins

Prof. Wayne W. Carmichael
Department of Biological Sciences, Wright State university

Technical Project Manager	**Wayne W. Carmichael**	Date_____
Salton Sea Project Manager	Milton Friend	Date_____
Salton Sea QA Coordinator	Barry Gump	Date_____

A2 Table of Contents

A3Distribution List

Salton Sea Authority
Wayne Carmichael
Wright State University-Office of Research and Sponsored Programs

A4Project/Task Organization and Responsibilities **(organizational chart)**

The project is part of the second round of funded projects and follows up as well as compliments the study entitled “An Environmental Reconnaissance of the Salton Sea”. The project was initiated by the Salton Sea Research Management Committee and is under the direction of the Salton Sea Authority. The project reports through the study Project Officer to the Executive Director of the subcommittee.
This investigation of the cause of eared grebe mortality is a cooperative effort with individual researchers at the National Wildlife Health Center in Madison, WI, and the Salton Sea Authority. Tahni Johnson, wildlife disease specialist contracted through SSA to work on the Wildlife Disease Prevention Program, has been designated to monitor the Sea for major avian disease outbreaks, fish kills, and algal blooms. Tahni Johnson, patrolling the sea by airboat, will be collecting water, algae, and bird specimens from the Salton Sea. The samples will be refrigerated and shipped overnight in coolers with ice packs, provided by WSU or the SSA. Sample collection records and maps of the site locations, complied by Tahni Johnson, will accompany the samples, sealed in zip-lock bags. The sample correspondence will include the sample abbreviation, date, time, GPS coordinates, weather, water color, odor, and any other information of significance to the study. Eared grebe samples will be collected (dead or sick), euthanized if necessary, necropsied and dissected by Johnson and shipped frozen to Chris Franson at NWHC in Madison, WI, for assistance with dissection and further analysis. Selected samples will sent from these tissues to WSU or in some cases may be sent directly to WSU from the SS (T. Johnson). Primary responsibility for receiving samples at WSU is a Masters student working on the project (Jennifer Flynn) who logs and processes the samples. She reports directly to Wayne W. Carmichael the project PI.

The sampling regime may be modified at any time to include additional samples, should incidents of obvious or unusual algae bloom material or bird mortalities occur. Overall progress will be monitored by the Project Officer, Milton Friend.

A5Project Definition and Background:

Bloom and mat-forming cyanobacteria from fresh, brackish, and marine waters may produce a wide variety of toxins including hepatotoxins, neurotoxins and dermatatoxins. The most frequently found cyanobacterial toxins encountered worldwide are the hepatotoxic microcystins and nodularins, cyclic peptides consisting of seven or five amino acids respectively. To date 65 different structural variants are known that, depending on the specific chemical structure, vary in potency from highly toxic to non-toxic, with most being very toxic (Harada et al., 1999). Microcystins have been characterized from planktonic *Microcystis, Oscillatoria/Planktothrix, Anabaena, Nostoc, Anabaenopsis,* and certain picoplankton genera, whereas nodularin is produced by *Nodularia spumigena* (Domingos et al., 1999). Of the three families of cyanobacterial neurotoxins, anatoxin-a has been found in *Anabaena, Oscillatoria,*

Aphanizomenon, homoantoxin-a in *Oscillatoria*, anatoxin-a(S) in *Anabaena*, and saxitoxins from *Lyngbya, Aphanizomenon, Anabaena, Cylindrospermopsis* and *Planktothrix.* Toxins causing severe dermatitis among contacting swimmers may be produced by benthic cyanobacteria such as *Lyngbya, Oscillatoria* and *Schizothrix* in marine waters. Collectively, the cyanotoxins have been responsible for continued widespread poisoning of wild and domestic animals plus human fatalities. Preliminary observations have verified that several toxic algal species are present in the Salton Sea that may cause wildlife mortalities, especially in sublethally stressed animals, rendering them more susceptible to disease. Beginning in January 1999, organic solvent extracts of phytoplankton samples, containing prymnediophytes, coccolithophores, raphidophyceae, and dinoflagellates, taken from the top 50 –100 cm of the water column were tested for toxic activity in brine shrimp and mouse assays by researchers at San Diego State University. Although some blooms showed toxicity towards invertebrates and none were active in the mice, this limited study was not sufficient in ruling out toxic algae, particularly cyanobacteria, as a factor in major mortality events. Results from eared grebe tissues collected at the SS in 1992 -1994 identified the cyanotoxin, microcystin(s) produced by cyanobacteria, in high enough concentrations to cause acute toxicity Carmichael et al., unpublished data). Enzyme Linked Immunosorbent Assay (ELISA) performed with extracts from 25 grebe samples of liver, gizzard and upper gastrointestinal tract tissues, had measured levels of microcystin(s) as high as 700 ng/g, well above the known levels of microcystins that cause acute lethality of about 200 ng/g (Carmichael et al., unpublished data). Forty-nine Salton Sea water samples of phytoplankton provided by the US Fish and Wildlife Service in 1995 – 96, contained levels of microcystins that ranged from not detected to 2 ppb (Carmichael et al., unpublished data). Although these low levels are not likely to cause acute toxicity, the toxin was associated with a planktonic cell smaller than 5 microns, possibly *Synechococcus*. This background data forms the basis for the hypothesis of this project: **Microcystins contribute to the eared grebe mortalities on the Salton Sea and the source of the organisms producing the microcystins are to be found in the picoplankton**. The first objective of this study is to identify cyanobacteria algal species from Salton Sea water samples including any cyanotoxins produced from these species that may contribute to the unexplained deaths of eared grebes. Cyanobacteria suspected of microcystin-production will be isolated and cultured for verification of toxicity and characterization of toxic components. The possibility also exists for examination of cyanotoxins other than microcystins that may be produced by certain cyanobacteria if specific producers are identified, ie. Anatoxin-a from *Oscillatoria* or PSPs from *Lyngbya*. The second objective is to determine the presence or distribution of toxins in selected eared grebes, specifically from tissue samples subject to microcystin concentration and effect. Monitoring will include the major inflows, including the New, Alamo, and Whitewater Rivers, and areas where the grebes are observed dying or exhibiting excessive drinking and preening behaviors. Control tissues from unexposed grebes from another area similar in ecological and physical conditions, possibly Mono Lake in California where no episodes of grebe mortality have been observed, are being sought through connections to other water resource monitoring organizations. These results will help to characterize the temporal and spatial distribution of algal species assemblages and abundances, with an emphasis on potentially toxic cyanobacterial species. These results will also contribute information to the U. S. Fish and Wildlife Service's goals for the Salton Sea: developing an understanding of the Salton Sea ecosystem and the factors driving the avian die-offs; cataloging known and potentially toxic algal species; suggesting recommendations on methods for interrupting these mortalities and the controlling harmful algal

blooms to minimize disease and potential risks to human and wildlife health; and developing methodology for ecosystem management for maximum sustainable biological and economic resources.

A6Project/Task Description: Throughout the year and especially during grebe winter residence from December to April, Tahni Johnson will conduct regular, periodic estimates of avian populations and their distribution on the SS. Sick grebes will be identified by observing signs of abnormal behavior such as excessive preening, exhibiting "drinking behavior", gathering at sources of freshwater and coming out of the water onto the shore. The locations of aggregates of birds and sampling sites will be identified by Global Positioning System (GPS) latitude and longitude measurements. Eared grebes that allow approach will be collected (dead or sick), euthanized if necessary, necropsied and dissected by Johnson or shipped frozen to Chris Franson at NWHC in Madison, WI, for assistance with dissection and further analysis. Frozen liver, gizzard, stomach contents, and intestines (1-2 grams or as much as possible) from 10 – 20 % of the total number of grebes sampled, including sick, dead, and control birds, will be shipped overnight to WSU for toxin analysis. Chris Franson, USGS National Wildlife Health Center, with assistance from Johnson, will conduct clinical and epidemiological investigations, including avian cholera, botulism, and serum chemistries of the grebes. Reports on their findings, including information on pulmonary, hepatic, and skin lesions observed during necropsies will accompany grebe tissues sent to WSU. All carcasses and grebe samples will be identified with the code 4586 followed by a sequential three digit accession number (i.e. 4586-001, 4586-002, etc.). Water and algae samples will be sampled bi-monthly along designated transects, 13 total, including four sites around the Whitewater River, four sites around the New River delta, three sites around the Alamo River, and two open water sites, at north and south ends of the Sea. The estimated time required for collecting all samples is two days.

Three surface water grab samples will be taken from the side of the air-boat, from the beginning, middle, and end of each transect and collected into 500-ml Nalgene bottles, supplied by WSU. A nylon plankton net and nylon rope (Carolina Scientific 15", ten mesh, 153 mm porosity) supplied by WSU will be used to collect plankton samples by towing for 2 –3 minutes at low speed from the airboat for each transect. Collected plankton tow samples will be transferred to 250-ml Nalgene bottles. The plankton net will collect larger algae and bloom material, while the grab samples will be used to collect smaller nannoplankton that might pass through the net. At the river openings near shoreline areas, the sediment surface layer will be sampled for benthic mat-forming cyanobacteria by scraping the sediment surface with a large metal spoon and placing the material into a 60-ml Nalgene bottle. All sample bottles will be labeled with an alphabetical code for the site area (Alamo = A, New = N, Whitewater = W, open = O), the transect number (1 – 4), location along transect (beginning = B, middle = M, and end = E), and an alphabetical code for the sample type (grab = G, plankton tow = T, sediment = S).

PROJECT TASK LIST AND TIMELINE

Task No.	Task	Responsibility	Start Date	Completion Date
1	Collect phytoplankton	Tahni Johnson	5/99	5/00

2	Collect grebe samples	Tahni Johnson & Chris Franson	Winter 99	Winter 2001
3	Process phytoplankton and grebe tissue	Jennifer Flynn & Wayne Carmichael	Winter 99	Winter 2001
4	Analyse for cyanotoxins	Jennifer Flynn & Wayne Carmichael	Spring 2000	Spring 2001

A7Quality Objectives and Criteria for Measurement Data

Data to be collected:

Task 1 &2 --No data will be generated for this part except a listing of samples and locations

Task 3 and 4--Culture of isolated cyanobacteria followed by analyses for cyanotoxins; extraction of tissues and analyses for cyanotoxins

Conditions under which data are to be collected:

Samples are received-logged in and examined microscopically for taxonomy.

Samples are filtered and processed for cyanotoxin analyses

Cultures are made and maintained in environmentally controlled rooms with necessary microbiological methods.

Cyanotoxin testing is done by a microcystin screen (E:LISA) followed by microcsytin analyses using HPLC and LC/MS.

DATA COMPLETENESS

Based upon initial tests in 1993 we will focus on the detection, quantitation and identification of microcystins in both phytoplankton and grebe liver/gut. We expect to be able to do these tasks with a good degree of completeness as we have enough experience to accomplish these goals. Other possible cyanotoxins such as neurotoxins will be selectively looked for but it may not be possible to completely identify these toxins.

Data Representativeness:

Sampling points, times and protocol have been set by the personnel sampling at the SS wildlife area. Tahni Johnson is responsible for this. Transects have been established and set using a GPS. Sample points along the transects have been set by our lab to represent the area appropriately. Sampling dates are designed to represent all seasons and possible conditions.

Tahni Johnson will also respond to event driven situations such as observed phytoplankton blooms or avian mortalities not associated with normal sampling locations.
Data Comparability:

A8Special Training Requirements/Certification

Jennifer Flynn is an M.Sc. student completing her degree on the project topic. She is trained in the detection and analyses of microcystins which is the focus of her thesis. Prof Carmichael has 30 years experience in the study of cyanotoxins and has written over 100 papers and book chapters on the topic. No special certifications are required.

A9Documentation and Records

All samples coming into the laboratory are logged into a notebook and stored in a designated location. The log-in data is also filed onto a PC data base. An action sheet is generated once the sample has been examined. This action sheet includes sample identification, taxonomy, processing and analyses. From the action sheets a data base of results and comments is generated. This file is kept both in PC and Apple format and stored on the PI 's computer and on the students computer. Summaries of this data base are filed with each progress report and will be put into the final report.

Section B: MEASUREMENT/DATA ACQUISITION

B1 Sampling Process Design (Experimental Design)

Sampling Design, Assumptions and Rationale:

Tahni Johnson, Salton Sea Wildlife Authority, is responsible for all sampling subject to certain input from the PI's laboratory. Chris Franson, Wildlife Disease Center, is responsible for most necropsy and shipment of tissue samples to the PI's laboratory. Isolation and culture of representative cyanobacteria from field water samples is the responsibility of Jennifer Flynn and Wayne Carmichael. Isolation and analyses of cyanotoxins is also their responsibility. Methods chosen for cyanobacteria culture are those that are published and have been in use in the PI's laboratory for 25 years. Isolation and testing or cyanotoxins are based upon literature methods and upon methods developed in the PI's laboratory for the SS matrix (i.e. saline to hypersaline plus grebe tissues). These methods are in use and will be published following completion of the current project.

Procedures for Locating and Selecting Environmental Samples: NA
Schedule for Project Sampling Activities: See A6

Classification of Measurements as Critical or Noncritical: NA

Validation of Any Nonstandard Methods: NA

B2 Sampling Method Requirements

Sampling Methods:

Types of Samples to be Collected: Phytoplankton grab and net samples, grebe liver and gut (necropsies sent frozen)

Sampling Method's Requirements:

Decontamination Procedures and Materials:

Autoclave residual water samples, disposal of residual tissue samples through the University Laboratory Animal Services (incineration), chemical oxidation (10% chlorine) of any cyanotoxin residuals.

Sampling System Failure Response and Corrective Action Process:

Sample volumes allow for replicate runs and SS sampling plan allows for resampling if needed.

B3 Sample Handling and Custody Requirements

Provide a list of measurement parameters and appropriate details for samples that will be collected and analyzed during the project. Additional rows can be added to the table using the "Table" pull-down menu on the toolbar.

PARAMETER TABLE

Parameter	Number of Samples	Matrix	Sample Preservation	Holding Time
Phytoplankton	50 (est)	Water, sediment	4 ^{0} C, -80^0 C, lyophilization	Project duration
Tissues	50 (est)	Liver, gut	-80^0 C	Project Duration

Custody Procedures
All samples are logged into a data base and location numbers assigned. Samples are logged out and original sample container initialed as to user. All samples are treated as hazardous in terms of handling and processing.

B4 Analytical Methods Requirements

Detection Limits and Goals for Precision and Accuracy

Method		**Toxin Type**	**Detection Limit**	**Goal for Precision % RSD**	**Goal for Accuracy % REC**

ELISA	SW, Tissue	Microcystin, nodularin	0.5 ppb[1]	< 15[1]	> 85[1]
PPIA	SW, Tissue	Microcystin, nodularin	1 ppb[2]	< 15[3]	> 85[4]
AA	SW, Tissue	Anatoxin-a(s)	1 ppb[5]	NA	NA
HPLC-MS	SW, Tissue	Cylindrospermopsin	0.2 ppb[6]	NA	NA
HPLC-FI	SW, Tissue	Anatoxin-a, saxitoxin, neosaxitoxin	Antx-a ppt STX ppt	Antx-a 2 ng NA[7]	Antx-a (NA) NA

[1] Chu et al. 1990
[2] An and Carmichael 1994
[3] PPIA %RSD based upon average of four replicates
[4] PPIA %REC based upon average of three replicates
[5] Antx-a(s) detection limit based upon standard curve using HPLC purified extract from Anabaena flos-aquae strain 525-17 Mahmood and Carmichael 1987; %RSD and REC not yet determined.
[6] CYL - To date only a GC/MS method is available for this toxin Eaglesham et al. 1999 . An HPLC method is being developed and can be applied to this toxin if appropriate.
[7] Antx-a detection limit from James et al. 1998, %RSD and REC not available- Will be developed using antx-a (standard synthetic toxin from Biometrics, Minnesota and toxic algae) to spike matrix samples and then determine % recovery; STX and other PSP's detection limit from Oshima 1995, %RSD and REC have not been determined in this laboratory but exist for other laboratories i.e. Oshima-Japan.

LC/ESI-MS Conditions:

Column: MetaChem Monochrom C18, 2 x 50 mm, 5 micron particle size
Mobile Phase: A) 0.1% formic acid in water B) 0.1% formic in acetonitrile
Gradient: 25 % B to 50% B in 5 minutes, with first minute diverted to waste
(Purge 20 column volumes with 50% B at end of run; equilibrate with 20 column volumes prior to run)
Temperature: 35 deg C (column heater to stabilize temperature)
Flow: 0.25 mL/min
Injection Volume: 20 micro liters
Selected Reaction Monitoring (MS/MS) Scan Experiments (minimum 3 replicates)
Limit of Detection: 300-500 picograms (on column)
Limit of Quantification: 0.5-1.0 nanograms (on column); high ppm (ng/g - ug/g) for samples; precision $\leq$ 15% (trace analysis for tissue samples); $\leq$ 5% for algae samples
Extraction Efficiency (SPE sample prep): 90%
Ionization Suppression from Tissue Matrix: 35-40% suppression in signal response
Combined reduction in recovery/response: 50%
Expected Weight of Tissue Samples: 1-2 grams
Baseline resolution of analytes, RT repeatability +/- 0.5%

B5 Quality Control Requirements

QC Procedures: LC/MS spectra are calibrated when using APCI/ESI using a calibration mix of caffeine, MRFA and Ultramark 1621per manufacturer instructions.

Field QC checks:NA

Laboratory QC checks: ELISA uses standards for internal checks. LC/MS is tuned for each run against a standard. Any deviations from normal performance are reported to the PI.

B6 Instrument/Equipment Testing, Inspection, and Maintenance Requirements

The ELISA plate reader is maintenanced per manufacturer recommendations for operation and calibration. The LC/MS is under warranty for the period of the contract. Service is performed per manufacturer instructions.

B7 Instrument Calibration and Frequency

ELISA plate reader uses an internal calibration for wavelength that is set with every run. LC/MS is tuned for each run and all spectra are run against standard cyanotoxins.

B8Inspection/Acceptance Requirements for Supplies and Consumables

WSU's Department of Environmental Health and Safety track all solvents and chemical wastes. A lab data base is maintained for all chemicals. Glassware is washed per a method for cleaning and decontamination of trace cyanotoxins and other organics.

B9 Data Acquisition Requirements (Non-direct Measurements)

The PI maintains an ongoing literature data base (through ISI) on cyanotoxins that numbers over 2500 references and is increased by 25-50 new references each month. We purify and maintain our own standards of all cyanotoxins except anatoxin-a (commercially purchased-Calbiochem) and cylindrospermopsin (purified and provided by Dr Glen Shaw, NRCET, Queensland, Australia).

B10 Data Management

Field collection data is handled by Tahni Johnson (SS Wildlife Refuge) who maintains the data base and provides voucher information for all samples. At WSU, graduate student, Jennifer Flynn logs in samples and maintains all data per the project. Summaries are provided to the PI who reports all progress to the SS Authority.

Section C: ASSESSMENT/OVERSIGHT

C1Assessments and Response Actions

The PI-Wayne Carmichael reviews all sample report sheets from the SS and advises the student on how they are to be handled. Any suspect data is discussed and suggestions made for a repeat or reexamination.

C2Reports to Management.

Section D: DATA VALIDATION AND USABILITY

D1 Data Review, Validation, and Verification Requirements

Tahni Johnson (SS) and Chris Franson at NWHC in Madison, WI, provide the sampling locations, details and accession numbers for water and bird samples. Reports on their findings, include bimonthly status reports on weather, temperature, waterbloom conditions, bird locations and mortality summaries. Information on bird tissues include pulmonary, hepatic, and skin lesions observed during necropsies plus other important inforamtion that accompanies grebe tissues sent to WSU. All carcasses and grebe samples are identified with the code 4586 followed by a sequential three digit accession number (i.e. 4586-001, 4586-002, etc.). Water and algae samples are sampled bi-monthly along designated transects, 13 total, including four sites around the Whitewater River, four sites around the New River delta, three sites around the Alamo River, and two open water sites, at north and south ends of the Sea. Three surface water grab samples are taken from the side of the air-boat, from the beginning, middle, and end of each transect and collected into 500-ml Nalgene bottles, supplied by WSU. A nylon plankton net and nylon rope (Carolina Scientific 15", ten mesh, 153 mm porosity) supplied by WSU is used to collect plankton samples by towing for 2 –3 minutes at low speed from the airboat for each transect. Collected plankton tow samples are transferred to 250-ml Nalgene bottles. The plankton net collects larger algae and bloom material, while the grab samples are used to collect smaller picoplankton that might pass through the net. At the river openings near shoreline areas, the sediment surface layer is sampled for benthic mat-forming cyanobacteria by scraping the sediment surface with a large metal spoon and placing the material into a 60-ml Nalgene bottle. All sample bottles are labeled with an alphabetical code for the site area (Alamo = A, New = N, Whitewater = W, open = O), the transect number (1–4), location along transect (beginning = B, middle = M, and end = E), and an alphabetical code for the sample type (grab = G, plankton tow = T, sediment = S).

Upon receipt of each sample it is logged into our WSU data base. It is then visually examined (plankton samples are identified by microscopy) and a decision is made on how to process and analyze the sample. Plankton samples are cleaned, filtered and freeze dried for microcystin analyses. Identification of microcystins is first by ELISA (as a screen) and then by LC/MS. Our LC/MS data base includes standard operating procedures (SOP) for all of the major and minor microcystin compounds. All results are reviewed by the PI and student and decisions are made as to acceptability of the data. Sample vouchers are maintained for repeat analyses-if needed.

D2 Validation and Verification Methods,

A quantitative estimate of microcystin concentration in any given sample is first done by ELISA. This value is used as a guide to further LC/MS analyses of the sample. Initially only a qualitative analyses by LC/MS is needed. This can be upgraded to a quantitative estimate of individual microcystins using an SOP that is developed for individual microcsytins as compared with the appropriate standard.

D3Reconciliation with Data Quality Objectives

Results are always reviewed and summarized in the context of the overall goal to determine whether cyanotoxins (especially microcsytins) are present in the Salton Sea and could contribute to bird mortality.