

Title: Cyanobacteria and their Toxicity to Humans, Wildlife, and Fish in the Salt River Reservoirs Arizona.

Project Summary

Beginning in March of 2004, fish kills occurred in the Salt River Reservoirs presumably as a result of nutrients brought in via the Rodeo-Chedeski Fire. Etiology of the kills implicated anatoxin-a, however, little of this toxin was found in the water even though it was found at toxic levels in fish stomachs. The half-life of anatoxin-a may only be a few hours. Other algal toxins such as, microcystin and cylindrospermopsin may play a role in these events. This proposal will isolate causative organism(s) so that early warning systems can be developed to protect humans and wildlife in these reservoirs.

Principal Investigators

Dr. William Matter (PI). SNR. wmatter@ag.arizona.edu
(520) 621-7280.

Dr. David Walker (co-PI, project contact). SNR/SWES/ERL. dwalker@ag.arizona.edu
(520) 626-2386

Dr. Fiona Jordan (co-PI). SWES/ERL. fiona@ag.arizona.edu.

Dr. Paul Zimba (co-PI). U.S.D.A.-A.R.S. (662) 686-3588. pzimba@msa-stoneville.ars.usda.gov.

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Matching Funds: Az.G&F: \$3000 (cash contribution) and \$4500 (in-kind lab analyses and labor). ADEQ: \$7500 (in-kind lab analyses and labor). USDA: \$4200 (in-kind, reduced lab fees and consultation with Dr. Zimba). Salt River Project: \$2000 (in-kind, labor and consultation)

Critical Regional Water Problem: The reservoirs in question are used as a vitally important drinking water source for the most populated area of the state e.g., the Phoenix Metro Region. They also receive exceptionally heavy recreational use. Additionally, they provide a critical water source for many species of wildlife both terrestrial and aquatic. Toxic events in these reservoirs affect each multiple use to varying degrees. Fisheries are certainly affected as large fish kills prompt concern from fishermen, recreationists, and municipalities and rightfully so. While certain toxins (e.g. anatoxin-a) are very short-lived and may not affect drinking water supplies farther downstream, they are very potent toxins and have been attributed to human deaths. Recreationists are especially prone to these toxins if they happen to be in the wrong place at the wrong time. Other algal toxins (e.g. microcystin, cylindrospermopsin, saxitoxin) are very environmentally persistent and may not only have locally toxic effects, but municipalities and treatment plants farther downstream. This problem affects regulatory and resource management agencies such as ADEQ, AzG&F, US F&WS, SRP, as well as municipalities receiving this water (cities of Phoenix, Scottsdale, Tempe, Mesa, Gilbert, Chandler, Peoria, Goodyear, and Glendale Arizona).

Results and Benefits: The data from this project will be used to identify causative organisms of toxin production in the Salt River reservoirs, as well as environmental conditions conducive for their growth. With this information, we can recommend management strategies to regulatory and water delivery agencies to alleviate the problem. Results from this project may also be used to develop an early warning system of toxin production.

Nature, Scope, and Objectives

Introduction to Cyanobacterial Toxins

Several species of algae can, under certain environmental conditions, produce novel compounds that exhibit potent biological activities generally considered to be secondary metabolites. Secondary metabolites are compounds which are not essential to the metabolism and growth of an organism and which are present only in certain taxonomic groups. Biosynthesis of secondary metabolites is common in bacteria as well as eukaryotic microbes and plants. Secondary metabolites, and their roles in the life history of the producing organism, are much debated. Many are considered to be chemical defenses which enable a competitive advantage over other species occupying a similar ecological niche (i.e. allelopathy) or they could discourage predation by higher trophic level organisms (i.e. zooplankters). Others may serve roles in chemical signaling while others are believed to be evolutionary relics. Secondary metabolites require elaborate biosynthetic pathways for their synthesis.

Many secondary metabolites are potent toxins responsible for a wide array of human illnesses, mammal and bird mortality, and extensive fish kills. Toxins of human health significance originate primarily from three classes of unicellular algae: dinoflagellates, diatoms, and cyanobacteria. Only a few of the dinoflagellates, out of the several thousands known, are toxigenic. Within the diatoms, only a single species, *Pseudo-nitzschia* produces a toxin impacting human health. Selected strains and species of all common cyanobacteria, however, have been proven to produce toxins.

Cyanobacterial toxins occur on a world-wide basis, over forty freshwater species have been implicated in toxic events (Carmichael, 1997), and are primarily a freshwater issue involving either hepatotoxins or neurotoxins. Anatoxin-a (Fig. 1), the most potent agonist of the nicotinic acetylcholine receptor found to date, is produced by certain strains of *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, (Fig. 2) and *Oscillatoria*, all species found in the Salt River reservoirs. Anatoxin-a causes a depolarizing muscular blockade. In the presence of anatoxin-a, cleaving by acetylcholinesterase can not take place thus causing the sodium channel to lock open, overburdening the muscle which eventually tires out with paralysis dyspnea, cyanosis, cardiac arrhythmia and eventual death soon to follow. Symptoms are very similar to organophosphate nerve agent poisoning and there is no known antidote. The LD₅₀ in mice (i.p.) is 200 µg/kg. Anatoxin-a is also known as Very Fast Death Factor with the irreversible onset of symptoms occurring in as little as 3-4 minutes.

Microcystin (Fig. 3), a potent hepatotoxin, are produced by certain species, and strains within these species, belonging to the genera *Microcystis* (Fig. 4), *Anabaena* (Fig. 5), *Nodularia*, *Nostoc*, and *Oscillatoria* (Fig. 6). There are at least 52 analogues of microcystin (Carmichael, 1997). Microcystin's LD₅₀ is (i.p. mouse) 50µg/kg and toxicity is initiated by depolymerization of the actin and microtubule cytoskeleton resulting in intrahepatic hemorrhage within hours and death induced by hypovolemic shock (Jochimson, 1998). Microcystins were found to be the causative agent in a 1996 outbreak in Caruaru Brazil in which 26 patients died of liver failure at a dialysis clinic (Jochimson, 1998). The clinic withdrew its water from a reservoir with an active bloom of *Microcystis*. Microcystins have also been proven to promote liver tumors in laboratory animals (Nishiwaki-Matsushima et al, 1992). Epidemiological studies in China show positive correlation between the presence of microcystin in water supplies and the incidence of human primary liver cancer (Yu et al, 1989). Both acute and chronic exposure to cyanobacterial hepatotoxins may pose significant human health risks.

In 2001, the PI's found *Cylindrospermopsis raciborskii* (Fig. 7), a recent non-native invader of the Western U.S., in Roosevelt reservoir. It has also been found in Florida lakes and the toxin it produces, cylindrospermopsin (Fig. 8), was attributed to alligator and fish die offs. Our discovery in 2001 is the first documented incidence in the state and one of only a few in the Western U.S. We notified state regulatory agencies with our finding and predicted that this species would probably spread to other areas of the state and would increase in number. Since that time, our predictions have become reality and *C. raciborskii* is now found in several lentic habitats throughout the state including all the major reservoirs surrounding the greater Phoenix

metro area. *C. raciborskii* is a relatively small species of cyanobacteria usually with a trichome width of only 2-3 microns (Fig. 7). They usually have a terminal akinete and occur in both a curled and straight morphology. Both morphologies have been found in Arizona often occurring together. It can dominate the phytoplankton numerically reaching hundreds of thousands of cells/mL. Unlike other potentially toxic species of cyanobacteria, *C. raciborskii* is reported to produce some level of toxin continuously. Chorus and Bartram (1999) estimated that a single *C. raciborskii* cell contains between 0.0041 and 0.0026 picograms of cylindrospermopsin which is equal to 1.5 to 5.5 mg of toxin/g of dried cells. Cylindrospermopsin is a hepatotoxin and a sulfate ester of a tricyclic guanidine substituted with a hydroxymethyluracil. It is becoming increasingly problematic in drinking water reservoirs. Cylindrospermopsins' main target is the liver but it can also affect other organs and extrahepatic lesions may occur in the heart, thymus, or kidney (Chorus and Bartram, 1999). Cylindrospermopsin causes intra-hepatocytosis by inhibiting the synthesis of protein and glutathione. It is a very potent hepatotoxin with an LD₅₀ (i.p. mouse) ranging from 20µg/kg over a 24 hour period to 2µg/kg over 5 days.

In 1979 in Palm Island Queensland, 149 persons were poisoned, mostly children, several of whom required hospitalization. The poisoning occurred after victims drank water from Solomon Dam following a copper sulfate treatment to try and eradicate a persistent bloom of cylindrospermopsis.

Cyanobacteria, Toxins, and Recent Fish Kills in the Salt River Reservoirs

The authors have been investigating water quality in all of the reservoirs surrounding the Phoenix Metro area since 1996 including phytoplankton dynamics and routine, systematic monitoring of microcystin, cylindrospermopsin, anatoxin-a, and saxitoxin. The authors have compiled the most comprehensive biological and water quality dataset within these reservoirs to date. Reservoirs routinely monitored include Roosevelt, Apache, Canyon, Saguaro, Bartlett, and Pleasant as well as river and/or canal sites above and below these reservoirs (Fig. 9, 10, and 11).

The Rodeo-Chedeski fire (Fig. 12) was a massive disturbance to the Salt River watershed which feeds the Salt River reservoirs. Large amounts of suspended sediment and ash, with attached algal nutrients, have been and are continuing to be washed into Roosevelt and downstream reservoirs during storm events. This is likely to be the scenario for years to come and it has resulted in punctuated eutrophication within these reservoirs. Autochthonous processes within the reservoirs may mean eutrophication proceeds unabated long after nutrient loading via the Salt River has diminished. This is not to say that these reservoirs were not already very productive pre-fire. The PI's are the only persons with continuous data from both pre- and post-fire for the Salt River and downstream reservoirs.

During August of 2001 in the up-reservoir reaches of Saguaro, there was a sudden die-off of large amounts of *Corbicula fluminea*, a non-native asiatic clam. We sampled the water and tissue of corbicula for various toxins for both AzG&F and ADEQ. Samples were submitted to Dr. Gregory Boyer at SUNY-CESF in Syracuse. At that time, we found levels of anatoxin-a at 120-140µg/L. These were the highest levels ever recorded by the reporting lab. Australia has an advisory limit for anatoxin-a of 3 µg/L.

Beginning in March of 2004, tens of thousands of fish began to die in the up-reservoir reaches of Apache. This constituted a major fish kill and involved multiple species. Suites of physico-chemical and synthetic organic samples revealed nothing out of the ordinary. Dissolved oxygen levels were more than adequate to sustain aquatic life. Algal biomass was only moderate. Fish kills continued to plague both Apache and Canyon, the next reservoir downstream, for the next few months. The PI's assisted state agencies (ADEQ, AzG&F, ADHS, SRP, Tonto National Forest) by collecting samples for toxin analyses and algae identification and enumeration, serving as the scientific lead and helping with public advisories.

On June 10th 2004, there was a massive fish kill in the upper reaches of Saguaro reservoir (Fig. 13 and 14) in the same area where large amounts of anatoxin-a were found in 2001. This event resulted in the death of a tremendous amount of fish of many different species ranging in size from threadfin shad (tens of grams) to very large bass, catfish, and carp weighing more than 5 kilograms. This event received much media attention and alarmed many anglers and

other recreationists. We were sampling on Canyon reservoir on June 9th, the day prior to this event. There were several moribund and dead threadfin shad on Canyon that day.

All aqueous samples that we took from Saguaro came back as non-detectable, however, fish stomach samples submitted from the fish kills showed toxic levels of anatoxin-a (Fig. 15). The short half-life of this toxin makes it extraordinarily difficult to detect in environmental samples as it is quickly degraded by sunlight and alkaline conditions; conditions not lacking on the Salt River reservoirs in June. Anatoxin-a may be best preserved in fish stomachs and fish may be important bioindicators of past toxic events. The fact that we found such high levels of anatoxin-a in this area in 2001 is probably due to very fortuitous timing. The problem of trying to obtain environmental samples of anatoxin-a is common. In 1999 and 2000, several dogs died after ingesting anatoxin-a in Lake Champlain. Toxicological results in all dogs proved the cause of death as anatoxin-a poisoning yet, over 500 samples taken within days after the event showed no detectable levels of anatoxin-a in the water (pers. comm. Dr. Greg Boyer, SUNY-CESF). Anatoxin-a is a fast-acting neurotoxin that often leaves nothing in its wake, besides dead animals.

In July of 2002, the first human death attributed to anatoxin-a toxicity occurred in Dane County Wisconsin. A 17 year old male went into shock and suffered a massive seizure before his heart acutely stopped just two days after swimming in a golf course pond where there was an active bloom of *Anabaena flos-aquae*. Another young male teen, who survived after swimming in the same pond, had severe abdominal cramping and diarrhea. Blood and stool samples from both teens showed the presence of the suspected causative organism, *Anabaena flos-aquae*, and toxic levels of anatoxin-a. Very similar to what we have found in the Salt River reservoirs, no anatoxin-a was found in aqueous samples.

Our proposed objectives in this study are to:

- 1) Collect, isolate, and purify axenically suspect algal toxin producers from the Salt River reservoirs.
- 2) Quantify which specific species, or strains, produce what type of algal toxin.
- 3) Identify toxin producers using molecular techniques.

Approach, Methods, Procedures, and Facilities

We intend to isolate and purify axenically suspect algal toxin producers from the Salt River reservoirs. We will sample from established sampling areas, as well as other areas suspect of harboring potentially toxic species of algae, within Roosevelt, Apache, Canyon, and Saguaro reservoirs. We will sample whole water samples from within the photic zone of each reservoir. Depth samples will be obtained using a weight-activated 4-liter Beta bottle. We will also concentrate planktonic samples using an 80 μ m Wisconsin-style zooplankton net fitted with a reducing cone and flowmeter so we can quantify volume passed through the net during any given tow. This will enable us to calculate number of cells/liter of water. Samples will be kept at 4°C until processing at the Environmental Research Laboratory the following day.

In order to account for successional changes in the phytoplankton community, we will sample seasonally for one year beginning during the summer of 2005. During this time, we will also be monitoring the water for algal toxins and will continue to work closely with state and federal regulatory agencies and utilities, (ADEQ, ADHS, AzG&F, SRP, US F&WS), all of which provide invaluable in-kind support.

Axenic purification of collected samples will follow protocols established by Sivonen et al (1990) and Vaara et al (1979). Briefly, the water samples are serially diluted onto selective BG-11 or Z8 media with and without nitrogen, enriched in the presence of cycloheximide (50 mg/L) to inhibit fungal growth and incubated under illumination. After enrichment, the culture will be transferred to a BG-11 or Z8 agar medium that has been scored. The separation of cyanobacteria from their adhering bacterial contaminants is facilitated, due to their gliding motility, by scoring the agar medium (Varra et al., 1979). Additionally, in order to obtain axenic cultures, we will enrich the culture in the dark for 48 h and add cycloserine (1 mg/ml) to kill any and all residual bacterial contaminants. Once separated from contaminating microorganisms, the cyanobacteria can be easily transferred and subcultured on fresh agar plates or liquid media and maintained axenically.

It may be necessary to micromanipulate the samples to physically separate and isolate colonies, filaments, or bundles of filaments belonging to conspicuous cyanobacterial morphotypes and culture axenically. Purified isolates will be identified morphologically by microscopy and verified for unialgal content. Once it has been determined that the cells have been isolated axenically, we will distinguish the uniqueness of each isolate by performing REP-PCR techniques like those described by Lyra et al. (2001). Those having identical REP-PCR fingerprints and similar morphology will be grouped together and a single representative isolate will be chosen for characterization with respect to toxin production. Lyophilized cultures of each unique isolate will be sent to the laboratory of Dr. Paul Zimba for pigment and toxin analyses. Additional tests may be conducted to verify toxin production in response to environmental stimuli such as photoperiod, temperature, grazing pressure (by zooplankton), varying nutrient levels, and introduction of other algal species to check for any allelopathic mechanism.

Toxin and pigment analysis will be performed by Dr. Paul Zimba of the USDA. For pigments, subsamples will be analyzed for chlorophyll and carotenoid content using HPLC methodologies (Zimba et al. 2003). Filter retained cells (GF/C or GF/F filter) will be extracted in 100% acetone at -4C for 8 hours. Pigment samples will then be filtered through 0.7µm porosity filters, ampulated and analyzed using a HP1100 HPLC system equipped with diode array and fluorescence detectors. Pigments will be identified using spectral libraries derived from standards, linear regression relationships of pigment concentration and peak area will be used to quantify pigments.

Microcystin content of field and culture samples will be assessed using HPLC/MS methodologies based on World Health Organization guidelines (Bartram and Chorus 1999). Filter retained water samples will be extracted in 70% MeOH, following sonication. After 4 hrs extraction, samples will be filtered using 0.7 µm porosity filters (Whatman, NJ), ampulated and analyzed using an HPLC system equipped with a mass spectrometer in addition to diode array and fluorescence detectors. Toxins will be identified using spectral libraries developed for the commercially available microcystin variants, and by using fragmentation patterns of unknown microcystins for identification (Zimba et al. 2002).

Anatoxin-a will be assessed using filter retained samples which will be extracted using 70% MeOH following sonication. After 4 hrs extraction, samples will be filtered using 0.7 µm porosity filters. Samples will be reacted with a fluorescent dye as part of a precolumn derivitization step, ampulated and analyzed using an HPLC system equipped with a mass spectrometer in addition to a fluorescence detector. Confirmation of anatoxin presence will include sample analyses without derivitization to identify presence of the 164 AMU toxin.

Cylindrospermopsin will be detected using standard HPLC methodology. Samples will be extracted in 20% MeOH, with diode array detection of the toxin using a Dionex Summit HPLC system. Confirmation of cylindrospermopsin will include mass spectrometry of positive samples using commercially available standards.

Bioactive peptides include a number of related compounds shown to have cytotoxic or neurotoxic properties (Harada 2004). Samples will be extracted in 5% acetic acid and analyzed by diode array detection on an HPLC system. Analytical standards have been requested from Dr. Harada's laboratory and mass spectrometry will be used for collaborative analyses.

Initially, toxin production will be determined for individual isolates cultivated in laboratory media. Second, toxin production will be determined for individual isolates (only those that scored positive in laboratory media) exposed to different environmental stimuli. For instance, toxin production will be verified for isolates cultivated in filtered (0.2 µm) reservoir water at ambient conditions. These isolates will also be cultivated in filtered water under varying environmental conditions such as changes in pH, TOC, light and temperature. Toxin content will be determined by lypholysis of the aqueous solution, with and without bacteria for a total of 3 samples each: 1) A subsample containing suspended cells and media. 2) The decanted liquid media/reservoir water after centrifugation (3000 X g) to pellet cells. 3) The cell pellet of cyanobacteria/isolate collected after centrifugation.

Molecular Characterization

DNA to be amplified in the REP-PCR reaction will be extracted from each isolate according to the following procedure: Extraction of chromosomal DNA is achieved by pelleting a 50 ml culture of cells. The pellet is resuspended in TE buffer, 1% SDS and 10 ug/ml proteinase K and incubated for 1 hr at 37°C to lyse the cells. This solution is mixed and centrifuged twice with an equal volume of phenol/chloroform in a Phase Lock Gel™ tube, respectively. Upon transferring the aqueous phase to a new tube, a 1/10 volume of sodium acetate and 0.6 volume of isopropanol are added to precipitate the DNA. The DNA is pelleted and washed with 70 % ethanol, dried, resuspended in TE buffer and quantified. Each 25 ul REP-PCR reaction contains 0.5 uM concentration of each primer (Versalovic et al., 1994), 25 mM concentration of each deoxynucleoside triphosphate (dNTP), 1X buffer consisting of 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂ (pH 8.9), 5.0% dimethyl sulfoxide (DMSO), 2.5 U of *Taq* DNA polymerase (Roche, Indianapolis, Ind.), and 30 ng purified and extracted DNA from each individual isolate. The PCR program consists of a 95°C for 5 min cycle, followed by 35 cycles of 95°C for 0.5 min, 45°C for 0.5 min, a single cycle of 72°C for 4 min and a single final extension step consisting of 72°C for 16 min. The fingerprints generated for each isolate are then visualized after electrophoresis on a 3.0% agarose gel (NuSieve 3:1 agarose; FMC BioProducts, Rockland, Maine).

Related Research

A number of studies have attempted to characterize the common cyanobacteria *Anabaena*, *Aphanizomenon*, *Microcystis* and other known algal toxin producers using molecular techniques but with varied success (Sivonen, 1990; Sivonen et al., 1992; Rouhiainen et al., 1995; Lyra et al., 1997; Beltran and Neilan, 2000; Lyra et al., 2001). For instance, Lyra et al. (Lyra et al., 1997; Lyra et al., 2001) found that neither the physiological (toxicity) characteristics nor the geographic origin of the microrganisms could be discerned using 16 S rRNA gene sequence similarities. More specifically, there was no difference in the 16 S rRNA gene sequences between the planktic, anatoxin-a producing *Anabaena* and the non-toxin producing *Aphanizomenon* strain. Similarly, Beltran and Neilan (2000) found no correlation between toxin production and 16 S rRNA gene sequences in *Microcystis* strains, however, using other molecular techniques, like REP-(repetitive extragenic palindromic) and ERIC-(enterobacterial repetitive intergenic consensus) PCR, Lyra et al. (Lyra et al., 2001) were able to differentiate between toxic and non-toxic strains (Fig. 16). Similarly, we propose to use REP-PCR to differentiate between cyanobacterial isolates and correlate this information with toxin production.

Training Potential

We estimate this project can support at least one, 0.5 time graduate research assistant and one 0.25 time student worker. This is an inter-disciplinary project and relevant fields of study and degree programs include, but are not limited to, soil, water and environmental science, school of natural resources, veterinary science, and environmental microbiology.

Information Transfer

The results from this project will be disseminated to all local, state, and federal regulatory agencies charged with overseeing the physical, chemical, and biological integrity of these and other watersheds. Examples include ADEQ, ADHS, AzG&F, US F&WS, SRP, and all downstream municipalities in the Phoenix Metro area receiving this water. We will also publish the results from this project in peer-reviewed journal articles such as Society of Environmental Toxicology and Chemistry and American Society for Limnology and Oceanography. We will also give presentations at meetings of the same or similar organizations.

Interaction with Water Centers

This project exactly matches the goals of the University of Arizona, National Science Foundation Water Quality Center whose mission statement is to, “conduct research that evaluates physical, chemical, and microbial processes that affect the quality of surface and subsurface waters utilized for potable supplies”. The reservoirs in question are drinking water supplies for the most populated area of the state and we are also evaluating how the physical and chemical attributes of these reservoirs has affected, and is affected by, microbial processes within them. We are informing and educating not only the public about water quality, but also state, federal, and local agencies in charge of water quality within these reservoirs.

Partnerships

We have formed strong partnerships with state and federal agencies. These agencies include:

AzG&F: Matching Funds = \$3000, in-kind contributions = \$4500 (lab analyses).

ADEQ: In-kind contributions = \$7500 (labor and field personnel, lab analyses).

USDA: In-kind contributions = \$4200 (reduced lab fees, consultation with Dr. Zimba).

Salt River Project: In-kind contributions = \$2000 (labor and field personnel, consultation).

US F&WS: Possible matching funds in 2006.

Citations

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Figure 1. Structure of Anatoxin-a

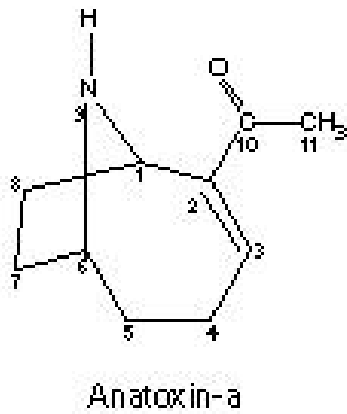


Figure 2. *Aphnizomenon flos-aquae* from Roosevelt reservoir



Figure 3. Structure of Microcystin.

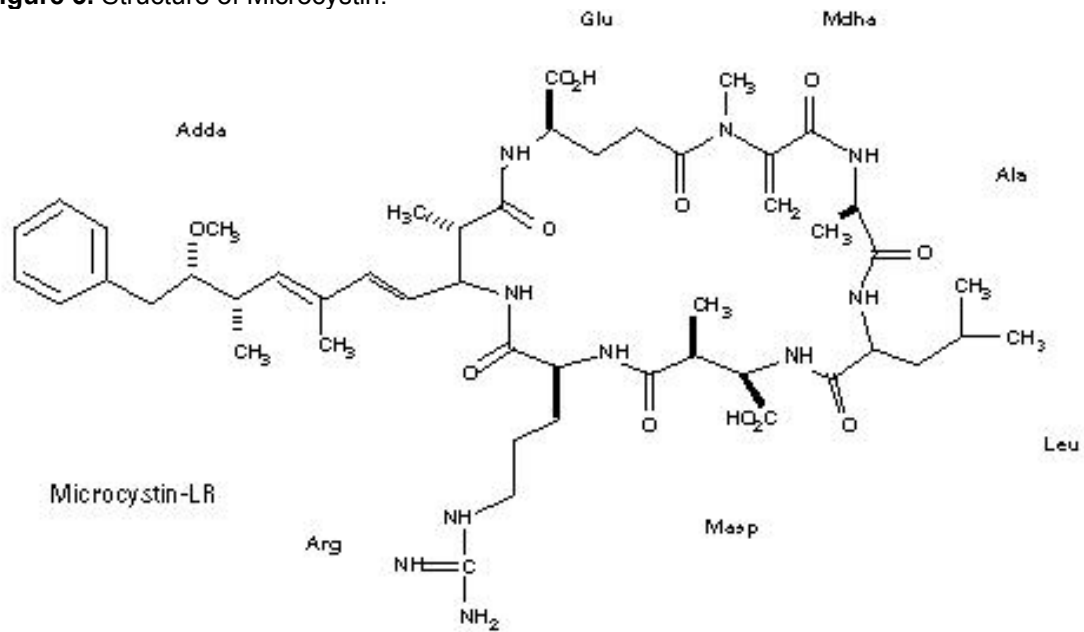


Figure 4. *Microcystis aeruginosa* from Saguaro reservoir

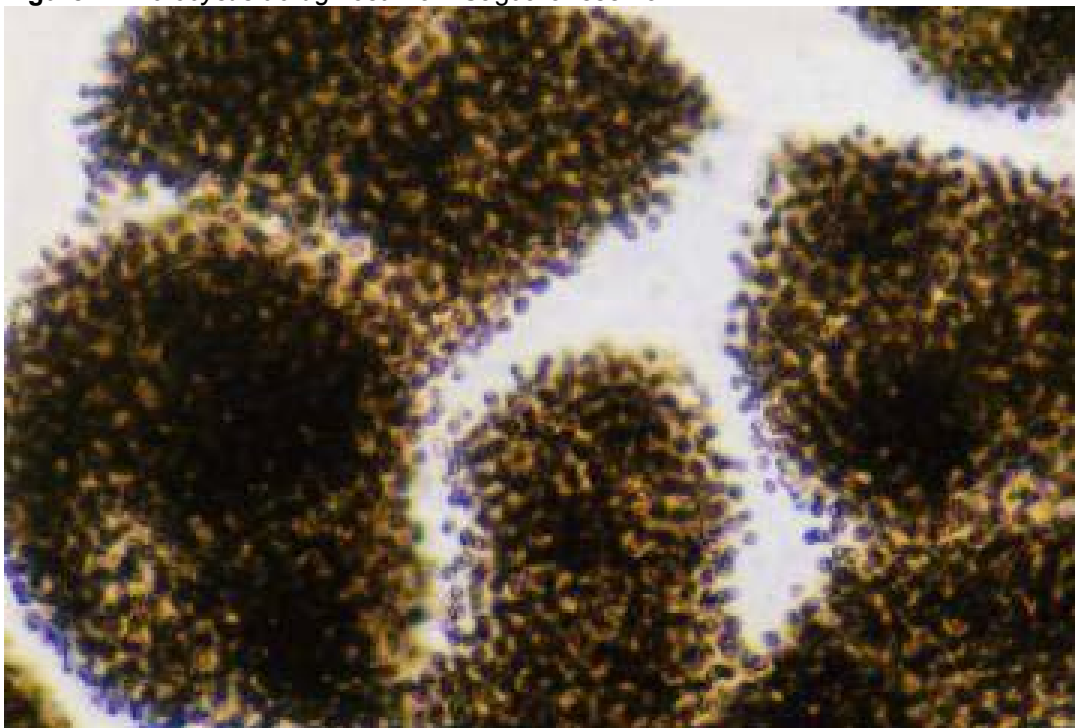


Figure 5. *Anabaena laxa* from Apache reservoir.

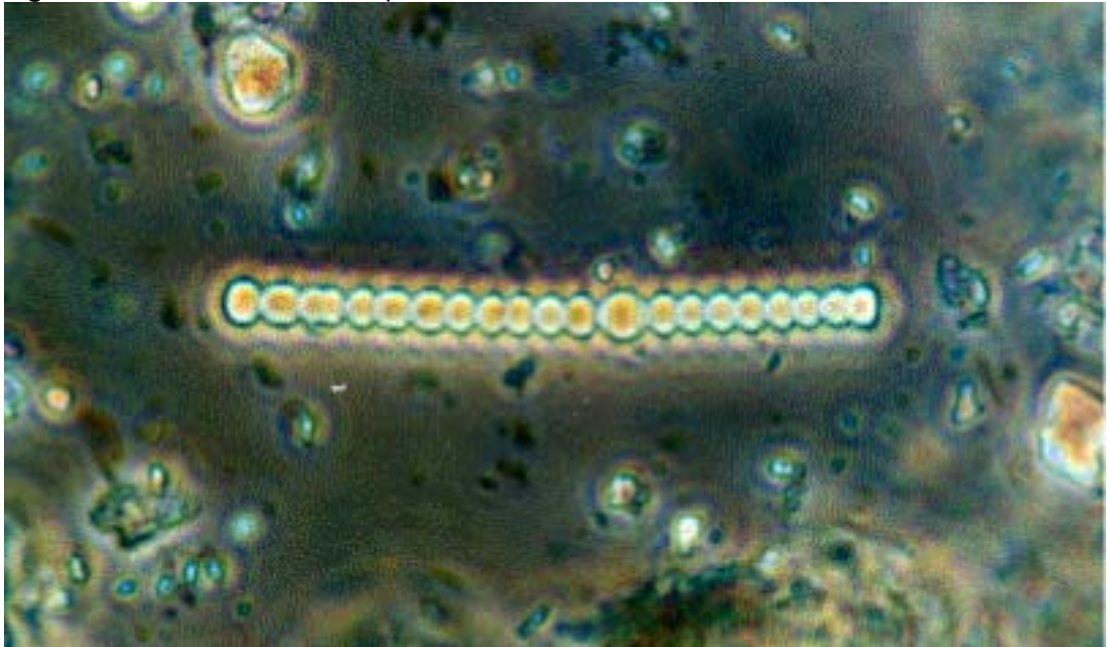


Figure 6. *Oscillatoria* sp. from the Salt River

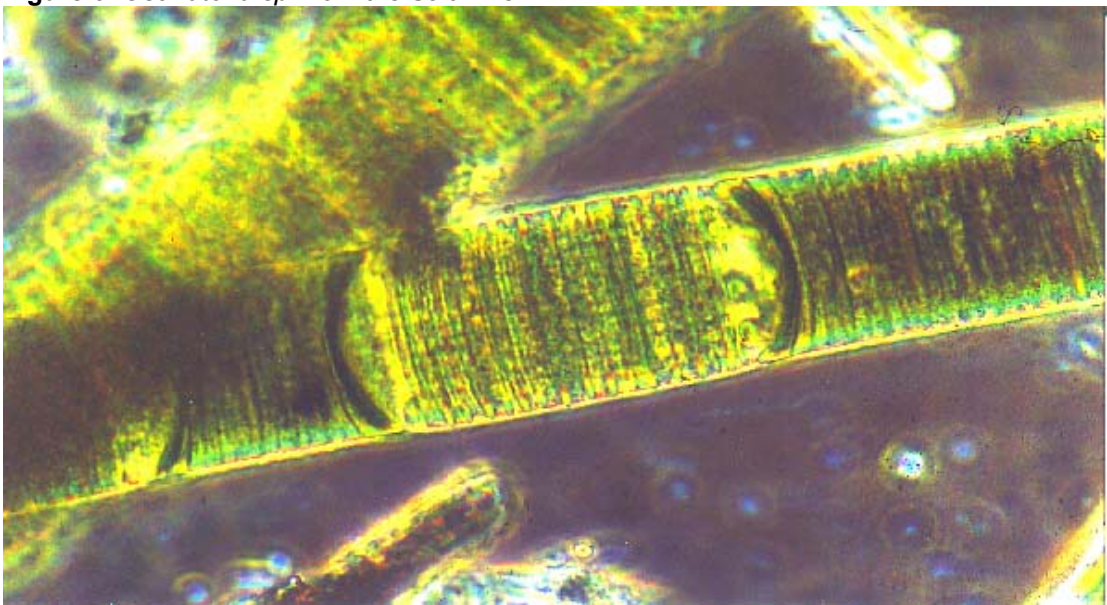


Figure 7. *Cylindrospermopsis raciborskii* in both curled and straight morphologies from Saguaro reservoir

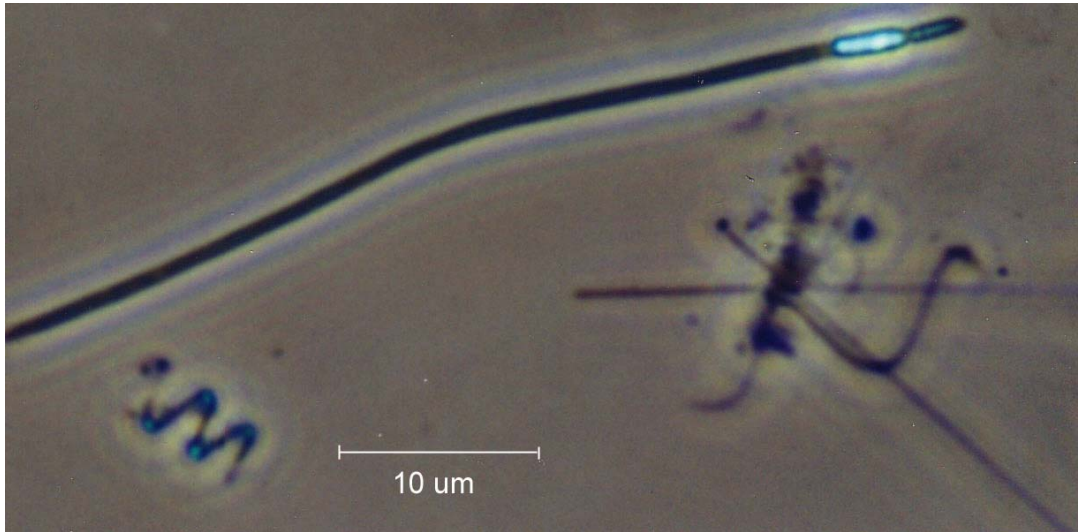


Figure 8. Structure of cylindrospermopsin

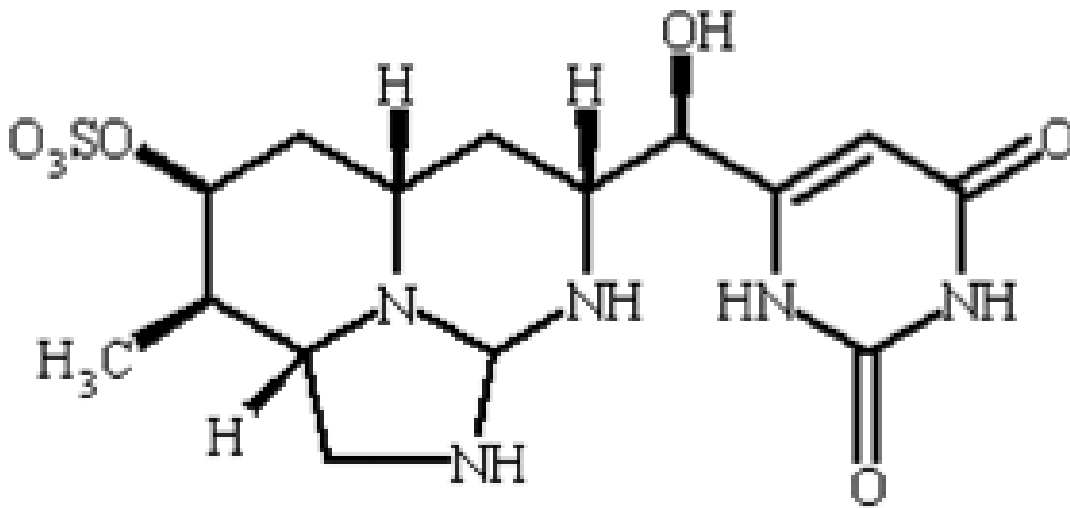


Figure 9. Reservoir, canal, and river sampling sites surrounding the Phoenix Metropolitan Area for the NSF/UA project “Comprehensive Watershed Management for the Valley of the Sun and Central Arizona Basins”

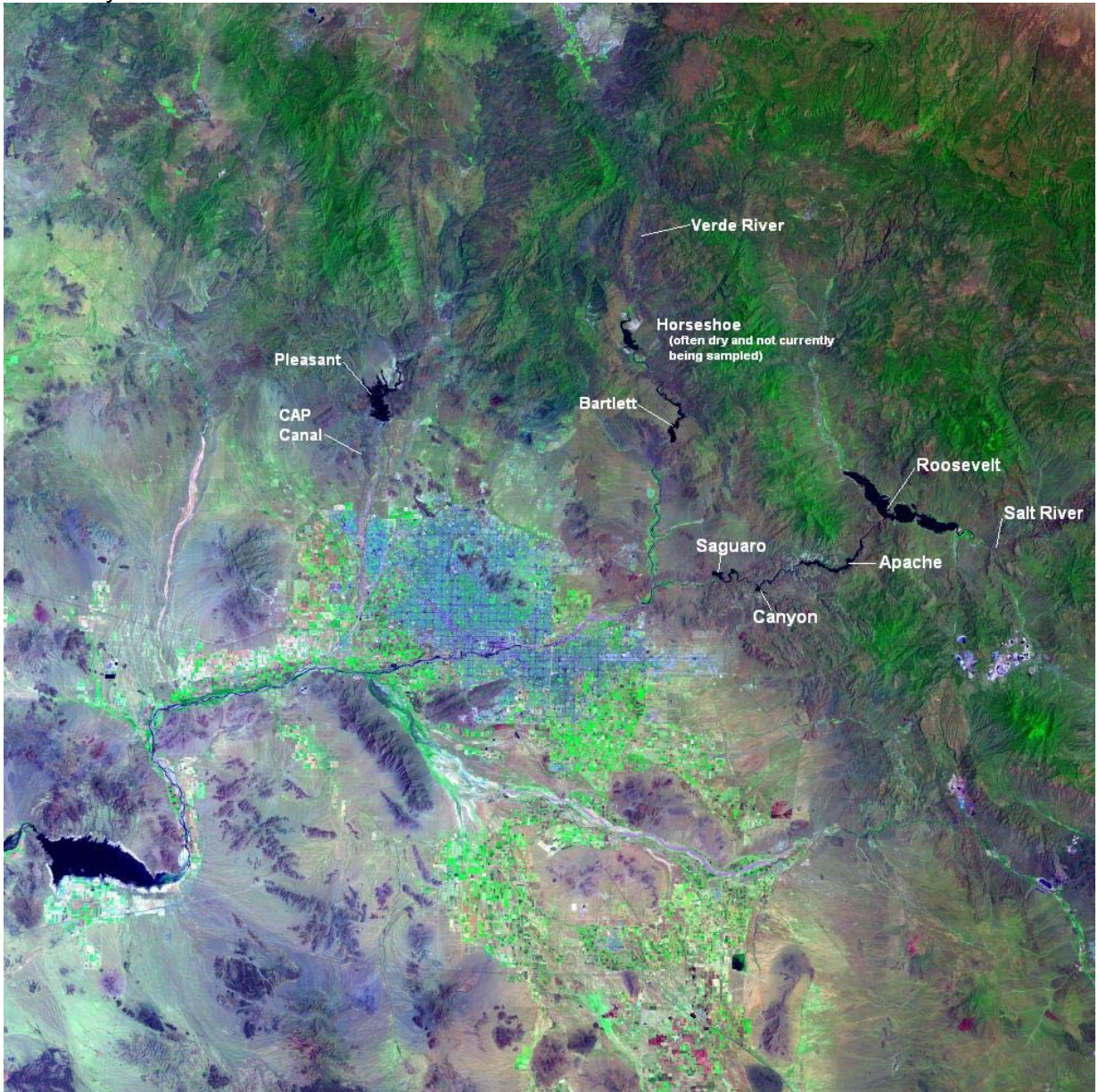


Figure 10. Roosevelt, Apache, and Canyon Reservoirs.

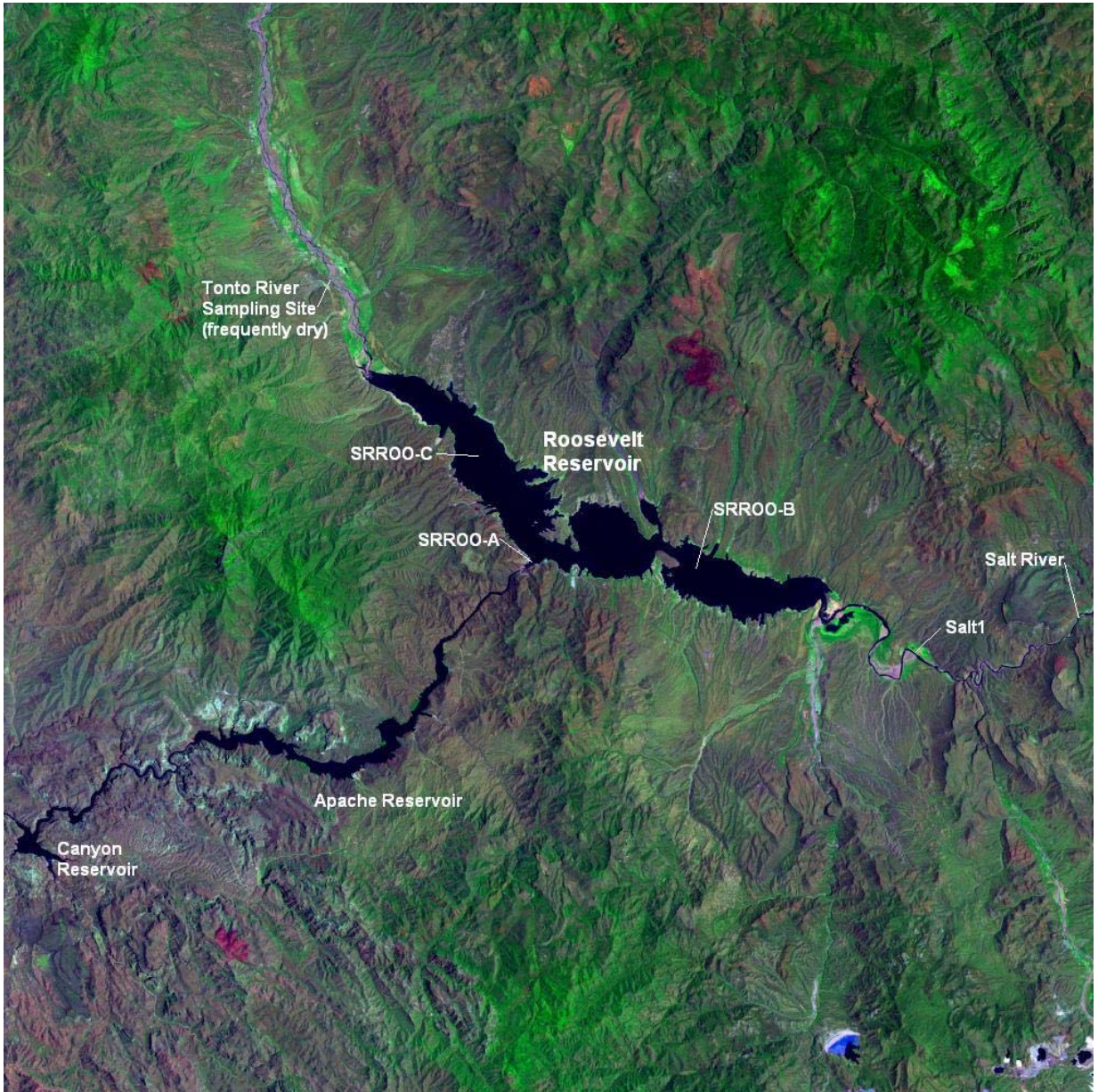


Figure 11. Apache, Canyon, and Saguaro Reservoirs

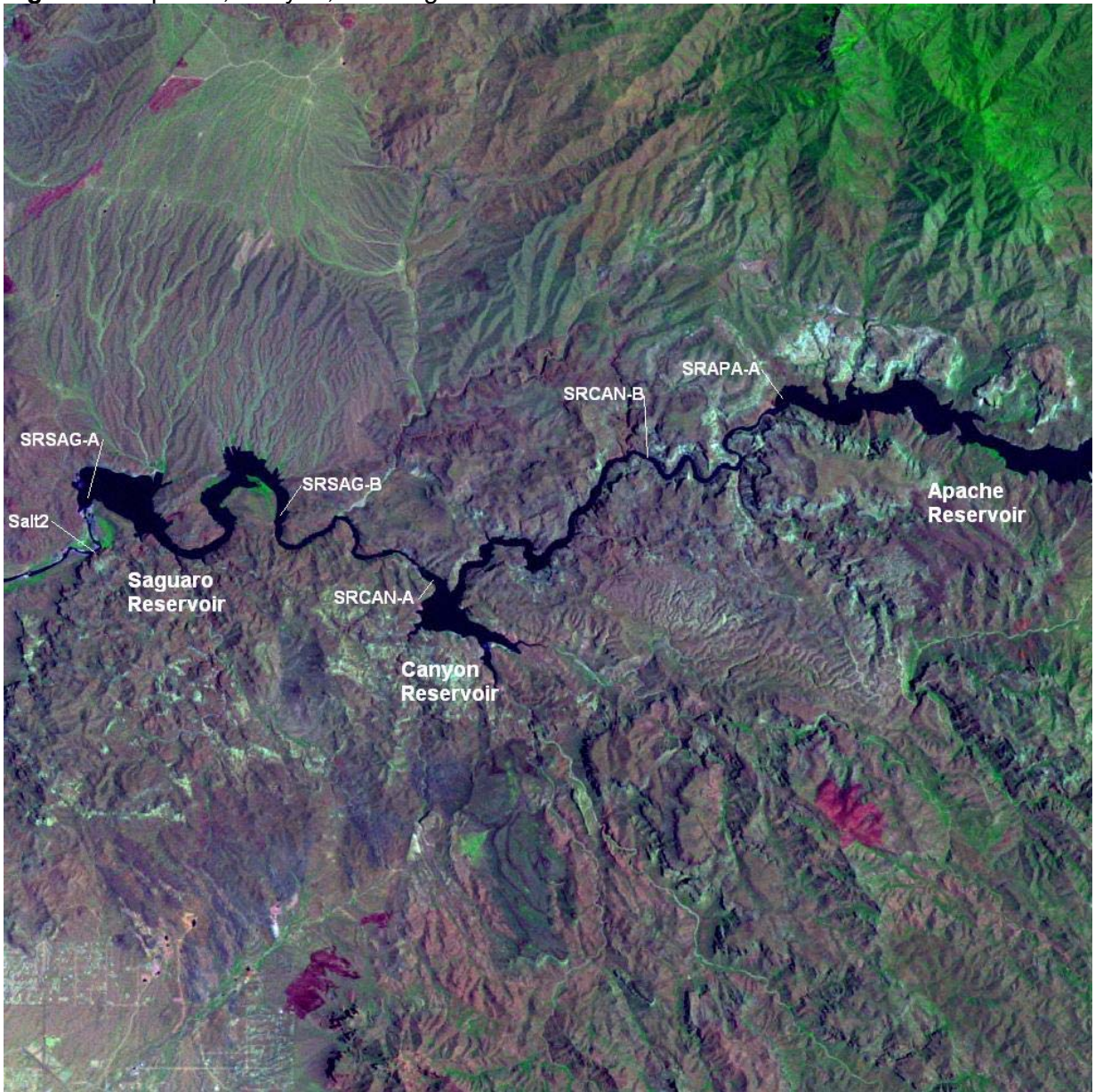


Figure 12. Burn area of the Rodeo-Chedeski Fire in relation to the Salt River and Roosevelt Reservoir.

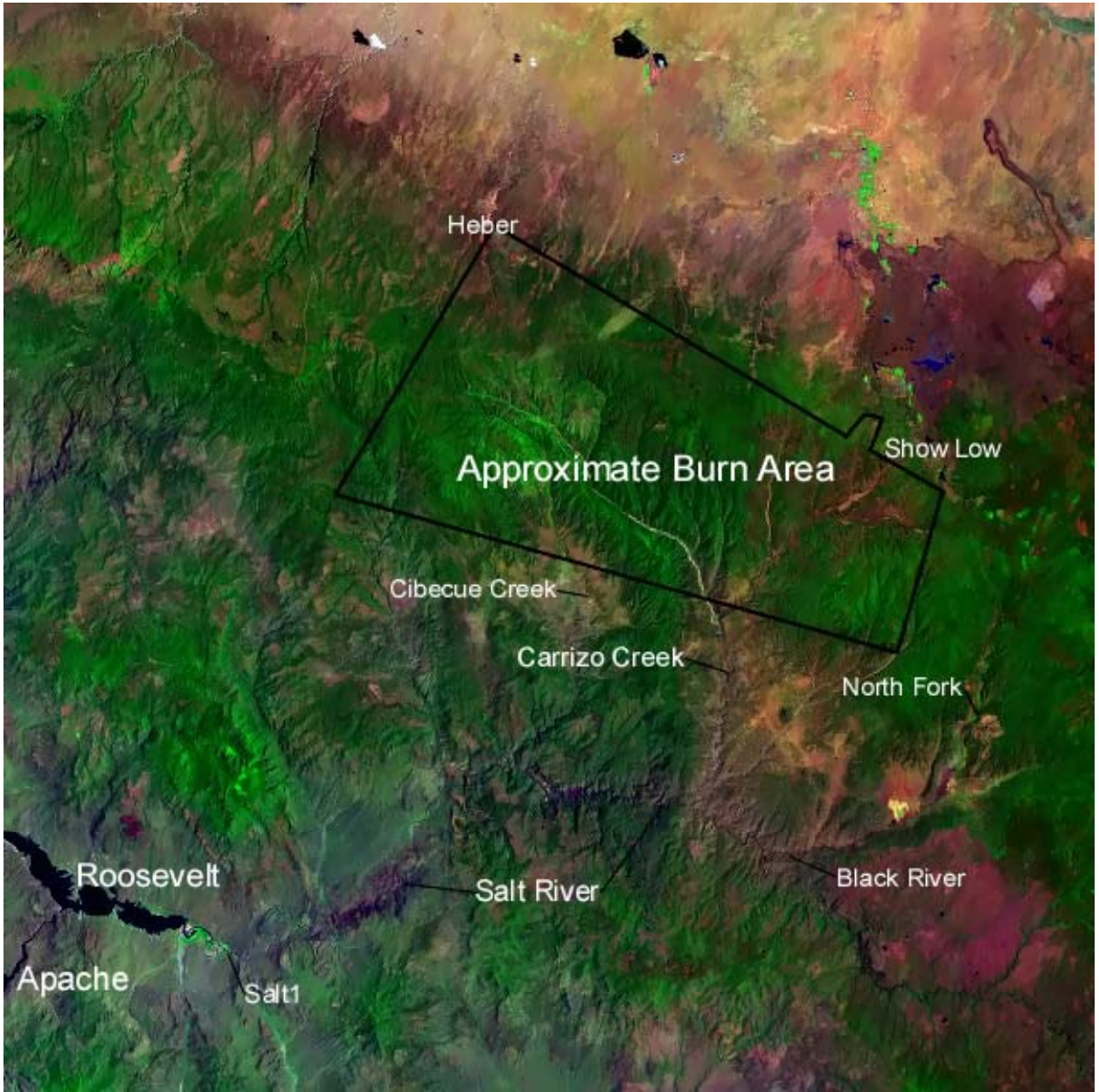


Figure 13. Fish kill in Saguaro reservoir on 6/10/04.



Figure 14. Fish kill on Saguaro reservoir on 6/10/04



Figure 15. Threadfin shad stomach contents being prepared for algal toxin analyses.



