Molecular and Genetic Ecotoxicologic Approaches to Aquatic Environmental Bioreporting

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Molecular and population genetic ecotoxicologic approaches are being developed for the utilization of arthropods as bioreporters of heavy metal mixtures in the environment. The explosion of knowledge in molecular biology, molecular genetics, and biotechnology provides an unparalleled opportunity to use arthropods as bioreporter organisms. Interspecific differences in aquatic arthropod populations have been previously demonstrated in response to heavy metal insult in the Arkansas River (AR) California Gulch Superfund site (CGSS). Population genetic analyses were conducted on the mayfly Baetis tricaudatus. Genetic polymorphisms were detected in polymerase chain reaction amplified 16S mitochondrial rDNA (a selectively neutral gene) of B. tricaudatus using single-strand conformation polymorphism analysis. Genetic differences may have resulted from impediments to gene flow in the population caused by mortality arising from exposure to heavy metal mixture pollution. In laboratory studies a candidate metal-responsive mucinlike gene, which is metal and dose specific, has been identified in Chironomus tentans and other potential AR-CGSS bioreporter species. Population genetic analyses using the mucinlike gene may provide insight into the role of this selectable gene in determining the breeding structure of B. tricaudatus in the AR-CGSS and may provide mechanistic insight into determinants of aquatic arthropod response to heavy metal insult. Metal-responsive (MR) genes and regulatory sequences are being isolated, characterized, and assayed for differential gene expression in response to heavy metal mixture pollution in the AR-CGSS. Identified promoter sequences can then be engineered into previously developed MR constructs to provide sensitive in vitro assays for environmental bioreporting of heavy metal mixtures. The results of the population genetic studies are being entered into an AR geographic information system that contains substantial biological, chemical, and geophysical information. Integrated spatial, structural, and temporal analyses of these parameters will provide invaluable information concerning environmental determinants that restrict or promote gene flow in bioreporter populations. — Environ Health Perspect 106(Suppl 6): 1395–1407 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl6/1395-1407beaty/abstract.html

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In the Rocky Mountain West, heavy metal pollution from contemporary and historic mining operations poses significant environmental problems. Since the discovery of gold and other minerals in the Rocky Mountains during the mid-1800s, mining activities have had a major impact on watersheds in this region. In Colorado, heavy metals from an estimated 5000 abandoned waste dumps or tailing piles impact >2600 km of streams (1,2). These hazardous waste sites expose humans, fish, wildlife, and other animals to multiple heavy metals. Mixtures of heavy metal contaminants can impact aquatic ecosystems at different levels of organization (2,3) and assessment of risks associated with exposure to such chemical mixtures is difficult (4). Long-term low-level effects of exposure to chemical mixtures are especially difficult to assess with currently available testing systems (4,5). Theoretically, organisms such as aquatic arthropods that experience long-term exposure to the mixtures and proceed through embryogenesis and development in the presence of the mixtures would be the most sensitive reporters of environmental contamination. This is not a new concept; however, novel approaches are now available to exploit such organisms for bioreporting. Indeed, the explosion of knowledge in molecular biology, molecular genetics, and biotechnology provides an unparalleled opportunity to use arthropods and other aquatic organisms as bioreporters (1–3,6–18).

Modern population genetic approaches permit analyses of perturbations in the genetic structure of bioreporter populations resulting from exposure to heavy metals and other environmental toxicants (19). In addition, molecular approaches permit the identification and isolation of endogenous metal-responsive (MR) genes in bioreporter species. These two approaches provide environmental bioreporting capability at the genetic, individual, and population levels and potentially provide unprecedented capability for bioreporting of heavy metal pollution. At Colorado State University (Fort Collins, Colorado), we are involved in a long-term project to develop novel approaches for the utilization of aquatic arthropods for bioreporting of mixtures of heavy metals. The technologies and approaches we developed for genetic analyses of mosquito vector populations and for identification of populations that pose exceptional risk of transmissible disease.
humans (20–28) are being exploited in our environmental toxicology studies on the Arkansas River (AR).

The principal goal of this research is to determine if intraspecific differences occur in arthropod populations in response to environmental pollution in the AR—California Gulch Superfund site (CGSS). Population genetic approaches are being used to test the hypothesis that reductions in intraspecific variability in arthropod bioreporter species result from impediments to gene flow in the population in response to heavy metal mixture pollution. Molecular genetic approaches are being used to determine if differential gene expression can be detected in arthropod bioreporter populations above and below the AR—CGSS. MR genes and regulatory sequences are being isolated, identified, characterized, and potentially exploited for environmental bioreporting. The population genetic and molecular genetic information is being incorporated into an AR geographic information system (GIS) to investigate associations between gene frequencies and environmental, biological, geophysical, and hydrologic factors.

Background

AR—CGSS

The upper AR basin is located in central Colorado between the Sawatch and Mosquito mountain ranges. The upper AR basin is a site of poor water quality, and many point and nonpoint sources of impact from past mining and metallurgical operations have been identified in the Leadville, Colorado, area. Heavy metals (e.g., Cd, Cu, Pb, and Zn) from California Gulch, a U.S. Environmental Protection Agency Superfund site, are discharged directly into the AR (Figure 1). Levels of Cd, Cu, Pb, and Zn exceed federal criteria levels for >400 km of the upper AR, and heavy metal toxicity to Ceriodaphnia dubia has been measured >50 km downstream from CGSS (1,2). Interspecific differences in aquatic arthropod populations have been demonstrated in response to heavy metal insult associated with the AR—CGSS. There are shifts in benthic community composition and reduced species diversity at sites located downstream from the CGSS (1,29). Thus, this study site provides a unique opportunity to investigate population and individual genetic responses to heavy metals in aquatic organisms.

Aquatic Arthropods as Bioreporters

Diptera (chironomid midges), Ephemeroptera (mayflies), and Trichoptera (caddis flies) have all been utilized for toxicologic studies (15,16,29–35) and for environmental bioreporting (36–50). Chironomids, mayflies, and caddis flies have common attributes that make them attractive for bioreporting: they are commonly found in most aquatic systems; they are relatively sedentary and thus representative of local conditions; they are benthic and thus closely associated with sediments; and they accumulate metals in a manner directly proportional to environmental conditions (33). The juvenile stages occur in aquatic environments; thus, critical periods of embryogenesis and development, which may require months or even years, occur in the presence of environmental contaminants. In addition, some are easy to propagate, manipulate, and rear in the laboratory. Importantly, they respond genetically to specific environmental conditions, and populations exhibit significant genetic heterozygosity (41). Representatives of the three groups are present in the AR—CGSS and considerable information is available concerning their abundance and distribution (1–3,51,52). However, little is known concerning their potential for bioreporting using molecular genetic approaches.

The response of insects to heavy metal insult has been the subject of much research. Insects exposed to heavy metals at sublethal doses exhibit pathologic effects on hatching, locomotion, respiration, feeding, growth, morphology, pupation, emergence, and oviposition (33,34,53,54). Concentrations and toxic effects of metals depend on the bioavailability of the metal (31,32,50,55,56) and the size, age, sex, and developmental stage of the arthropod (38,57–60). Resistance to heavy metals varies with different genotypes and strains within a species (19,61).

Problems associated with using benthic organisms for biomonitoring, such as natural seasonal and longitudinal changes in benthic community composition, bioavailability of the metals, size and feeding habits of insects, lack of direct correlation between metal burdens in insects and in the stream (1,3,15,45,52,62–64), can be partially addressed by monitoring community structure and integrating field and laboratory studies (2). Genetic ecotoxicology provides an exciting new approach to complement and enhance existing bioreporting approaches. For example, in contrast to ephemeral conditions in the stream, the genetic and molecular biological response of arthropods reflects ongoing and cumulative exposure of the arthropods to the metals. If the genetic response can be exploited for
bioreporting, it will provide a powerful tool for the study of environmental pollution.

Population Responses to Heavy Metal Pollution

Organisms respond in a number of ways to heavy metal exposure (Figure 2). Organisms that survive exposure often develop tolerance through a variety of molecular genetic mechanisms. Because tolerant individuals have a selective advantage over susceptible individuals in polluted environments, the frequency of tolerance genes will increase in exposed populations over time. Evidence of this in insects comes from studies of the metallothionein (MT) genes in Drosophila melanogaster (65,66). The expression of MT genes is regulated at the transcriptional level, and the promoter regions contain elements responsive to metal ions (67). In Drosophila, there are two MTs (MTo and MTn) that share little similarity (66). MTo is 43 amino acids and MTn is 40, but they share only 11 amino acids (67). The two MT genes are differentially transcribed during development—MTo early in embryogenesis through the third larval instar and MTn later in embryogenesis and throughout development (17). It has been proposed that MTo is involved in metal ion homeostasis and that MTn is related to detoxification processes. Populations of Cd-tolerant D. melanogaster were selected over several generations in the laboratory. Larvae that were homozygous for the tolerance gene produced twice as much MTn mRNA as susceptible flies and a duplication of the MT cistron was described (53,66). This duplication was subsequently discovered in field populations and its frequency was correlated with Cd and Cu used in insecticide and fungicide applications. No duplications were detected in tropical Africa; low frequencies were detected in Australia, Japan, and Egypt; and North American and European populations had frequencies as high as 50% (68).

Metal tolerance has been best studied in plants, especially those associated with mine tailings (13,14). Mechanisms of tolerance vary widely among plant species, and metal-tolerant and susceptible individuals can exist in populations located only a few meters apart (14). Although susceptible alleles would be rapidly selected against in exposed individuals, tolerant alleles should flow freely into unexposed populations. Perhaps severe selection is imposed for the maintenance of tolerance in exposed individuals whereas tolerance genes may be selected against in unexposed populations. Similar mechanisms have been suggested for the maintenance of susceptibility and refractory genes in populations of insects exposed to insecticides (69). Numerous studies have noted rapid increases of resistance alleles in insecticide-treated populations but with similar rapid decreases in resistance alleles following removal of insecticides or in insects collected from adjacent unexposed areas (70). Such studies suggest that pollutants may act as barriers to gene flow in natural populations, and as populations become better adapted and more tolerant of pollutants, barriers to gene flow may increase.

Population Genetic Approaches for Environmental Bioreporting

Molecular techniques have revolutionized our ability to analyze the breeding structure of animal populations and the genetics of many species. Techniques using polymerase chain reaction (PCR) have been especially useful in studies of insect populations (26). The techniques reveal extensive genetic polymorphisms in taxa that were previously refractory to population genetic analysis either because they lacked genetic polymorphisms by traditional genetic techniques or were too small to be analyzed (e.g., immature stages of insects). PCR-based techniques including RAPD PCR and AP PCR and amplification of specific genes or sequences are powerful tools for population genetic analyses.

Biomarkers for Environmental Bioreporting

Specific genetic responses may also be exploited as biomarkers for environmental hazards and may provide mechanistic understanding of the response. Many genetic responses, which are readily identifiable using molecular approaches, have been detected in organisms or cells exposed to a variety of mutagens, carcinogens, and other genotoxins. For example, gene amplification is a commonly detected molecular response to chemical stress. The copy number of the gene is increased, thereby increasing the gene product involved in neutralization of the insult. The molecular basis of organophosphorus pesticide resistance in Culex quinquefasciatus mosquitoes is due to the amplification (250X) of a detoxifying esterase gene (71). Gene amplification also occurs in mosquito cells transformed with an antibiotic (hygromycin) resistance gene and selected for hygromycin resistance (72). Mutations in the transcriptional unit (e.g., in gene or genes or associated regulatory sequences) could also yield phenotypes with increased resistance to environmental contaminants. Changes in regulatory sequences could increase transcript copy number; changes in exon sequences could result in gene products that more effectively neutralize the insult.

Heavy metals induce a variety of candidate biomarker genes in exposed organisms, e.g., MT, stress or heat shock genes. Although the MT genes are the best known and characterized (67,73), undoubtedly many MR genes in organisms may be exploited as molecular biomarkers. For example, our studies to identify MR genes in mosquitoes identified a protein that was expressed in Cu-exposed individuals. We have not yet detected induction of genes with products in the size range of MT, but we have detected a number of other MR genes. Thus, although MT genes are likely candidates for development of heavy metal bioreporters, other MR genes (e.g., heat shock genes) may prove more useful in some arthropods (36,74–76). Species-specific MR genes may be present in each arthropod species selected for bioreporting.

Molecular approaches can be readily exploited for detection of all of these potential genetic biomarkers. For example, copy number of specific MR genes can be readily detected by Southern blot and subsequent densitometric analysis (72). A variety of techniques are now available to permit detection of intramolecular changes, even single nucleotide changes, in genes. One technique that has proven exceptionally useful is single-strand conformation polymorphism (SSCP) analysis (27,77). The technique is based on the principle that electrophoretic mobility of a single-strand DNA or RNA molecule in a non-denaturing gel is dependent on both its size and shape. A number of stable shapes or

Figure 2. Populations of organisms respond continuously to stressors.
conformations are formed when secondary base-pairing occurs among nucleotides on a single DNA strand. The length, location, and number of intrastrand base pairs determines secondary and tertiary structure of a conformation. Point mutations that affect intrastrand interactions may therefore change the shape of a molecule and thus alter its electrophoretic mobility. The mobility of a renatured single-strand DNA molecule is sensitive to point mutations. SSCP can detect 99% of point mutations in DNA molecules 100 to 300 bp in length and 89% of mutations in molecules of 300 to 450 bp in length (78).

Geographic Information Systems Analysis
Geographic information systems provide technologies, statistical analyses, and functions that permit management and analysis of complex information systems and large databases while retaining spatial and temporal integrity (79). This study provides an unprecedented opportunity to effect a marriage of GIS and population genetic approaches to environmental monitoring. An extensive GIS database of the AR basin is already available (80) and will be augmented with the population genetic results.

Genetic Ecotoxicologic Studies of the AR–CGSS
The overall hypothesis of these studies is that population genetic and molecular genetic approaches using indigenous arthropod species will provide an important new approach to environmental bioreporting of metal and chemical mixture pollution in the AR–CGSS.

Population Genetic Studies
Introduction. As noted previously, there has been a recent revolution in the types and diversity of genetic markers for use in population genetics (26). These markers have dramatically increased the resolution with which we can analyze gene flow, genetic diversity, and phylogenetic relationships among populations of a species. Furthermore, these techniques have enabled population geneticists to target specific regions of the genome for analysis. For example, genetic variation in a presumptive MR gene could be detected with SSCP analysis. By analyzing genetic variation in many individuals from metal-contaminated and unpolluted environments, we can test for the effects of natural selection in increasing or decreasing the frequency of certain genes in metal-contaminated environments. However, frequently we have no a priori knowledge of candidate MR genes and there may be many physiological and developmental mechanisms that condition adaptation to the presence of metals. Furthermore, a species may lack the ability to adapt to metals and may simply leave a contaminated environment or die. In these cases, it is more appropriate to focus on the general genetic diversity of an organism as an indicator of the whole organism response to metal exposure. If perturbations in the breeding structure of the population are identified using neutral genes, specific genetic responses to heavy metals can then be identified in the bioreporter species using molecular genetic approaches. Neutral genes can include regions of nuclear genes such as introns and repetitive regions as well as organelle genes from the mitochondrial genome.

We are examining genetic diversity in the 16S mitochondrial tRNA of Baetis tricaudatus as a measure of the total genetic diversity in the species in the AR. We have used this region in population genetic analyses of a number of arthropod species (27,81–83). There is a large amount of variation in this gene within a species and the distribution of alleles fits the infinite allele model, suggesting that it is selectively neutral.

Examination of neutral genes provides clues to the genetic history of a population. If a population occurs in a region of metal exposure and has reduced genetic variability relative to populations of the same species from unexposed areas, this is strong circumstantial evidence that the population has historically been severely reduced or destroyed by metal exposure. Alternatively, a population that has tolerated metal exposure or has adapted to the presence of metal may have genetic diversity similar to unexposed populations. These trends in genetic diversity may shift seasonally in species with annual cycles of reproduction or in species with overlapping age structures. For example, benthic drift from unpolluted upstream sites or oviposition by females from unexposed downstream sites would increase the genetic diversity at a site on an annual or even monthly basis. However, this might be offset by periodic flushes of heavily contaminated waters (e.g., during spring runoff) through that site. Changes in genetic structure of exposed populations have been used in biomonitoring studies as evidence of metal impact (16,84), and lower genetic diversity has been associated with reduced fitness (85) and increased susceptibility to other stressors (86–88).

Materials and Methods
Materials. Unless otherwise specified, materials were molecular biological grade from Sigma Chemical Company (St. Louis, Missouri).

Study Sites. Bioreporter arthropods were collected from the AR sites upstream and downstream from the CGSS near Leadville, Colorado (Figure 1). Water quality, sediment metal levels, and macroinvertebrate communities were surveyed from 1989 to 1993 at the 10 stations along the AR from Climax to Buena Vista (7). Metal levels in water and sediments were greatly elevated immediately below CGSS but decreased with distance downstream because of adsorption/absorption and dilution from tributaries. The sample area was approximately 50 km long, with a decrease in elevation of 866 m. The study sites on the Cache La Poudre River (CLPR) were selected to correspond to the AR study sites and provided a control for gene flow in the bioreporter species in a relatively pristine stream. Sampling regimens were similar for the two stream study sites.

Bioreporter Species. Although we are focusing on mayflies in initial analyses, three bioreporter species from different taxa were collected from the selected study sites on the AR and CLPR: mayflies, B. tricaudatus; midges, Chironomus spp. (Orthocladiinae); and caddis flies, Brachycentrus americanus. The differing life histories and biology of the three species may result in differing efficacy as bioreporter species. Bioreporter arthropods were collected three times (late summer, early summer, late summer) from the AR and the CLPR and stored in 80% ethanol for analysis. We will focus on herein on genetic analysis of B. tricaudatus collected in August 1995; 50 specimens were collected from each location, placed in 80% ethanol, and transported to the laboratory. DNA was extracted from the head of each individual arthropod using a modification of the technique described by Coen et al. (89). The remainder of the organism was used for taxonomic verification.

Molecular Markers. We amplified a 406-bp region of the 16S mitochondrial genome with oligonucleotide primers that are conserved in all arthropods (89,90). Amplified 16S genes were analyzed for point mutations using SSCP analysis. Each novel sequence or haplotype of the 16S was
sequenced to test the sensitivity and specificity of the SSCP analysis and to assess phylogenetic relationships among haplotypes. Information collected on the number of haplotypes and the frequency of each haplotype was used to calculate the Shannon diversity measure as an indicator of genetic diversity at each site (81). This analysis allowed us to test the hypothesis that genetic diversity in *B. tricaudatus* is lower in metal-polluted regions of streams than in reference streams or in unpolluted regions of the same stream.

**Polymerase Chain Reaction.** The forward and reverse primers were added to 1 x PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 15 mM MgCl2, 0.1% gelatin [w/v], 1.0% Triton X-100 and 200 μM dNTPs) to a final concentration of 1 pmol/μL. The PCR solution was dispensed to 96-well plates in 24-μl aliquots. Light mineral oil (1 drop) CI-1000 (UV) ultraviolet light emitted 260 nm light was layered on top. The rack was placed in a CL 1000 oven emitting ultraviolet (UV) light (Ultra-Violet Products, Upland, California) for 10 min to destroy possible carryover DNA products. Template DNA (5 ng) was added through the oil to the mixture and the 96-well plate was inserted into a 96-well adapter on top of the thermal cycler.

Amplifications were carried out in a PTC-100 programmable thermal cycler (MJ-Research, Watertown, Massachusetts) with the following cycle program: 1) 95°C for 5 min, 2) 90°C for 20 min, 3) 92°C for 1 min, 4) 48°C for 1 min, 5) 72°C for 2 min, 6) cycle to step three 10 times, 7) 92°C for 1 min, 8) 54°C for 35 sec, 9) 72°C for 2 min, 10) cycle to step seven 30 times, 11) 72°C for 7 min, and 12) 4°C for 24-h (overnight) storage. The initial 80°C step was used to load the Taq polymerase (1 unit). The polymerase was not added at room temperature because Taq polymerase can extend nonspecifically bound primer to cause artifacts that are subsequently amplified during PCR. A negative control, without template DNA, was used in all amplifications. Upon completion of the amplification, samples were maintained at 4°C. Amplified products were resolved by electrophoresis alongside reference size markers on 1.2% 89 mM Tris base, 89 mM borate, 2 mM EDTA, pH 8.3 (TBE) agarose gels containing 0.5 μg/ml ethidium bromide. DNA fragments were visualized with UV light.

**Single-Strand Conformation Polymorphism Analysis.** Conditions for resolution of point mutations using SSCP have been optimized and published (26). Polycrylamide gels 16 x 20 cm in size and 0.4 mm in thickness were poured and run with the Hoeffer SE600 system (Amersham Pharmacia Biotech, Piscataway, New Jersey). The outer glass plate was first treated with bind silane (Sigma) to adhere the gel to the plate for staining. The opposite plate was treated with Sigmacote (Sigma). Optimal sensitivity to point mutations was obtained using 5% acrylamide gels with 2% cross-linking. Immediately prior to pouring the gel, the gel solution was filtered through three layers of Whatman paper #1 (Whatman International, Maidstone, England). Ammonium persulfate and *N*,*N*'-tetramethyl-ethylenediamine were added to initialize polymerization.

From each 25-μl PCR reaction, 1 μl was removed to a 500-μl tube and 9 μl loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added. The tube was tapped to mix the contents, spun down, heated to 95°C for 5 min, then plunged directly into ice. From this, 5 to 10 μl (approximately 150 ng DNA) was loaded directly onto the SSCP gel. Electrophoresis was performed at 4°C for 5 to 6 hr with constant amperage (20 mA) with 0.5 X TBE in both upper and lower buffer chambers.

DNA bands were resolved using silver staining. All staining procedures were carried out in 250-ml volumes. Following electrophoresis, the glass plate with the adhered gel was removed to 10% acetic acid in a shallow plastic tray and placed on a rotary shaker for 20 min. The fixative was saved for later use in terminating the developing reaction. The gel was rinsed three times in double-glass-distilled water (ddH2O) with 2 min agitation per wash. Fresh ddH2O was used in each wash. The gel was transferred to a staining solution containing 0.15% (w/v) silver nitrate and 0.15% (v/v) 37% formaldehyde in ddH2O and aged for 30 min. The gel was removed from the staining solution, drained, and rinsed for no more than 20 sec in ddH2O. The gel was then placed in developing solution and aged. The developer was a 3% sodium carbonate solution chilled to approximately 4°C; immediately prior to placing the gel in the developer, 37% formaldehyde and sodium thiosulfate were added to concentrations of 0.15 and 0.0002%, respectively. As soon as bands became clearly resolved, the fixative solution from the first step was poured directly into the developer and aged for 3 min. The gel was then rinsed twice in ddH2O and the glass plate and gel were allowed to air dry. The gel was scanned and the image was both printed and stored on 100-Mb Zip disks (Iomega Corp., Roy, Utah). The gel was scored and results entered into a database.

**Double-Stranded Cycle Sequencing.** All novel haplotypes were sequenced using double-stranded sequencing to test the sensitivity of our SSCP analysis (genes with different SSCP patterns should have different sequences). A pair of haplotypes, each originating from a different mayfly, with identical SSCP patterns was sequenced to test the specificity of the SSCP analysis (genes with identical SSCP patterns should have identical sequences). Sequence information also allowed us to infer the amount of sequence divergence among different haplotypes and infer phylogenetic relationships among the different haplotypes. All sequence data were collected by automated sequencing with an Applied Biosystems model 377 automated sequencer in Macromolecular Resources (Colorado State University, Fort Collins, Colorado).

**Data Analysis.** A Shannon diversity measure was calculated from the frequency and number of haplotypes at each collecting site (81). Similarity was calculated using Nei's unbiased genetic distance (81). Sequences were initially aligned using the program CLUSTALV (91) and then aligned on established structures for the 16S mitochondrial DNA genes (92,93). Distance, maximum parsimony, and maximum likelihood methods were used in phylogeny reconstruction.

**Results**

**Sensitivity and Specificity of Single-Strand Conformation Polymorphism.** Single-strand conformation polymorphism analysis of 406 bp of the 16S mitochondrial rDNA gene in *B. tricaudatus* in the AR has detected 10 haplotypes in 854 individuals analyzed to date (94). The nucleotide sequence of the 406-bp 16S DNA region obtained from 29 individual mayflies (representing 2 to 3 samples of each haplotype) was determined; the sequences revealed that SSCP analysis failed to find a single point substitution in only 1 of 406 pairwise sequence comparisons. Sensitivity of the SSCP analysis under our experimental conditions is therefore 99.8%.

**Genetic Diversity of a Neutral Gene.** Our hypothesis has been that populations of mayflies from regions on the AR with moderate to high levels of metal exposure will have reduced genetic diversity relative to mayflies from regions of low metal
exposure on the AR and along the CLPR. To test this hypothesis we examined 50 mayflies collected from each of four locations on 10 August 1995 on the upper AR (Figure 1). The first site, EF5, was located upstream from the CGSS on the East Fork of the Arkansas River, downstream from the Leadville Mine drainage tunnel. The second site, AR1, was located upstream of the CGSS, a point source for metal pollution. Site AR3 was located directly below the CGSS and was heavily exposed to metal pollution. AR5 was located further downstream from CGSS and was a site with significant metal pollution. Six haplotypes of 16S mitochondrial rDNA were detected at EF5 and AR1, whereas five haplotypes were detected at AR3 and four at AR5 (Figure 3A).

**The CGSS as a Barrier to Gene Flow among Populations of Sensitive Species.** One cause of reduced genetic diversity in mayflies at impacted sites would be the reduction in downstream migration across heavily contaminated sections of stream. The frequencies of each haplotype at adjacent sites can be used to test for similarity. If there is a barrier to gene flow, sites will be less similar to each other than expected for adjacent sites across the section of stream below the CGSS between AR3 and AR5. Pairwise comparisons of similarity for sites above and below the CGSS suggest the presence of a barrier to gene flow. The smaller the number, the more similar the sites. EF5 versus AR1 (0.153), EF5 versus AR3 (0.087), and AR1 versus AR3 (0.227), are more similar than AR3 versus AR5 (0.417), AR1 versus AR5 (1.202), or EF5 versus AR5 (1.209) (chi-square = 258, df = 21, p < 0.0001). AR3 is directly at CGSS and can be populated by mayflies from upstream. AR5 is below AR3 and is separated by a stretch of contaminated water.

**Shannon Diversity Across Sites.** Genetic diversity can be an indicator of health of a population in impacted areas. Reduced diversity in exposed populations relative to unexposed populations could result from high mortality. Sediment levels of zinc for each site at collection are shown in Figure 3B. However, haplotype number alone is a poor indicator of total diversity because mayfly nymphs with novel haplotypes could drift into sites from unpolluted upstream regions.

The Shannon diversity measure reflects the number and frequency of each haplotype and is

\[ D = -\sum_{i=1}^{n} p_i \log_2 p_i \]

The number of haplotypes is \( n \) and \( p_i \) is the frequency of each haplotype. Shannon diversity measures are listed by sampling location in Figure 3C. Genetic diversity is greatest at AR1 and reduced at EF5, AR3, and AR5. This pattern supports our original hypothesis. The greatest genetic diversity was detected in a relatively unexposed location.

**Effect of Geographic Features.** This pattern also suggests that other factors may influence genetic diversity at a site. The population density of *B. tricaudatus* is shown in Figure 3D. Population density increased as we went downstream, directly correlated with stream size and inversely correlated with elevation (95). Genetic diversity was not correlated with population density at the impacted sites.

**Discussion**

Our preliminary results demonstrate the potential for genetic ecotoxicology for bioreporting and suggest that genetic diversity at a site is correlated with metal exposure. The greatest amount of haplotype diversity was detected at AR1, an upstream site with minimal metal exposure. Diversity decreased downstream from this site where the CGSS enters the AR. Diversity then increased at distances further downstream from the CGSS (96). Analyses of samples from additional sites and collections made at different times of the year, as well as of alternate genes, are in progress. The results will then be incorporated into the AR GIS to test associations between genotypes, diversity, water

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**Figure 3.** Biological, genetic, and environmental results from sites on the Upper Arkansas River in fall 1995. Sites are shown in order of location on the river with furthest upstream site on the left. The upstream site, EF5, is below the Leadville Mine drainage tunnel, AR1 is above the CGSS, AR3 is directly below the CGSS, and AR5 is downstream from CGSS. (A) Frequency of 16S mitochondrial rDNA haplotypes, \( n = 50 \) per site. (B) Sediment zinc, mean of three samples. (C) Genetic diversity of *B. tricaudatus* populations, calculated from the frequencies in A. (D) Population density of *B. tricaudatus*, mean of five samples.
quality, and geophysical characteristics of the AR–CGSS, as suggested by preliminary results. We anticipate that critical information concerning environmental determinants of the breeding structure of aquatic bioreporter species will be forthcoming from these analyses. For example, Lake Creek enters the AR below AR6; softer water from the creek could make metals more bioavailable to *Baetis*, thereby reducing genetic diversity at AR7. This genetic ecotoxicologic approach may reveal previously unrecognized impediments to gene flow in natural populations as well as environmental features that could influence genetic diversity and be important factors in land use planning. These features include elevation, stream size, and distance from a source of colonizing mayflies. These results are consistent with a model in which many mitochondrial haplotypes have evolved since *B. tricaudatus* arose as a species. At least eight of these are represented in the upper AR basin in areas that are relatively free of metal exposure. [These haplotypes do not represent recent mutation events from metal exposure, as five of these haplotypes were present in the CLPR populations sampled in the fall of 1995 (94).] An array of haplotypes migrates downstream as mayfly nymphs drift to colonize new habitats. However, most mayflies that drift into regions of metal exposure are probably killed and haplotype diversity is reduced. Haplotype diversity may increase downstream as a consequence of genetically diverse populations entering the lower AR through nymphs drifting in from minimally exposed tributaries or from upstream migration of adults from areas of low exposure.

*B. tricaudatus* is a useful organism with which to demonstrate the effects of pollution on genetic diversity. It is a cosmopolitan species in the AR drainage and other drainages in the western United States. It is relatively simple to identify in all immature stages. Most important, our results suggest that it is sensitive to heavy metal exposure and that the degree of exposure is correlated with genetic diversity. Future studies with other potential bioreporter species that are tolerant or sensitive to heavy metals may be equally productive.

**Molecular Genetic Studies**

**Introduction**

Modern molecular techniques also permit geneticists to target specific MR genes for analysis. MR genes can be amplified directly from a single organism and genotypic frequencies in metal-contaminated or uncontaminated environments can be ascertained. Population genetic analysis of such genes theoretically provides a better approach to identifying specific environmental restrictions to gene flow. Genetic variation in specific MR genes can be readily identified by SSCP or sequence analysis. In this regard, we are isolating and characterizing genes that are differentially expressed in chironomid, caddis fly, and mayfly bioreporter species at the AR and CLP sites. The rationale for these studies is that gene expression will differ qualitatively and/or quantitatively in heavy-metal-exposed and naive bioreporter populations. These studies will demonstrate the feasibility of using MR genes from aquatic insects as biomarkers in molecular biological approaches to metal pollution monitoring of aquatic ecosystems. Characterization of the MR genes will also provide a fundamental understanding of the underlying mechanism of gene regulation by heavy metals as well as facilitating the development of practical molecular-based assays for biomonitoring heavy metal pollution in water.

**Materials and Methods**

**Bioreporter Species.** Initial studies to identify MR genes were conducted in the mosquito (*Aedes aegypti*). Information obtained for mosquito MR genes has greatly facilitated identification of homologs of these genes in environmentally relevant organisms from the AR–CGSS (e.g., midges, mayflies, and caddis flies). *Chironomus tentans* is being used as a model species in laboratory studies to define MR genes in midges. There are more than 2500 *Chironomus* species in North America (97). *Chironomus* species usually account for at least 50% of the combined macroinvertebrate species composition on any given freshwater ecosystem (97). Chironomids are common and have a holarctic distribution, they are relatively sedentary and thus representative of local conditions, and larvae may accumulate metals in a manner directly proportional to environmental conditions (41,57,98). *C. tentans* is easy to rear under laboratory conditions and is a representative species for a variety of aquatic ecosystems. As noted previously, *B. tricaudatus* is widely distributed in the AR–CGSS and CLPR and is the subject of population genetic investigations. We are also isolating MR genes from this bioreporter species.

**Rearing and Heavy Metal Challenge of Bioreporter Species.** *C. tentans* were reared as described by Woods et al. (41), Nebeeker et al. (57), and Harrhay et al. (98). *A. aegypti* were reared as described by Raynes-Keller et al. (99). The basic protocol was to expose *C. tentans* or *A. aegypti* larvae to predetermined sublethal doses of heavy metals (41,57,98). Each experiment was conducted in duplicate; 50 to 100 fourth instar larvae were placed in receptacles filled with 200 ml dechlorinated tap water containing either 50 or 500 ppb Cd (or other metals such as Hg) and Cu, or their binary and trinary combinations, in the case of *A. aegypti*, or no metal. Samples (larvae) were collected after 24-hr exposure. Preliminary studies with *A. aegypti* revealed that this exposure time was adequate for the induction of MR genes. After exposure larvae were harvested and nucleic acids were isolated. The challenge doses and regimens were selected based on the published data on the biotoxicologic effect of Cd on *C. tentans* (41,57,98) and our data demonstrating the effect of Cd on *A. aegypti* (99).

**Construction and Screening of cDNA Libraries.** To prepare cDNA libraries, larvae from bioreporter species were exposed to heavy metals (Cd, Cu, and Hg). A control group was not exposed to heavy metals. Using standard mRNAs were isolated from each challenged and control larvae of each species (100). A cDNA library was constructed using the same protocols we used to construct cDNA libraries of *A. aegypti* and *Simulium vittatum* (101). The cDNA libraries were constructed using the lambda ZapII XR cloning kit (Stratagene, La Jolla, California) from 5 μg of twice oligo dT selected poly A (pa*) RNA isolated from *C. tentans* exposed to selected heavy metals or from 5 μg of twice-selected pa* RNA isolated from *B. tricaudatus* larvae from AR3 (Figure 1).

The quality and representativeness of the library was assessed by the number of independent clones obtained per microgram of vector DNA (>10⁶ pfu/μg), the number of actin cDNAs obtained (approximately 1% of the plaques should contain this cDNA), the number of nonrecombinant clones (<5% of the clones should be nonrecombinant), and the size of the cDNA inserts (more than 500 bp). Approximately 1 × 10⁶ independent clones of each library were transferred to Gene Screen nylon membranes as described by the manufacturer (NEN Dupont, Boston, Massachusetts). The membrane-bound
lambda cDNAs were screened with 32P-radiolabeled DNA probes made from A. aegypti MR cDNAs under low stringency conditions, as follows: First, blots were prehybridized in a solution containing 50 mM Tris, pH 7.5, 1% sodium dodecyl sulfate (SDS), 0.9 M NaCl, and 100 μg/ml sheared and denatured herring sperm DNA for 30 min at 37°C. Second, approximately 4 × 10^5 dpm/ml of labeled and denatured probe was added to the prehybridization mixture and incubated at 37°C for an additional 16 hr. Third, blots were washed twice in 2 × SSC at RT for 5 min, 2 times at 37°C in 0.2 × SSC/1% SDS for 30 min (1 × SSC = 150 mM NaCl, 14 mM sodium citrate; RT, room temperature), then dried and exposed to x-ray film. Putative positive clones were rescreened at a lower density until plaque-purified clones were obtained. Selected lambda cDNA clones were transformed into plasmid by the automatic excision technique as described by the manufacturer (Stratagene, La Jolla, California). The nucleotide sequence of cDNA clones was determined by double-strand DNA sequencing using the femtomole kit according to manufacturer’s instructions (Promega, Madison, Wisconsin).

Characterization of Chironomus tentans Metal-Responsive Genes: RNA Analyses. For Northern blot analysis of metal-induced transcripts, RNA was isolated from C. tentans larvae or organs by the one-step guanidinium thiocyanate method (100). Five micrograms of RNA (101) was denatured at 80°C for 5 min in a solution containing 50% deionized formamide and 6% formaldehyde and electrophoresed on a 1.5% agarose/0.66 M formaldehyde gel. RNAs were blotted onto a Gene Screen nylon membrane following manufacturer recommendations (NEN Dupont). The RNAs were cross-linked to the filter by UV irradiation according to manufacturer’s recommendations (Stratagene). Probes were hybridized and washed using highly stringent conditions, as follows: First, Northern blots were prehybridized in a solution containing 50 mM Tris (pH 7.5), 1% SDS, 0.9 M NaCl and 100 μg/ml sheared and denatured herring sperm DNA for 30 min at 65°C. Second, 4 × 10^5 dpm/ml of labeled and denatured probe was added to the prehybridization mixture and incubated at 65°C for an additional 16 hr. Third, blots were washed twice in 2 × SSC at RT for 5 min, two times at 65°C in 0.2 × SSC/1% SDS for 30 min, then dried and exposed to x-ray film. The A. aegypti actin cDNA (102) was used as an internal standard for transfer and concentration variability of biomarker analyte in the solid phase (nylon membrane). MR and actin (internal standard) analyte abundance was quantified by densitometric analysis.

Results
Molecular Cloning of Abundantly Transcribed Metal-Responsive Genes from Aedes aegypti. The A. aegypti larval cDNA library was screened with radiolabeled cDNA probes made from pa^RNA isolated from metal-exposed and unexposed larvae or with a radiolabeled degenerate 21-base oligonucleotide primer corresponding to nucleotides coding for the first seven amino-terminal amino acids of the D. melanogaster MT gene (103). Three independent MR cDNA clones, designated pB1.3.3, pB6u, and pB6d, have been recovered by differential screening (104). Additionally, two independent MR cDNA clones, designated pmtl.2.10 and pmt3.0.4, have been recovered with the D. melanogaster MT probe and are being characterized.

Northern blot analysis indicated that both pB6u and pB6d were exclusively transcribed in the larval midgut, which is a critical organ for absorption and processing of nutrients. Relative to the controls (Figure 4, lane 1), the activity of the gene corresponding to clone pB6d was induced to the same extent by all metals alone or their combinations, regardless of the metal concentration. Gene pB6d may prove to be a sensitive reporter of heavy metals in general. In contrast, the gene corresponding to clone pB6u was induced differentially by different metals and combinations of metals (Figure 4). Note that there was no basal transcription of gene pB6u in nonexposed larvae (Figure 4, lane 1). None of the genes corresponding to the cloned cDNAs (pB1.3.3, pmt3.0.4, pmt1.2.10, pB6u, and pB6d) were induced by heat-shock treatment (30, 37, or 42°C for 24 hr), by exposure to acidified water (pH 3), or by food deprivation (104). Taken together, the results indicate that the A. aegypti cDNA clones pB6u and pB6d are bona fide MR genes. Detailed descriptions of these studies, procedures, and controls, etc. will be forthcoming (104).

The nucleotide sequence of a 1034-bp cDNA corresponding to gene pB6u has been determined. The translated product of gene pB6u is 200 amino acids and has two cysteine-rich domains flanking a proline-serine/threonine-rich domain (PT box). Comparison of the translated protein sequence of gene pB6u against other known protein sequences revealed significant similarity to other invertebrate midgut-specific proteins. Overall, gene pB6u has the greatest similarity to the moth Trichoplusia ni mucin gene (105). The molecular cloning and characterization of the A. aegypti mucin gene will be described elsewhere (104).

Metal-Responsive Genes from Chironomus tentans and Baetis tricaudatus. A C. tentans cDNA library was constructed from pa^RNA isolated from metal-exposed fourth instar larvae. The library contained 3.4 × 10^6 pfu/ml (independent clones), with < 1 × 10^4 pfu/ml nonrecombinant clones (background), and the average insert size was > 1 Kb in length. The C. tentans cDNA library was screened with radioactive cDNA probes made from pa^RNA isolated from metal-exposed and naive larvae (differential screen) and with radioactive DNA probe made from the A. aegypti pB6u gene (similarity

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Metal induction of Aedes aegypti genes pB6u and pB6d. Northern blot analysis of total RNA (10 g/lane), in duplicate samples (a, b), isolated from fourth instar larvae that were exposed for 24 hr to 10 and 1 ppm copper (lanes 2, 3); to 1, 0.1, and 0.01 ppm cadmium (lanes 4, 5, 6); 0.5, 0.05, and 0.005 ppm mercury (lanes 7, 8, 9); 1 + 10 ppm cadmium plus copper (lane 10); 0.5 + 10 ppm mercury plus copper (lane 11); 0.5 + 1 ppm mercury and cadmium (lane 12); 10 + 1 + 0.5 ppm copper plus cadmium plus mercury (lane 13); and no metal (lane 1). Blot was hybridized with radioactive DNA probes made from pB6u and pB6d cDNAs. Final wash was with 0.2 × SSC/0.1% SDS at 65°C for 30 min. The exposure was for 14 hr at −70°C using an intensifying screen.
 Several putative MR clones were isolated (plaque purified).

A B. tricaudatus cDNA library was constructed from larvae collected from AR2 and AR3 above and below the CGSS (Figure 1). pA+ RNA was isolated from AR2 and AR3 larvae. The library contained $1.4 \times 10^6$ pfu/ml (independent clones), with $<1 \times 10^4$ pfu/ml nonrecombinant clones (background), and the average insert size was $>1$ kb in length. The B. tricaudatus cDNA library was screened with radioactive cDNA probes made from pA+ RNAs isolated from either AR2 or AR3 larvae (differential screen) to identify genes whose transcripts were more abundantly expressed, or preferentially expressed, in larvae from AR3 (more polluted). Several AR3-specific clones were isolated (plaque purified).

The C. tentans and the B. tricaudatus cDNA libraries were screened with a radiolabeled DNA probe made from the A. aegypti pB6u gene at low stringency conditions (1M NaCl, 37°C). Several independent MR cDNA clones were recovered from each library. Preliminary Northern blot analysis indicates that the C. tentans and B. tricaudatus MR cDNA clones correspond to genes whose transcripts are more abundant in organisms exposed to heavy metals. For example, the C. tentans gene C610B transcript is more abundant in larvae exposed to 1 ppm Cu or 0.5 ppm Cd (Figure 5; compare lane 1 to lanes 2 and 4). The M62Bu gene from B. tricaudatus is more abundantly transcribed in organisms collected from a heavy-polluted site AR3 than in organisms collected from the more pristine site AR2 (Figure 6; compare lanes 1 and 2). The C. tentans (C610B) and B. tricaudatus (M62Bu) genes are currently being characterized.

**Discussion**

These studies are delineating MR genes in environmentally relevant bioreporter species. Once genes are identified and characterized, this information can be used in population genetic studies as described previously to provide information about specific genetic responses of populations to heavy metals. The genetic information can also be exploited for improved biomonitoring for heavy metal pollution and for development of novel bioreporting systems.

We are in the process of isolating and characterizing the promoters and DNA regulatory sequences from these candidate MR genes. Briefly, genomic clones will be isolated by screening a C. tentans genomic library with probes made from the C. tentans MR cDNAs. The nucleotide sequence of the genomic clones will be determined. Nucleotide sequence similarity comparison of the C. tentans MR promoters with other known DNA sequences present in the gene bank(s) will allow us to pinpoint putative promoters and other DNA regulatory elements. The information gained in these studies will serve as the baseline for the fine characterization of C. tentans MR promoters by deletion and scanner mutation techniques in vitro. As regulatory sequences of MR genes are identified, they will be characterized functionally by deletion and linker-scanner mutations. An appropriate reporter gene such as luciferase (Luc), CAT, or β-galactosidase, will be cloned downstream of the regulatory region ("Inducible Molecular Bioreporters of Heavy Metals"). These studies will help us to delineate the specific DNA sequence responsible for metal responsiveness. The isolation and identification of MR DNA regulatory elements and genes would provide the basis for future transgenic arthropod approaches for reporting metal pollution in water. For example, recent progress in transposable element-mediated transformation of non-drosophilid dipterans (108–110) could eventually lead to a scenario in which transgenic C. tentans could be transformed with a construct containing an MR promoter upstream from an reporter gene and exposed to aquatic environments. The activity of the reporter gene could be assessed to monitor metal pollution or to assess the efficacy of remediation efforts.

**Inducible Molecular Bioreporters of Heavy Metals**

**Introduction**

One goal of our research is to develop novel molecular bioreporter systems for ready and rapid assay for heavy metals. We previously developed a sensitive in vitro MR bioreporter system for the assay of heavy metals using the Drosophila MT promoter, which will be described briefly (111,112). Information derived in our ongoing studies of MR genes and the regulatory sequences for the respective transcriptional units described for aquatic bioreporter species from the AR–CGSS will be exploited to develop new and specific molecular reporter systems for an in vitro assay for the presence of heavy metals.

**Materials and Methods**

**Construct.** The plasmid pMT-2 Luc (Figure 7A) was engineered to contain the Drosophila MT promoter and the firefly Luc reporter system (112). This plasmid also carried the heat shock promoter 70 hygromycin resistant gene, which facilitated selecting transformants. This plasmid was introduced into Aedes albopictus C6/36 cells, and stable transformants were selected by growth in medium containing hygromycin.

**Results**

**Induction of the pMT-2 Luciferase Construct with Copper Sulfate.** To characterize the pMT-2 Luc bioreporter system, pMT-2-Luc transformed C6/36 cells were challenged with medium containing 13 ppm copper sulfate. Cells were incubated at 28°C and harvested from separate flasks at 0, 12, 25, 48, and 60 hr. Five micrograms protein of the cell lysate diluted to a total volume of 20 μl was added to 100 μl Luc assay buffer. Single photon counts per minute per sample were determined in a scintillation counter (Beckman, model LS1701, Fullerton, California). A 12-fold
**Induction of the Construct with Heavy Metal Mixtures.** Six heavy metals (As, Cd, Cr, Pb, Hg, and Ni) and four organics (benzene, chloroform, phenol, and trichloroethylene) detected in contaminated water (113) were then investigated for their ability to induce the pMT-2 Luc reporter system (Figure 7A). The cells (80% monolayer) were subjected to the doses of heavy metals or organics (0.1×, 0.5×, 1.0×, and 10×) detected in contaminated water (4) and incubated at 28°C for 12, 24, 48, and 60 hr. Only 0.5× (19 ppm) Pb+2 and 10× (3 ppm) Hg+2 induced Luc protein expression (Figure 7B). Maximum levels were observed after 48 hr (Figure 7B). Several metals were assayed for their synergistic and antagonistic effects in vitro. Multiple binary and trinary combinations, at all concentrations, of metals and/or organics were exposed to LucC5 cells for 48 hr. Of these combinations 13 ppm Cu+2 + 19 ppm Pb+2 demonstrated the greatest synergistic effect, a 14-fold increase in Luc protein expression (Figure 7B). In contrast, antagonistic effects were observed when 13 ppm Cu was combined with 5 ppm benzene, 3.4 ppm phenol, 3.8 ppm trichloroethylene, and 1.5 ppm chloroform; expression was decreased 9-, 5-, 10-, and 6-fold, respectively (data not shown).

Long-term effects to LucC5 cells exposed to 13 ppm Cu, 19 ppm Pb, and 3 ppm Hg were investigated using sequential dose experiments. LucC5 cells were challenged with the heavy metal(s). After incubation for 48 hr, cells were washed and L-15 growth medium containing the original concentration of the heavy metal was added. After an additional incubation at 28°C for 48 hr, the cells were harvested and analyzed as described above. Maximum levels of Luc protein induction were observed after the first heavy metal dose for all three metals and mimicked the results seen after a 48-hr exposure. A decline in specific activity was observed after the second and third doses. These results may be attributable to long-term exposure of the cells to heavy metal(s).

**Discussion**

These studies demonstrate that an MR regulatory region from an insect (*D. melanogaster*) can directly express a reporter gene in the cells of another insect (*A. albopictus*) in a predictable and appropriate manner. These studies lay the groundwork for the in vitro characterization of identified MR genes from our selected AR-CGSS bioreporter species and for utilization of sensitive and specific promoter constructs into in vitro bioreporter systems.

**Summary**

The overall goal of this research is to apply molecular and population genetic approaches to exploit arthropods as bioreporters of heavy metal mixture pollution in the AR-CGSS. Modern molecular approaches permit analyses of perturbations in the genetic structure of bioreporter populations and of endogenous MR genes in individuals resulting from exposure to toxic heavy metal mixture in the environment. Population genetic analyses of neutral genes have revealed significant restrictions in genetic heterozygosity in *B. tricudatus* populations associated with heavy metal exposure. Molecular genetic analyses have identified MR genes in environmentally relevant bioreporter species, which are being investigated as potential genetic bioreporters of heavy metal mixture pollution. Identified MR elements may be exploited to develop sensitive molecular bioreporting systems. The genetic information pertaining to the aquatic species is being incorporated into the GIS program to reveal associations between gene frequencies and environmental factors. Thus population and molecular genetic responses of bioreporter species are being correlated with environmental and ecological information in an unprecedented manner. This program may become a model for environmental studies of Rocky Mountain and other aquatic ecosystems.

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