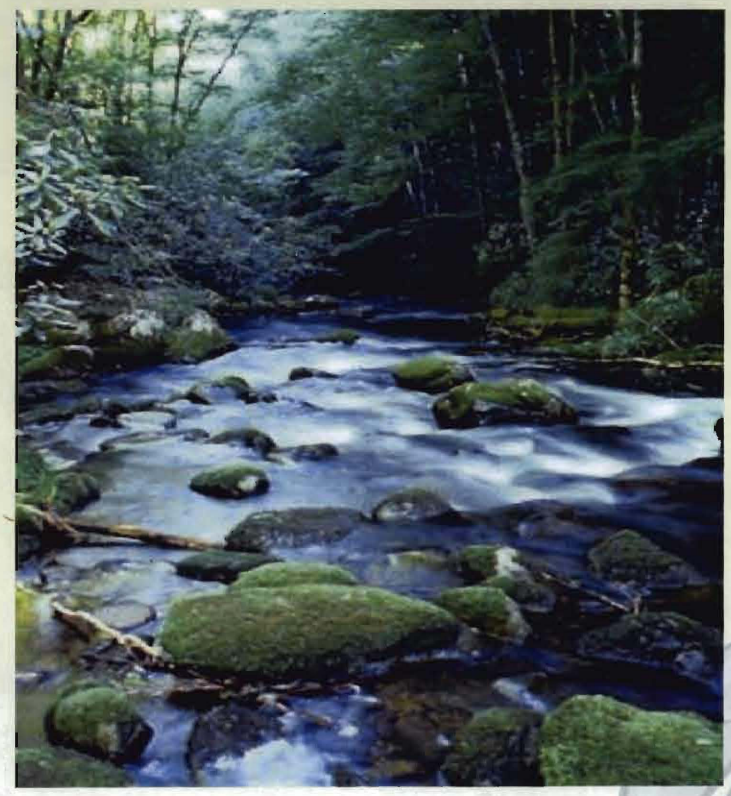
Assessing Environmental Health of East Tennessee Watersheds: Potential Influence of Procedural Artifacts on the Medaka Embryo-Larval Test for Devlopmental Toxicity



College Scholars Senior Project

Submitted by Meghan DeFord

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ASSESSING ENVIRONMENTAL HEALTH OF EAST TENNESSEE WATERSHEDS: POTENTIAL INFLUENCE OF PROCEDURAL ARTIFACTS ON THE MEDAKA EMBRYO-LARVAL TEST FOR DEVELOPMENTAL TOXICITY

by

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Abstract

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Oak Ridge National Laboratory has been evaluating the environmental health of East Fork Poplar Creek (EFPC) in Oak Ridge, Tennessee for more than a decade. One aspect of the ongoing monitoring effort is utilizing Japanese medaka in embryo-larval tests to evaluate developmental toxicity of EFPC water. Historically, water samples from EFPC have consistently resulted in high mortality in embryonic and larval medaka raised in the water. However, the toxicity tests have been resulting in significantly decreased mortality in recent years. This study aims to examine the possible influences of laboratory artifacts on these test results, namely potential changes over time in the sensitivity of the medaka to contaminants, and a concurrent change of experimental procedures. If the influence of these laboratory factors can be discounted as affecting medaka mortality in the tests, then the possibility of improved water quality in EFPC can be directly investigated. The results of the study indicate that laboratory artifacts are probably not involved in the change in mortality results currently observed in EFPC.

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INTRODUCTION

East Fork Poplar Creek (EFPC) in Oak Ridge, Tennessee is a receiving stream for discharges for the Department of Energy Y-12 National Security Complex. The stream is most noted for historical contamination of mercury, but is also impacted by a mixture of low-level contamination from other heavy metals and organic compounds (Loar 1989). Over the last decade, the stream has been subject to ongoing, extensive monitoring by the Y-12 Biological Monitoring and Abatement Program (BMAP) of Oak Ridge National Laboratory (ORNL). One component of BMAP is an embryo-larval test for developmental toxicity, in which fertilized fish eggs are collected and grown experimentally in treatment waters (e.g., water from EFPC sites) to document abnormalities and mortality. Since 1990, water collected from regular study sites established along EFPC has been assessed via embryo-larval tests using medaka (*Oryzias latipes*), a fish utilized by the Environmental Protection Agency as an experimental animal (Beniot 1991). These laboratory fish are unique in that their eggs take an average of nine to twelve days to hatch. The extended incubation period of medaka allows eggs to be exposed to the treatment water samples much longer than other species of fish before they progress to the fry stage. This extended exposure to toxicants in the water during early growth stages can greatly affect medaka survivability and therefore aids in evaluating the effects of developmental toxicants such as mercury.

Since testing began, the embryo-larval tests have been characterized by high mortality (typically 65-95%) of medaka raised in EFPC water from the specified testing sites. However, in recent years, mortality at all sites has abruptly decreased to less than 35%, a significant departure from the past results, as shown in figure 1.

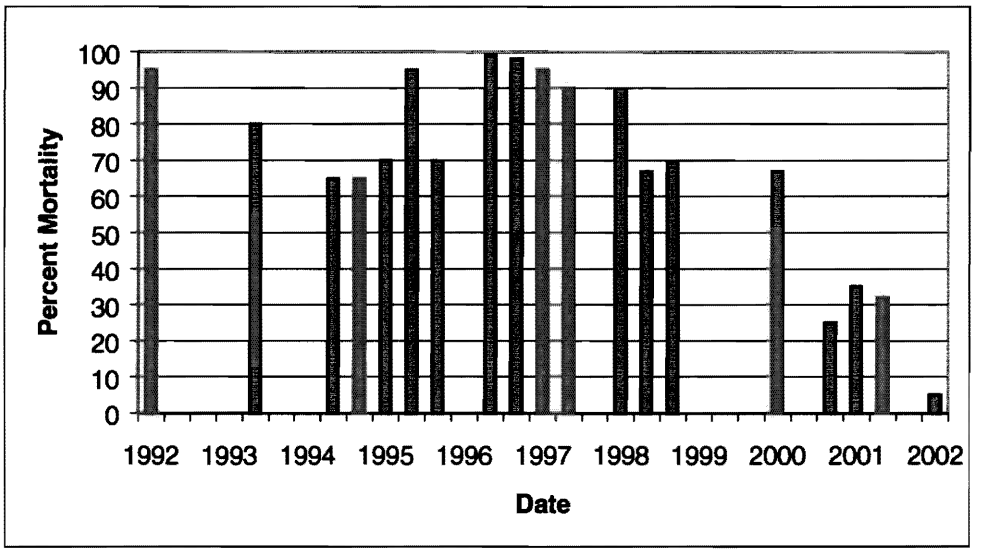


Figure 1. Mortality summaries of medaka toxicity tests from 1992 to 2002 on the East Fork Poplar Creek watershed. Adapted from "Bioindicators of Reproductive Competence," 2002 BMAP report.

A decrease in mortality of this magnitude may reflect several potential possibilities. The most optimistic possibility is that ecological conditions of EFPC have improved considerably, and the toxicity of the water has indeed decreased. Unfortunately, no ready explanation for such a decrease in toxicity is apparent, as discharges from Y-12 have not changed significantly, during the period of marked decreases in observed toxicity in this test. An absence of data suggesting broadscale environmental changes in EFPC requires reexamination of laboratory practices associated with the

test, namely possible changes in the sensitivities of laboratory stocks of medaka or concurrent changes that occurred in experimental procedures, specifically a change in test containers.

Laboratory experiments at ORNL have utilized stocks of medaka originally devised from the same supplier throughout the testing period. In the past, new stocks of fish have been received from the supplier, but for many generations, older fish have been replaced by their offspring bred in the laboratory. The possibility of changes in the integrity of the medaka fish stock over time must therefore be addressed. The fish at the ORNL laboratory are kept in six separate living streams supplied by a continuous flow of dechlorinated process water. Each living stream houses a population of medaka from the same hatch date. Either means of repopulating the medaka stock has the slight potential to lead to a change in sensitivity in laboratory populations over time. If this is the case, mortality statistics can potentially change over time, regardless of any compositional variation in the test waters. The potential for varied sensitivities between the medaka used in the past and the medaka used for current embryo-larval tests is one focus of the current study.

Changes in the laboratory protocol for embryo-larval tests were also implemented and standardized during the period of time when the test results showed such dramatic improvement. When the medaka toxicity tests commenced in 1992, eggs were raised in 7-milliliter borosilicate glass vials. In the year 2000, general fish larval

development tests at ORNL, using not only medaka, but also zebrafish (*Danio rerio*) and fathead minnows (*Pimephalus promelas*), switched from using the vials to plastic 24-well plates. The new media allowed greater ease in checking the test and changing water samples, in addition to associated cost savings. At the time of this switch, studies were completed to ensure that results would be similar between the glass vials and the plastic well plates. However, the potential for altered results needed to be reexamined in light of the observed concurrent decrease in mortality.

Several important factors may result from this change in test media, thereby affecting test results. Glass vials and plastic well plates can both potentially adsorb metal contaminants from the water, effectively preventing their influence on the developing embryo and causing a false negative for the metal during analysis. Another possibility is that volatile environmental contaminants may escape from the unsealed plastic 24-well plates, whereas they may not escape from the sealed glass vials. Organisms raised in glass vials can therefore experience greater exposure to volatiles than those raised in plastic well plates, potentially resulting in higher mortality. In short, if decreased medaka mortality results from changes in experimental media, incongruities observed between these two media have the potential to falsely indicate changes in toxicity in the watersheds, or at least changes in animal responses to the water.

An additional question concerning laboratory media was raised during the progression of this project. In 1999, heavy metal toxicity tests were conducted to establish relative sensitivities and attribute certain abnormalities to specific metals. These tests utilized untreated polystyrene 24-well tissue culture plates (Odom 1999). Based largely on the ease of this series of tests, laboratory procedures opted to begin using untreated well plates instead of glass vials. However, at some undocumented point between 2000 and present tests, these well plates (with positively charged wells) were inadvertently replaced with tissue culture treated plates (with negatively charged wells), raising the possibility that test results could have been affected by this change in media as well, especially concerning metal ion contaminants in water samples. This issue is briefly addressed in the project as well.

This project is designed to examine two potential alternative explanations for the apparent reduction in toxicity noted in medaka embryo-larval tests of water from EFPC, in an effort to ultimately determine the actual cause for this reduction. Current status of the ORNL medaka stock is inspected holistically by determining the viability of eggs that are produced and their reactions to toxicity tests. More importantly, the methodology of embryo-larval test protocols is evaluated by establishing effects of using glass vials versus plastic 24-well plates on medaka mortality. If these possible artifacts can be discounted as a cause of the decreased mortality in the medaka embryo-larval tests, then the environmental factors can be investigated.

MATERIALS AND METHODS

Medaka Embryo-Larval Test Procedures

Adult medaka are kept in living streams with continuous flow-through dechlorinated process water. The laboratory accommodating the living streams is windowless, and lighting is controlled by means of a timer with a fifteen hour photoperiod, beginning at 0600 and ending at 2100. Temperatures are monitored at 22-23 °C; to promote egg production, temperatures are increased to 24-26 °C at least 24 hours prior to egg collection. Fish are fed both newly hatched brine shrimp (*Artemia*) and flake food (Aquatox) twice per day, at approximately 0800 and 1500 hours.

To conduct the embryo-larval tests, medaka eggs must first be collected. Approximately thirty minutes after the morning feeding, females are netted and egg masses removed by gently rolling the eggs from the ventral surface until the mass comes free. Using a dissecting microscope, the eggs are separated from each other with watchmaker's forceps and placed into a petri dish containing distilled water. Separated eggs are randomized by swirling the water and then set aside in an incubator at 27 °C. After a maximum of two-hours, viable eggs are selected with the aid of a dissecting microscope and a transfer pipet, and transferred into a plastic petri dish containing the test water. By placing eggs directly into test water, later dilution of test water is avoided. Eggs are then placed individually into wells or vials

containing one milliliter of test water. The tests are kept in an incubator at 27 °C. Additional test water is kept refrigerated, and small allotments are warmed to approximately 27 °C for water changes, which occur every other day. Water changes ensure that the developing embryo does not deplete nutrients and dissolved oxygen in its milliliter of water and die as a result. With regular water changes, if mortality occurs, it can be attributable to toxicity of the water. Reconstituted water (Franson 1992; Odom, 1999) is used for the control.

Embryo-larval tests examine a group of twenty medaka embryos per treatment, raised until forty-eight hours after hatching or death occurs, but not longer than twenty-one days. The embryos are observed daily, and checked for status in hatching and mortality as well as abnormalities in development. After the first observation of a successfully hatched fish, it remains in the test for only two additional days before being removed so as to avoid artifactural death from starvation as a consequence of yolk absorption. Fish that are partially hatched are not counted as hatched; individuals have to be completely free of the chorion. Fish that have died but are obviously hatched were counted in the hatch data even though they may have never been observed as live hatches.

Once collected, data is statistically evaluated for changes in populations. A lowest observed effect concentration (LOEC) and a no observed effect concentration (NOEC) are calculated, respectively, by determining the lowest concentration with

results that significantly differ from the control and the highest concentration with results not significantly different from the control. In addition, the concentration that results in fifty percent mortality of the test organisms (LC_{50}) is also calculated. To detect significant differences in experimental media, chi-squared tests are performed using the mortality data, in which vials and plates containing aliquots of the same environmental water samples are paired against each other (e.g., control vial with control plate). With some of the tests, data are suitable to be grouped together and entered into the statistical software JMP, which expand the statistical tests (e.g., t-test) that could be run on the data. In general, one to two deaths per test are normal, and differences of this magnitude are not considered noteworthy. However, mortality above ten percent in the control invalidates the test.

Evaluating Sensitivities of Medaka Populations

Data comparing the sensitivity of the current medaka stock to the medaka stock used in earlier tests were obtained by collecting eggs and subjecting them to a copper sulfate toxicity test run in untreated 24-well plates. In 1999, a variety of heavy metal toxicity tests, including those using copper, were conducted in untreated 24-well plates to establish relative sensitivities to the metals. These tests relied on eggs from medaka ancestral to those currently raised in the laboratory. By comparing present copper sulfate toxicity results with the 1999 data, any changes in fish sensitivities to copper, a standard reference toxicant, could be examined.

In addition to copper sulfate tests, a potassium chloride test was conducted on each living stream to check for possible differences among the current populations. Because eggs from all five living streams may not be utilized in a specific embryo-larval test, the potassium chloride test was conducted to ensure that the populations in each living stream have comparable sensitivities and yield similar results. Results were considered individually in order to compare NOEC, LOEC, and LC_{50} among all five current populations. Because the required size of this test so large scale, it was run in well plates.

Differences between Experimental Media

Whereas the primary concern regarding the experimental media was raised as a result of significant change in medaka mortality in water from EFPC, water from Pigeon River and White Oak Creek watersheds were used in addition to EFPC to assess differences between glass vials and treated 24-well plates in this project. Embryo-larval tests were conducted in both lab media using water from locations that have been extensively tested in the past. These watersheds contained different contaminants of particular environmental concern, allowing a more detailed look at specific environmental influences coupled with the potential effects of laboratory protocol.

Water from three locations along EFPC was collected. Water from these locations is of interest because it contains notable mercury contamination resulting from the

upstream influence of the Y-12 National Security Complex. The first site, Station 17 (kilometer 23.5), reflects toxicity from Y-12 before the water enters public domain. Jackson Farm (kilometer 14) is a considerable distance downstream from the plant, allowing an examination of spatial distribution of contamination. Further downstream is the third site, Country Club (kilometer 10), which has additional input of a sewage treatment plant located upstream, between it and Jackson Farm. Three tests were run on this watershed.

The second watershed tested was White Oak Creek (WOC), also located in Oak Ridge, Tennessee. Water from three sites, kilometers 2.3, 3.8, and 6.8 were used. Kilometer 6.8 is a reference site located upstream of ORNL, while the lower two sites receive runoff and wastewater discharge from ORNL. WOC 3.8 is located directly below settling ponds for radioactive waste; therefore possible contamination attributable to leaching from the soils and groundwater movement can be checked at this site. WOC 2.3 is below radioactive burial grounds and trenches with radioactive contaminants, as well as other contaminants such as trichloroethylene. One test was conducted on WOC for this study.

Finally, water from Pigeon River in Canton, North Carolina and Denton, Tennessee was assessed with a single test. Three locations, above the paper mill plant (kilometer 104), below the paper mill plant (kilometer 103), and Denton (kilometer 26), were chosen for this test to reflect their relative position to Blue Ridge Paper

Company (formerly Champion Papers, Inc.). These sites are of interest because the potential effects of paper and pulp mill effluent (e.g., bleached kraft mill effluent) the Pigeon River watershed. Again, additional contaminants may play a role as well.

Toxicity tests were also conducted with increasing concentrations of heavy metals to determine if mortalities varied among the media. The copper sulfate test previously discussed in the medaka population section was conducted to additionally test this potential difference. Besides the untreated well plates required for that test, glass vials and treated well plates were also used to ensure no further effects were influencing the results.

RESULTS and DISCUSSION

Evaluating Sensitivities of Medaka Populations

The primary objective for this line of testing was to establish whether or not the present medaka stock have changed significantly in their responses to contaminants in recent years. In other words, could a change in medaka sensitivity to toxicants explain the decreased toxicity observed in recent years when EFPC water was tested? Two tests were conducted to address the question of potential change in sensitivities of laboratory medaka to environmental contaminants.

A copper sulfate toxicity test was conducted with two purposes: (1) to illustrate whether medaka sensitivities to copper sulfate had changed since 1999, a year in which medaka populations were sensitive to the EFPC water, and (2) to determine whether the choice of test media, glass vials, treated 24-well plates, and untreated 24-well plates, affected the results of toxicity tests with this reference toxicant.

The 1999 copper sulfate test was run in untreated plates and compared graphically to 2002 untreated well plate data in figure 2. The 2002 test utilized pooled eggs from three of the five living streams (two older populations and one middle-aged population). The data represented in figure 2 revealed no significant difference between the effects of copper sulfate on the current medaka population to that