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May 4, 2012

Dr. Neil Carriker
Program Manager, Special Projects
Tennessee Valley Authority Kingston Ash Recovery Project
Kingston, Tennessee

Subject: Preliminary Evaluation of 2011 ORNL Results: ***Fish Early Life Stages – In vitro spawning study***

Dr. Carriker,

I am pleased to submit to you a preliminary evaluation of 2011 *Fish Early Life Stages Task: In Vitro Spawning* results. The attached memorandum covers the spring 2011 fish in vitro spawning study conducted in support of the TVA Kingston Ash Recovery Project at several locations in the Emory and Clinch Rivers. This personal communication is meant to provide a provisional assessment of the results of this study. Data and conclusions may be subject to revision as statistical analyses continue, and thus conclusions should be considered preliminary prior to the issuance of a draft report.

We hope this preliminary update is useful to you. If you have any questions or require additional information, please don't hesitate to call me at (865) 574-9387.

Sincerely,

Mark S. Greeley, Jr.
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Manager, ORNL Environmental Toxicology Laboratory
Environmental Sciences Division
Oak Ridge National Laboratory

cc: M. J. Peterson, ORNL
D. Jones, ARCADIS

Preliminary Evaluation of 2011 ORNL Results:

Fish Early Life Stages Task: Spring 2011 in Vitro Spawning Study

May 4, 2012

Introduction

On December 22, 2008, a dike containing fly ash and bottom ash at the Tennessee Valley Authority's (TVA) Kingston Fossil Plant in East Tennessee failed and released a large quantity of ash into the adjacent Emory River. A byproduct of coal combustion, coal ash contains a variety of metals and other elements, particularly selenium, capable of influencing the reproduction and/or early development of exposed fish populations (Lemly 1993; Besser and others 1996). Elevated body burdens of selenium in female fish in a coal-ash contaminated lake have been associated with increased frequencies of atretic oocytes (dead or damaged immature eggs) and reductions in the overall numbers of developing oocytes. Similarly, a number of published studies have reported increased incidences of developmental abnormalities in larval fish exposed to selenium through maternal transfer to the developing eggs either in the laboratory or in contaminated bodies of water (Woock and others 1987, Lemly 1999, Jezierska and others 2009), including sites where coal ash from power plants is the primary source of selenium contamination.

To address the prospective effects of the Kingston fly ash spill on the early life stages of fish exposed in situ in the Emory and Clinch Rivers, fish from the spill site and other locations within these river systems were evaluated for their potential to develop selenium-induced deformities by an adaptation of the in vitro spawning procedures of Janz and Muscatello (2008). Fish were collected during the spring 2011 breeding season from five sites in the Emory and Clinch Rivers (ERM 8.0, ERM 3.0, ERM 0.9, CRM 8.0, and CRM 1.5) (Fig. 1), with redear sunfish (*Lepomis microlophus*) being the preferred species for this study because of relative abundance at each of the study locations and a body size sufficient to provide the requisite number of eggs for developmental trials. Similar in vitro spawning trials were also attempted with bluegill sunfish (*Lepomis macrochirus*), a species which is known to be particularly susceptible to the selenium constituent of fly ash, although trials with this species were limited by the small number of female bluegill sunfish in appropriate reproductive condition able to be collected for these purposes.

Female fish in spawning or near-spawning condition were collected by electroshocking from each study site and brought to ORNL for spawning trials. Mature eggs for fertilization purposes were obtained either directly from field-collected fish or from fish injected with a spawning hormone solution in the laboratory to induce final egg maturation. Milt was pooled from at least four different males from the same sites and kept on ice until used to fertilize the ovulated eggs (Fig. 2). Target sample sizes were four clutches per species per site (as recommended in Janz and Muscatello 2008), although in the case of redear sunfish we successfully spawned a minimum of six clutches from each of the five study sites. Developing embryos were subsequently raised in replicate incubation chambers through hatching and yolk absorption in water from both the respective sites of origin and reference water (from ERM 8.0) in a two-way (crossover) ANOVA experimental design. Test end points included fertilization success, survival, hatching success, and incidences of developmental abnormalities through the yolk-absorption stage of development. For the purposes of data analysis and presentation, developmental

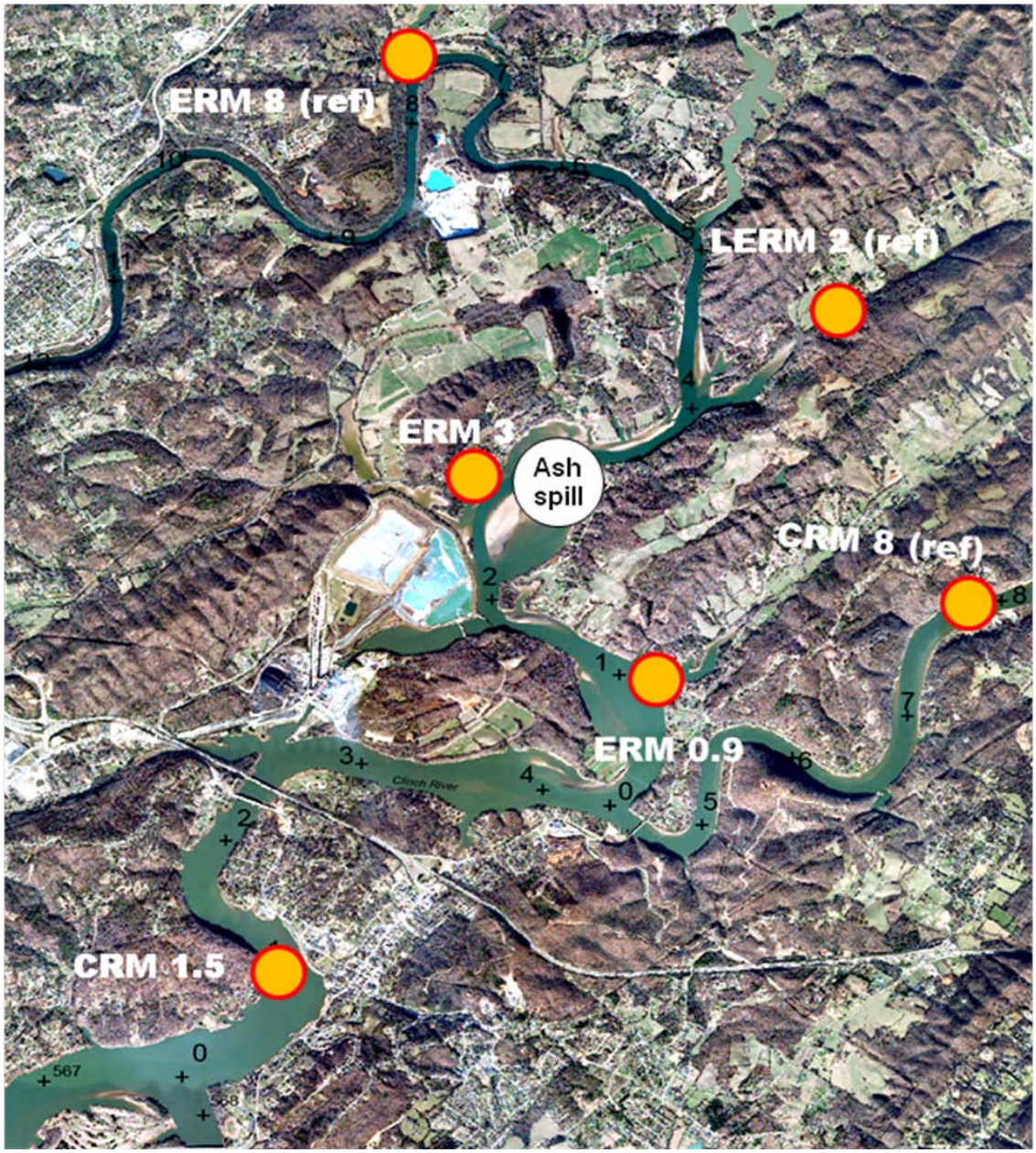

Figure 1. Map showing fish sampling locations in the Emory and Clinch Rivers for in vitro spawning trials. ERM = Emory River Mile; CRM = Clinch River Mile; LERM = Little Emory River Mile latter site not used in this study).

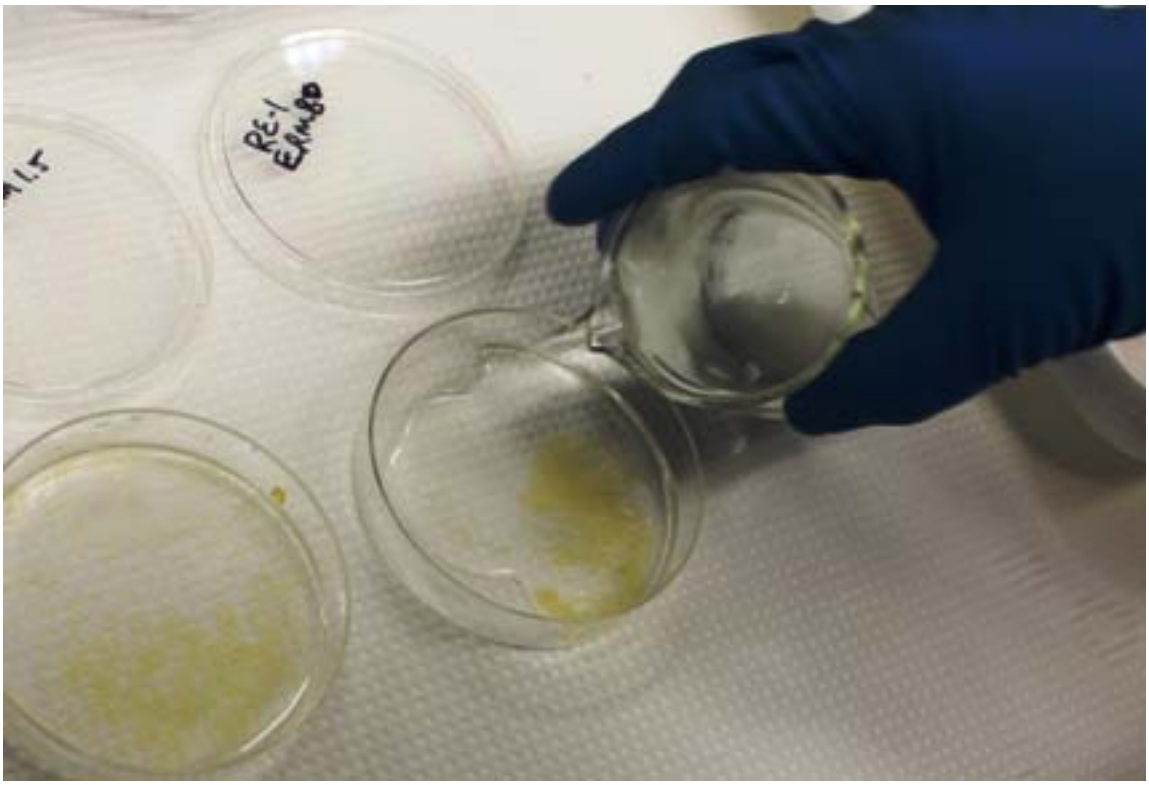

Figure 2. Water being added to redear sunfish eggs following in vitro fertilization to complete the embryo activation process.

abnormalities scored at the time of test termination were considered lethal and added to the final totals for mortality (EPA 2002).

Preliminary Results

After considerable trial and error, a total of 43 clutches of redear sunfish eggs from five locations were successfully fertilized and cultured in the laboratory (Table 1) in the spring of 2011 by methods adapted from Janz and Muscatello (2008) and EPA (2002). Six other clutches of eggs were prepared and followed for 24 hrs before being discarded due to a lack of successful fertilization and/or early development. For the purposes of this preliminary evaluation, these failed trials were not included in the data summary.

Average embryo-larval survival in these redear sunfish tests ranged from a low of 74% in the case of ERM 0.9 embryos cultured in site-water to a high of 94% for CRM 1.5 embryos cultured in site-water (Table 1). Relative survival in reference water from ERM 8.0 varied from a high of 89% for ERM 8.0 embryos to a low of 77% for ERM 3.0 embryos.

Table 1. Survival of redear sunfish embryos and larvae during 7-d laboratory tests following in vitro spawning of fish collected from study sites in the Emory and Clinch river systems (TN) during the spring 2011 breeding season. Preliminary results as of May 4, 2012.

Location[a]	Number of Spawning Trials	Embryo-Larval Survival[b] (mean % ± SEM)	
		Site Water	*Reference water*[c]
ERM 8.0	7	–	89.4 ± 3.7
ERM 3.0	14	80.1 ± 6.3	77.4 ± 7.1
ERM 0.9	8	73.9 ± 7.2	87.3 ± 2.9
CRM 8.0	6	78.2 ± 5.3	83.2 ± 4.3
CRM 1.5	8	94.3 ± 0.8	86.6 ± 3.5

[a] ERM = Emory River Mile; CRM = Clinch River Mile
[b] Survival in site water or reference water at end of 7-d test; larvae with abnormalities on day 7are considered dead for test purposes
[c] Sterile-filtered water from ERM 8.0

The somewhat lower average survival and higher variability associated with ERM 3.0 embryos in these trials were largely due to poor test results from two specific clutches of eggs out of the total of 14 tested for this site (data not shown). Similar poor results were occasionally observed in isolated clutches from other sites as well. Whether these were due to actual site differences in embryo fitness or embryo contaminant burdens or instead due to another factor such as the relative age of the eggs used the in vitro spawning trials is not clear at this time.

In addition to the redear sunfish spawning trials, a total of 10 clutches of bluegill sunfish eggs from four different locations were successfully fertilized with pooled milt from males from the same locations and cultured in the laboratory by the same methods employed with redear sunfish (Table 2). One other clutch was followed for 24 hrs then discarded due to a lack of successful fertilization and/or early development. For the purposes of this preliminary evaluation this failed trial was not included in the data summary.

Embryo-larval survival in the limited number of successful bluegill trials varied from a low of approximately 81% in ERM 3.0 embryos raised in reference water to approximately 96% in pooled embryos obtained from fish from three different sites (ERM 8.0, CRM 8.0, and CRM 1.5) that were also raised in reference water (Table 2).

Further in vitro spawning efforts with bluegill sunfish were significantly hampered by an inability to collect sufficient female bluegill sunfish in the correct reproductive condition from most of the study sites. In addition, the relatively small size of the female bluegill sunfish that were collected appeared to be highly detrimental to the post-electroshocking survival of these fish in the laboratory for these in vitro spawning purposes.

Preliminary Conclusions

- Redear sunfish were successfully spawned in vitro during the spring of 2011 from the five study sites.
- The average survival of redear larvae free of developmental abnormalities was >70% for all study sites with both sunfish species, an unexpectedly high rate of survival considering the developmental nature of these in vitro spawning procedures as applied to these particular fish species.
- The average survival of redear larvae free of obvious developmental abnormalities was >70% for ERM 3.0, ERM 0.9, and CRM 1.5, the 3 sites located downstream of the fly ash spill, in particular.
- Redear sunfish embryo-larval survival associated with parental fish from CRM 8.0 does not differ significantly from survival associated with fish from lower Emory River locations

Table 2. Survival of bluegill sunfish embryos and larvae during 7-d laboratory tests following in vitro spawning of fish collected from study sites in the Emory and Clinch river systems (TN) during the spring 2011 breeding season. Preliminary results as of May 4, 2012.

Location[a]	Number of Spawning Trials	Embryo-Larval Survival[b] mean % ± SEM	
		Site Water	*Reference water*[c]
ERM 3.0	6	87.0 ± 3.0	81.2 ± 3.9
Other sites[d]	4	–	95.8 ± 0.5

[a] ERM = Emory River Mile; CRM = Clinch River Mile
[b] Survival in site water or reference water at end of 7-d test; larvae with abnormalities on day 7are considered dead for test purposes
[c] Sterile-filtered water from ERM 8.0
[d] Includes clutches of eggs from 2 females from ERM 8.0, 1female from CRM 8.0, and 1 female from CRM 1.5

Considerations and/or Uncertainties

Redear sunfish embryo-larval survival associated with certain study sites, including ERM 3.0, may prove to be significantly reduced in comparison with fish from the upstream reference site at ERM 8.0 once statistical analyses are eventually finalized

This investigator suspects from his personal anecdotal experiences with these initial spawning trials that the specific reproductive status of the female sunfish supplying mature eggs for spawning trials – whether collected with ripe eggs before spawning, during spawning, or post-spawning from the field or induced through hormone injections in the laboratory – may possibly be one of the most significant factors in the subsequent success of an experimental clutch, due possibly to relationship of this factor with the post-maturational age of the eggs used in these spawning trials.

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