#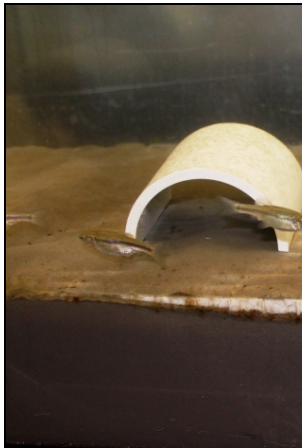 EVALUATING THE EFFECTS OF THE KINGSTON COAL ASH RELEASE ON FISH REPRODUCTION AND EARLY LIFE STAGES: LONG-TERM EXPOSURES TO ASH IN THE LABORATORY

OAK RIDGE
National Laboratory

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April 2014
Final

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EVALUATING THE EFFECTS OF THE KINGSTON FLY ASH RELEASE ON FISH REPRODUCTION AND EARLY LIFE STAGES: LONG-TERM EXPOSURES TO FLY ASH IN THE LABORATORY

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1. INTRODUCTION

On December 22, 2008, a dike containing coal 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 contaminants of potential concern including mercury, arsenic, and selenium. Selenium in particular is known to be capable, when at sufficient concentrations and in specific forms, of impacting the reproduction or early development of exposed fish populations (Lemly 1993 & 1999; Besser and others 1996, USEPA 2004).

The primary route of selenium exposure to larval fish appears to be uptake by adult female fish through the food chain and subsequent maternal transfer to the developing eggs (Woock and others 1987, Coyle and others 1993, Lemly 1999, Moscatello and others 2006). However, fish eggs are also capable of concentrating heavy metals and other environmental contaminants directly from water-borne exposures during embryonic development (Devlin 2006, Jezierska and others 2009) and from direct contact of fertilized eggs and developing embryos to contaminants in surface water and sediments (Woock and others 1987, Coyle and others 1993, Jezierska and others 2009).

Associated studies conducted by researchers at Oak Ridge National Laboratory (ORNL) in conjunction with TVA examined the bioaccumulation of metals in fish tissues (Adams and others 2012), and the health (Adams and Fortner 2012) and reproductive condition (Greeley and others 2012a) of fish exposed to fly ash in the Emory and Clinch Rivers downstream of the Kingston Ash Release. The potential effects of the ash release on fish reproduction have also been addressed in a relatively short-term laboratory exposure study (Greeley and others 2012b) and by an in vitro spawning study conducted on fish sampled from sites downstream and upstream of the ash release (Greeley and others 2012c).

Previous short-term laboratory study conducted by ORNL focused on the effects of direct contact exposures of eggs and embryos of the fathead minnow (*Pimephales promelas)* to river bottom sediment containing ash from the Kingston ash release in 7-d toxicity tests adapted from a standard USEPA embryo-larval survival and teratogenicity test (method 1001.0 in

EPA 2002). The current study focuses on the potential effects of much longer experimental exposures to fly ash on both the reproduction of adult fathead minnows and the early developmental success of offspring under controlled laboratory conditions.

2. APPROACH AND METHODS

Objectives

The original objectives of the project, as conceived in collaboration between TVA, project risk assessors, and ORNL, were to examine the potential effects of the Kingston Fly Ash Release on the reproduction of adult fish and the developmental success of their offspring through both direct and food chain exposures of bluegill sunfish (*Lepomis macrochirus*) to ash under controlled laboratory conditions. Once it was determined in pilot studies that the characteristics of the ash delivered to ORNL was not conducive to long-term laboratory studies with adult bluegill sunfish of appropriate breeding size, the current alternative study with fathead minnows was adopted to complement both a previous short-term laboratory embryo-larval exposure study with this species (Greeley et al. 2012b) and an in vitro spawning study with sunfish exposed to ash in the Emory and Clinch Rivers (Greeley et al. 2014). The objective of the present study was to determine the potential effects of direct long-term contact and water-borne exposures to ash from the Kingston Fly Ash Release on the survival and reproduction of adult fathead minnows and the early development of offspring under controlled laboratory conditions.

Ash and Sediment Samples

Bulk ash samples were collected by TVA personnel with an excavator from a drainage area adjacent to the TVA Kingston Fossil Plant settling basin on March 18, 2010 (Fig. 1) and stored in 30-gallon drums in a refrigerated truck at <6°C until delivery to ORNL on May 6, 2010. Ash was stored refrigerated at ORNL, then mixed on a powered barrel roller to reincorporate pore-water before initial use to line the bottom of exposure tanks (Fig. 2). Water was then slowly added to the static tanks and ash allowed to settle before a slow flow-through supply of water was eventually maintained to each tank. Ash was allowed to further settle for varying amounts of time from days to weeks before introductions of fish were attempted.

Once it was determined that the original study design with bluegill sunfish was impracticable, and an alternative study design using fathead minnows was adopted, reference sediment was obtained by TVA from the upper Clinch River (CRM 189.0) and transferred to ORNL on August 4, 2010. Reference sediment was stored refrigerated at ORNL, then re-mixed before use with a stainless steel drill attachment to reincorporate pore-water (Fig. 3).

Figure 1. Map showing source location for ash (WABULK318) used for long-term exposure studies conducted by ORNL. Original map supplied by TVA, and includes additional sampling locations for sediment used in a separate short-term exposure study (Greeley and others 2012b).

Figure 2. Initial experimental design for bluegill sunfish exposures with ash layering the bottom of multiple rectangular fish tanks at ORNL.

Figure 3. Sediment sample from the lower Emory River is shown being mixed at ORNL with a customized stainless steel bit attached to a variable-speed drill (from Greeley and others 2012b) similar to mixing procedures with current reference sediment samples.

Species

   Bluegill sunfish - For initial studies and stocking the experimental tanks, male and female bluegill sunfish were collected in the spring of 2010 by TVA and ORNL personnel using boat electrofishing from sites in the Emory and Clinch Rivers both upstream and downstream of the ash spill site. Sunfish were transferred to the ORNL Aquatic Ecology Laboratory by ORNL personnel in large aerated coolers containing site-water, then gradually acclimated to UV-treated and dechlorinated flow-through process water in the laboratory and maintained in large indoor holding tanks until needed.

   Pilot studies with bluegill sunfish of both genders and various sizes and involving various numbers of fish soon indicated that the ash bottoms installed in the large experimental tanks did not harden or crust over, unlike prior expectations, and instead remained very light and "fluffy" and susceptible to immediate suspension into the water column with the introduction of even the smallest of fish. Because such a situation would be both physically detrimental to fish health in long-term exposures and incompatible with ORNL wastewater discharge practices, various strategies were attempted to enable exposure of fish to ash in experimental tanks while maintaining water quality and limiting the physical discharge of ash through ORNL wastewaters.

   Eventually, a combination of a polystyrene grid supporting a 1000 micron nitex nylon screen over the top of the ash layer (Fig. 4) proved sufficient to allow the passage of water and some ash through the screening while generally dampening down the immediate "cloud" of ash moving into the water column that occurred otherwise with any fish movement near the ash. However, no strategy other than totally covering the ash with an impervious layer of material was sufficient for use in the case of even the smallest adult male bluegill sunfish, which in both startle behavior and typical fish-to-fish interactions tended to rapidly and very powerfully flick tail fins in close proximity to the bottom of the tanks and thus significantly disturb the ash bottoms regardless of the dampening strategy (other than the afore-mentioned use of an impervious barrier). Therefore, in consultation with TVA, it was eventually decided that the mutual characteristics of the light and soft ash and a relatively large and aggressive fish such as male bluegill sunfish were incompatible with the continuation of the original study design. With significant time and effort having already been spent on the bluegill study plan, an alternative test species was sought to pursue at least a subset of the original objectives.

Figure 4. Comparison of two fish large tanks at ORNL containing ash overlaid with nitex screening supported on a polystyrene grid. Tank on the left had several bluegill sunfish introduced just prior to the picture being taken; fish were introduced to the tank on right approximately 30 minutes earlier (note suspended ash in the water column and on top of PVC tiles in tank to the right).

Fathead minnows – One likely candidate for an alternative fish species to bluegill sunfish for studying the effects of long-term exposures to ash on fish reproduction under controlled laboratory conditions was already present at ORNL (Fig. 5). The ORNL Environmental Toxicology Laboratory maintained cultures of fathead minnows that supplied embryos for use in a previous study of the short-term effects of fly ash on fish early development (Greeley and others 2012b). The fathead minnow is a small and well-characterized native fish widely used for aquatic ecotoxicity evaluations and readily maintained in breeding colonies in the laboratory (U.S. EPA 2002). It was quickly determined that minimal dampening with nitex screening supported by polystyrene grids was sufficient to allow contact of fathead minnows with ash while maintaining water quality and limiting the potential for ash discharges to the ORNL wastewater streams.

Figure 5. Male and female fathead minnows (*Pimephales promelas*). Male (top) has the typical secondary sexual characteristic of a prominent dorsal epithelial pad (not present in female at lower left) for which the species is named.

Final Study Design

The final study design using fathead minnows closely followed standard practices for maintaining breeding colonies of this species for use in supplying fish larva for toxicity testing. Fifteen 20-gallon glass aquaria were cleaned and randomly distributed on aquarium racks that were currently housing other breeding tanks of minnows. In August 2010, 5 aquaria were lined with layers of ash newly-mixed from barrels of ash (Fig. 6) maintained in cold-storage since initial transfer to ORNL custody in May 2010. Five other aquaria were lined with equivalent layers of reference sediment, while the remaining 5 aquaria were designated as controls lacking either ash or sediment. Each tank including the controls was then further lined with a100--micron nitex screen supported by a polystyrene grid that was gently pressed into the surface of the ash or sediment layers (if present). Aquaria were supplied with a slow flow-through supply of UV-treated water sufficient for two complete water changes per day, an air-supply, and a heater to maintain temperatures at ideally 25°C ± 1°C. Aquaria were then gently aerated for approximately two weeks to allow complete ash and reference sediment settling before the introduction of test fish.
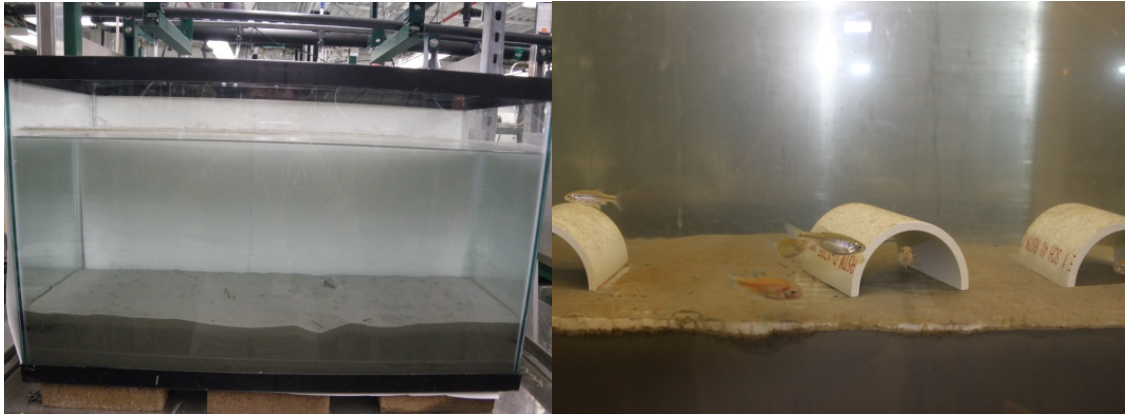
Figure 6. Final experimental design for long-term fathead minnow exposures to ash from the Kingston ash spill. Picture at left shows settled ash layering the bottom of an aquarium; aquarium to right has bottom layer of ash overlaid with nitex screen supported with a polystyrene grid. Note absence of ash in the water column and only thin layer of ash on top of screening and spawning tiles. Actual test aquaria were operated routinely with two spawning tiles per tank.

At test initiation, two clean PVC "spawning tiles" made from pipes cut in-half and roughened up slightly on the underside were placed at opposite ends of each aquarium. Nine female fathead minnows and two males of equivalent age cohorts were placed in each tank and allowed to acclimate for a week before the test was officially begun. During the acclimation period, the few fish that appeared sick or distressed from handling were replaced. The test began in early September with the placement of fresh spawning tiles in each tank.

For the purposes of monitoring cumulative egg production per aquaria, spawning tiles were replaced every 2-3 days (before hatching would typically occur in this species) and pictures taken of the underside of the removed tiles to document the numbers of attached eggs or embryos (Fig. 7). Embryos were then removed and discarded and tiles cleaned for later use. In order to determine the relative percentages of viable embryos among the aquaria, for a period of 8 weeks during the middle of the test period tiles were routinely soaked in a dilute solution of methylene blue for 30 minutes after removal from aquaria and prior to pictures being taken. Unfertilized eggs or damaged or dead embryos take up the vital dye and can be discerned in pictures by a residual blue tint.
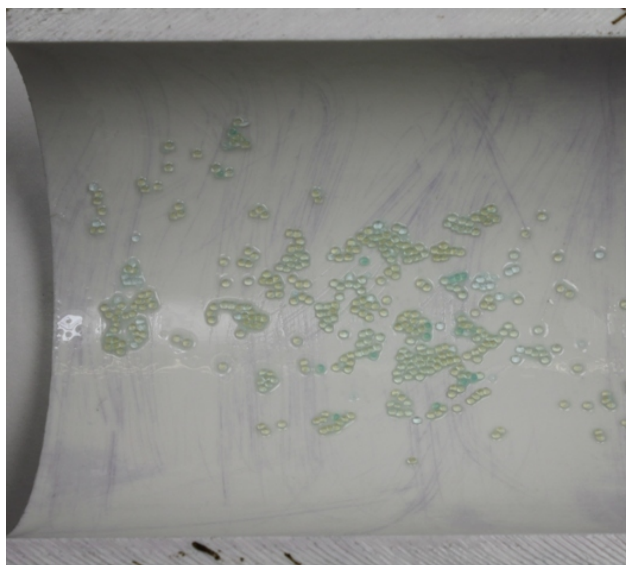
Figure 7. PVC spawning tile from a fly ash long-term exposure tank with attached fathead minnow embryos. Note slight blue tint in some eggs indicative of non-viable embryos following brief treatment with a methylene blue solution.

Although egg production on tiles stopped being monitored on a routine basis after 120-days of exposure, fish remained in the aquaria for approximately three additional weeks while developmental tests were conducted on embryos removed from the tiles. Test procedures were adapted from an EPA fathead minnow 7-d embryo-larval survival and teratogenicity test method for effluents and receiving waters (method 1001.0 in EPA 2002). Fertilized eggs were collected from spawning tiles placed in the aquaria on the prior afternoon, ensuring that embryos were ≤ 24-hrs old at the time of collection. Eggs were allowed to water-harden in aquarium water for approximately 1-hr after collection, then viable embryos < 24-hr old as indicated by developmental stage were transferred to dishes containing water from either the aquarium of origin or a control aquarium. Water was pre-filtered as needed through a 0.45 µmm or larger pore-size filter and subsequently sterile-filtered at 0.2 µmm prior to storage at ≤ 4° C in the dark for the duration of a test.   Before use in initiating tests or in daily water changes of test chambers, aliquots of stored water were brought to 25 ± 1 ° C in a water bath and/or temperature –controlled environmental chamber. Following randomization of the fertilized eggs, 10 embryos were transferred to each of 4 replicates of both aquarium and control water. For each test, embryos and larvae were incubated in environmental chambers maintained at 25 ± 1 ° C with a 16L:8D photoperiod for 7-days through the absorption of the larval yolk sac without feeding. Embryos were scored daily for survival, hatching success, and developmental abnormalities, and dead

embryos or larvae were removed and discarded, and water was changed daily. At test termination, approximately 60% of the test solution was removed from each test chamber without disturbing the remaining embryos and larvae, following which an overdose of a fish anesthetic, tricane methanesulfonate (MS-222), was added to each chamber to immobilize and euthanize the test organisms. Embryos and/or larvae were removed from the test chambers with a transfer pipette and placed in clear polystyrene dishes for final scoring of survival and developmental abnormalities, followed by archival storage of remaining test organisms in formalin.

Following test termination, males and females from each tank were euthanized in MS-222, weighed, and gut matter and gonadal tissues removed prior to compositing carcasses by gender for metal analyses. Ovaries were also composited by aquaria for metals analyses. Filtered and unfiltered water samples were composited by aquaria at test termination as well.

Statistical analyses

Homogeneity of variance of egg counts was verified by a Levene's Test (Sokal and Rohlf 1981), an F-distribution test that compares the ratios of the variances from two independent sample populations. If variances proved to be homogeneous, a 1-way Analysis of Variance (ANOVA) was used to test for site effects for the various reproductive parameters. If the ANOVA rejected a null hypothesis of equal means, then the Holm-Sidak or equivalent method was used to test for significant site differences from the reference in response parameters. For data with non-homogeneous variances, differences between means were examined by the Kruskal-Wallis One Way Analysis of Variance in Ranks followed by Dunn's Pairwise Multiple Comparison Procedures. In all cases, the significance level for rejecting the hypothesis of equal means between sites was set at $\alpha = 0.05$.

Endpoints of the fathead minnow embryo-larval toxicity tests were based on mortality, incidences of developmental abnormalities, and total number of dead and deformed embryos and/or larvae (EPA 2002). For the purposes of data analysis and presentation, any developmental abnormality scored at the time of test termination was considered lethal and those embryos and larvae were added to the final totals for mortality. Survival in control water was compared with survival in water from reference sediment- and ash-containing aquaria by 1-way ANOVA and a Holm-Sidak pair-wise comparison procedure at $p = 0.05$.

3. RESULTS AND DISCUSSION

The number of eggs produced per test aquarium during the 120-day exposure study varied considerably between aquaria both within and among treatment groups (Fig. 8). Thus, although the average number of eggs produced per aquarium was actually greatest in the ash-containing aquaria (Fig. 9), the relatively high degree of variability within treatment groups precluded the occurrences of any statistically differences between treatment groups.

Percentages of dead and damaged embryos or unfertilized eggs were greatest in two of the reference sediment-containing aquaria as compared to controls or ash-containing tanks (Fig. 10), but the high degree of variability between aquaria within the experimental groups again precluded seeing any statistically significant differences among treatment groups (data not shown). Furthermore, the aquarium with the lowest percentage of non-viable eggs among all aquaria also contained reference sediment (Fig. 10).

Survival of fathead minnow embryos and larvae during the 7-day embryo-larval laboratory tests that followed 120-day experimental exposures also did not differ significantly among experimental groups (Table 1). However, it should be cautioned that it is impossible to know with this experimental design which parents actually contributed to any particular spawning trial, so pseudo-replication is possible. Furthermore, the reproductive condition of the adult fathead minnows that supplied the embryos for this testing could well have not been optimal after 4-months of sustained reproductive effort without any rest period, although this supposition is not necessarily supported in females at least by their still relatively large and well-developed ovaries at test termination (Fig. 11).

No statistical differences in survival were observed between treatment groups at test termination (data not shown). Furthermore, there were no obvious differences between groups in mean ovary sizes relative to body size (gonadosomatic indices, or GSIs: Fig. 11), again suggesting the surviving females at least were in relatively good condition at the end of the experiment.

Not unexpectedly, mercury and selenium were not detected in aquarium water at test termination (Table 2). However, arsenic was detected, and although still low was statistically higher in fly ash-containing aquaria as compared with control aquaria. Arsenic, mercury (adjusted for sample type), and selenium (not surprising, given the absence of a food chain component in the final alternative study design) in whole body and ovary composites did not differ significantly

across treatment groups, although in both sample types arsenic did tend to be higher in fish from fly ash-containing aquaria as compared with the other experimental groupings.
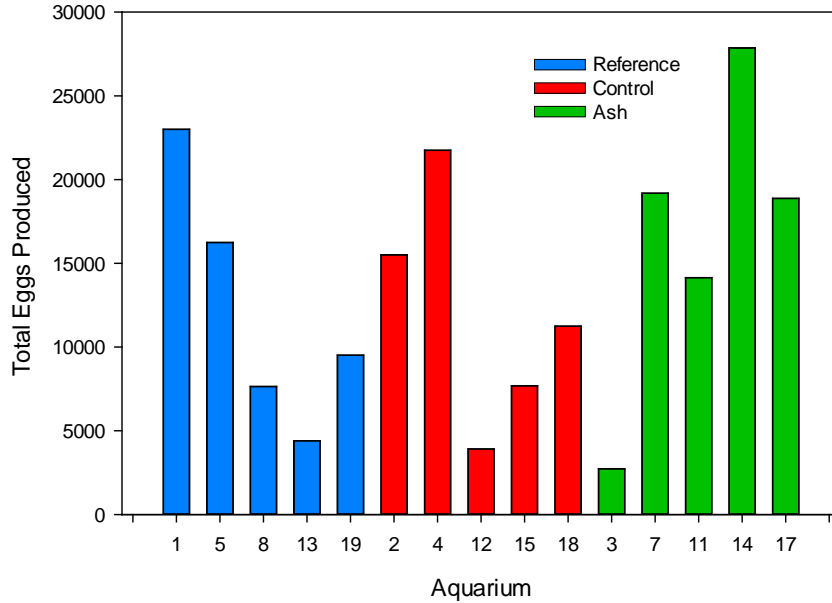
Figure 8. Number of eggs produced per aquarium during the 120-day fathead minnow exposures. Numbers on x-axis indicate random placement of each tank in experimental design.
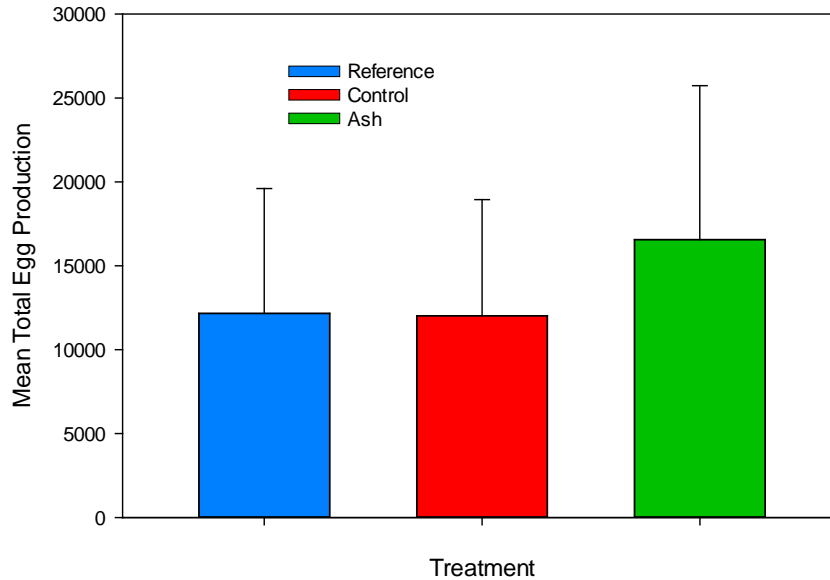
Figure 9. Average number of eggs produced by each treatment group during the 120-day fathead minnow exposures. Error bars indicate standard deviation.

Figure 10. Percentages of non-viable eggs produced per aquarium during the 120-day fathead minnow exposures. Numbers on x-axis indicate random placement of each tank in experimental design.
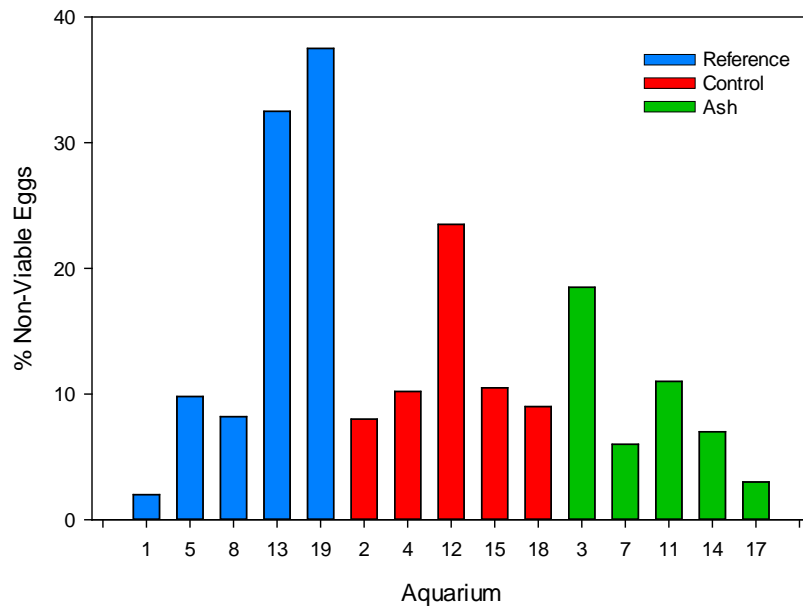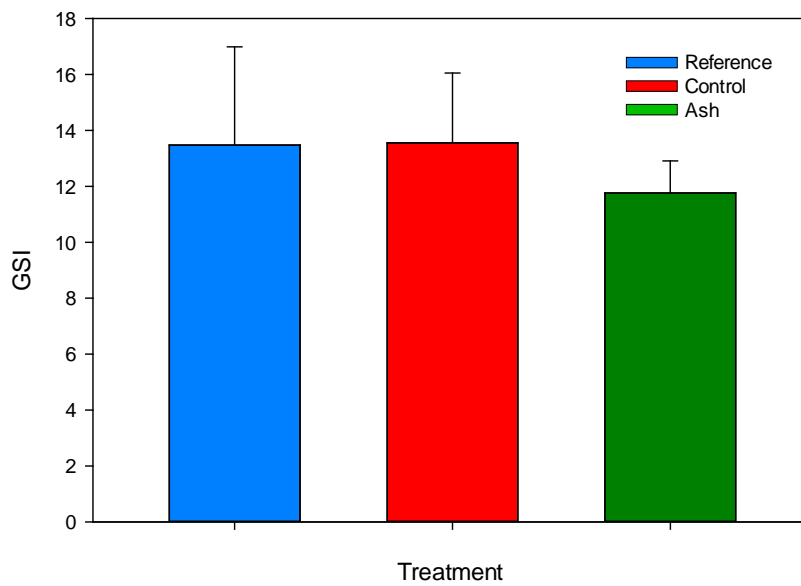

Figure 11. Mean gonad size relative to body size (GSI or gonadal-somatic indices) of female fish in each treatment group by the end of the 120-day fathead minnow exposures. Error bars indicate standard deviation.

Table 1. Survival of fathead minnow embryos and larvae during 7-day laboratory tests following 120-day direct contact and water-borne exposures of parental fish to coal ash.

Treatment	Number of Spawning Trials	Embryo-Larval Survival[a] (mean % ± SEM[b])	
		Control Water	*Aquaria Water: Reference Sediment or Ash*
Control	8	92.5 ± 2.9	88.8 ± 2.1[c]
Reference	7	86.4 ± 3.2	88.9 ± 2.6
Fly Ash	17	90.4 ± 4.6	92.5 ± 3.0

[a] Survival in control or treatment water at end of the 7-d test; larvae with developmental abnormalities on day 7were considered dead for test purposes
[b] SEM = standard error of the mean
[c] Incubation water taken from exposure aquaria

Table 2. Selected metal analyses conducted on composited water samples from treatment aquaria (mg/L) and composite ovaries and whole bodies (mg/kg) of female fathead minnows at the termination of the experiment. Presented as the means of 5 aquaria per treatment. Analyses by Pace and TestAmerica*; nd* = non-detects. Asterisks indicate significant differences from controls at p<0.05.

| Sample Type | Metal | Treatment Group | | |
		Control	Reference	Ash
Water (unfiltered)	Arsenic	0.00033	0.00150	0.00150*
	Mercury	*nd*	*nd*	*nd*
	Selenium	*nd*	*nd*	*nd*
Water (filtered)	Arsenic	0.00033	0.00049	0.00152*
	Mercury	*nd*	*nd*	*nd*
	Selenium	*nd*	*nd*	*nd*
Ovary	Arsenic	0.117	0.113	0.142
	Mercury	0.0182	0.0172	0.0174
	Selenium	1.920	1.780	2.040
Whole body	Arsenic	0.227	0.240	0.271
	Mercury	0.0982	0.0819	0.0963
	Selenium	0.906	0.835	0.920

4. SUMMARY AND CONCLUSIONS

The results of this study indicated that fathead minnows were able to survive for up to 120-d in the laboratory with continuous contact and water-borne exposure to coal ash from the Kingston coal ash release site, supporting the relatively low direct toxicity of this ash to this fish species as previously suggested by the results of an earlier short-term embryo-larval laboratory study (Greeley et al. 2012b).

The results of this study further demonstrated that fathead minnows are capable of successfully reproducing following extended exposures to fly ash in the laboratory. Egg production in fly ash-containing aquaria actually tended to be greater than in control or reference sediment-containing aquaria, although the degree of variability within treatment groups was too great for between-group differences to be statistically significant.

Egg viability and embryo-larval survival (along with incidences of developmental abnormalities) were not reduced in fly ash-containing aquaria as compared with reference sediment-containing or control groups, although caution should be taken in the interpretation of these results due to concerns with possible pseudo-replication.

Thus the current study does not provide evidence of potential reproductive or developmental impacts to fish from direct exposure to fly ash, but also does not address potential impacts in contaminated water-sheds from food chain-mediated mechanisms of exposure.

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