DEPARTMENT OF THE INTERIOR
U.S. FISH AND WILDLIFE SERVICE
REGION 5

FY11 ENVIRONMENTAL CONTAMINANTS PROGRAM
OFF-REFUGE INVESTIGATIONS SUB-ACTIVITY

Final Report

VA - Investigation of In-Stream Contaminant Impacts to Endangered Mussels in the Upper Tennessee River Basin
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II. INTRODUCTION

This report provides the results of a three year investigation that assessed the effects of water quality contaminants and degradation *in-situ* to freshwater mussels in the upper North Fork Holston River (NFHR) of the upper Tennessee River Basin (UTRB). Coursing a landscape predominantly used for grazing livestock, row crop tobacco, and timbering operations, the upper NFHR incurs sedimentation and pollutant inputs that contribute to water quality and substrate degradation. The UTRB supports a tremendous diversity of freshwater mussels (Parmalee and Bogan 1998) and the greatest diversity of fish in North America (Etnier and Starnes 1993). From 1915 to 1997, as many as 21 mussel species have been documented in the NFHR upstream of Saltville, Virginia. Jones and Neves (2000) documented the occurrence of 15 species. Dinkins (2007) documented nine species extant in the far upper reaches of the NFHR. At this time, the upper NFHR is home to two Federal endangered, two Federal candidate, and two Federal proposed mussel species.

Between 1995 and approximately the mid-2000's, malacologists observed and recorded a significant mussel die-off between NFHR Mile (M) 88 - 94. Single water chemistry samples did not indicate contaminants as causative. Henley (2003) documented trematode infestation in mussels in the die-off zone. Mussel viscera showing advanced cercarial development were characterized by almost complete absence of gametogenic activity and atrophy of connective and muscle tissues. Mussel viscera were primarily devoted to cercarial support to the exclusion of gamete production. Impacts of this magnitude were noted for rainbow mussel (*Villosa iris*) and slabside pearlymussel (*Lexingtonia dolabelloides*), a Federal candidate for listing (Henley 2003).

This Environmental Contaminants (EC) special study investigation was undertaken to identify and assess stressors, including effects of contaminants and trematodes, to mussels in the upper NFHR. Growth and survival of mussels caged in-stream was monitored at sites where the die-off occurred, upstream of the die-off area, and in an adjacent watershed. In-stream mussel exposure to contaminants was evaluated by comparing contaminant body-burdens relative to known benchmark thresholds (when available). Histological evaluation was also conducted. In the NFHR, this study represents the first time a native mussel species was deployed *in situ* for an extended period of time to investigate stressor effects.

III. Methods

Site selection

Two study sites (Site 1: NFHRM 88.8 and Site 2: NFHRM 95.3) were located within the NFHR reach where the mussel die-off had occurred and also bracketed agricultural lands (crops and pasture). A reference study site (Site 3: NFHRM 98.9) was chosen upstream of all discharges except for non-point source runoff from pasturing (Figure 1). Site-specific watershed characteristics were obtained from the Multi-Resolution Land Characteristics Consortium's National Land Cover Database (2006). Site 1 is in a fifth order reach of the NFHR and drains an area of 197.2 square miles. Sites 2 and 3 are in a fourth order reach of the NFHR and drain areas of 130.3 and 102.3 square miles, respectively. The drainage feeding the three NFHR sites is predominantly forested and predominant land use is pasture (Table 1). Although crops comprise only a small portion (less than 0.5%) of the total land use upstream of each site, Sites 1 and 2 were directly adjacent to crops buffered by a narrow band of forest approximately 10 meters

wide. All sites historically hosted significant mussel beds. Reference Site 3 still hosts a diversity and abundance of mussels (Dinkins 2007), and substrate type at all three sites is considered suitable for the native mussel fauna (Jones and Neves 2007).

Figure 1. Study site locations

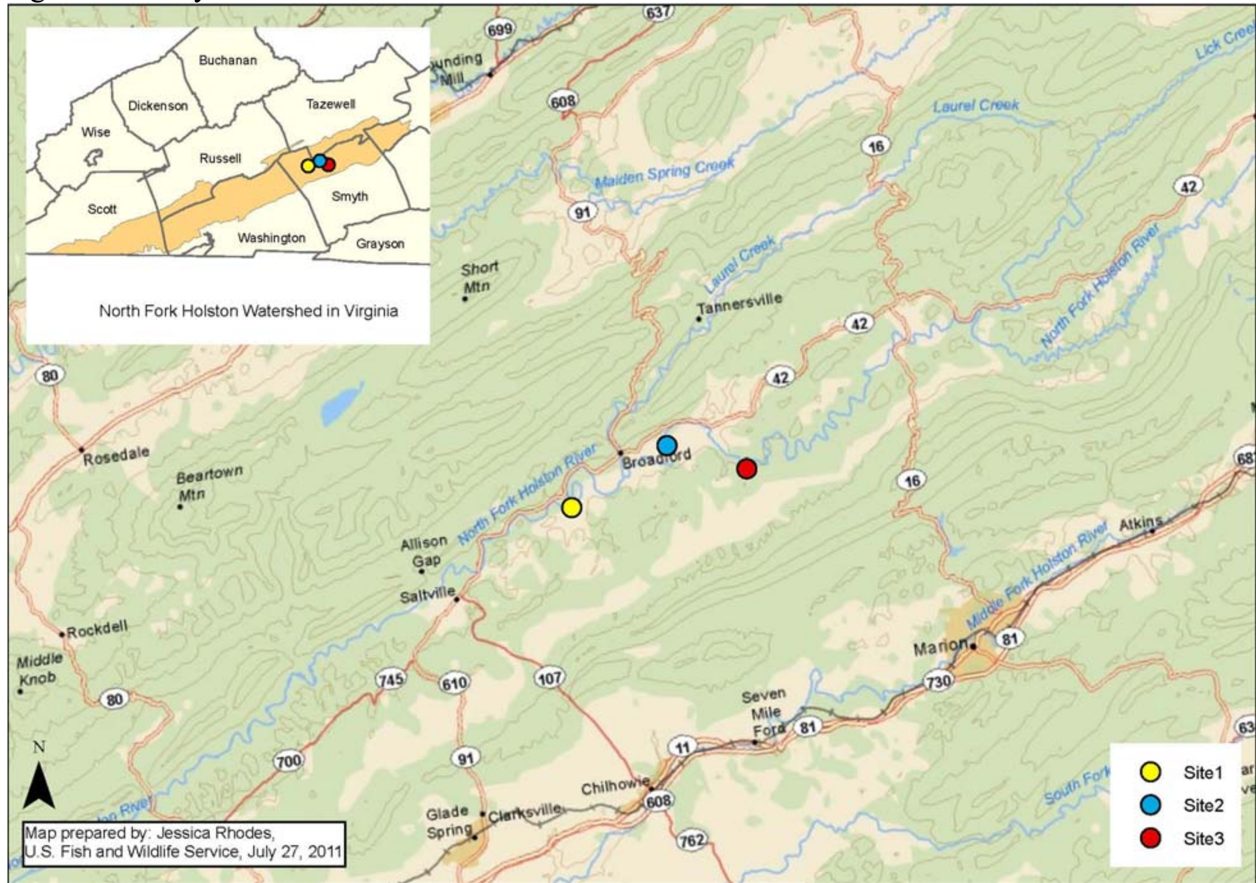


Table 1. Predominant land use by study site

	Percent Forest	Percent Pasture
Site 1	65.1	22.2
Site 2	61.0	27.7
Site 3	62.0	25.8

Mussels

In January 2007, rainbow mussels (*Villosa iris*) from an UTRB stream, Copper Creek in Russell County, Virginia, were propagated at the Freshwater Mollusk Conservation Center (FMCC) at VA Tech and in June 2007 transferred to the Virginia Department of Game and Inland Fisheries Aquatic Wildlife Conservation Center (AWCC) in Marion Virginia. Eighteen month-old adult rainbow mussels (individual mean size 16.0 millimeters, mm) were deployed in Barnhart silos (Figure 2) at three sites in the upper NFHR for 14 months (June 2008 - November 2009). Control mussels were captive-held for the study duration in silo chambers at the AWCC. There were twenty mussels per silo chamber, and four silos were deployed per site. Silos were visited

monthly to clear sediments from the silo chambers, ensure mussels were alive and that the silos were still in place (Figure 3). Mussel growth was monitored four times per year (April, June, August and October) using digital calipers (Mitutoyo ABSOLUTE Digimatic 500, Mitutoyo America Corp., Aurora, Illinois), to measure to the nearest 0.01 mm the length of each mussel at Sites 1, 2, 3, and the AWCC Control site . In November 2009 at the end of the in-stream monitoring period, all silos were pulled from the river and all silo chambers with the live mussels were transported within one hour in a cooler with river water to the laboratory for length measurements and soft tissue harvesting for body burden analysis and histopathology evaluation.

Water Quality

During each site visit the surface water quality parameters, pH, temperature (degrees Celsius), conductivity (micro-Siemens per centimeter), total dissolved solids (milligrams/liter, mg/l), and dissolved oxygen (mg/l)) were measured with a YSI 556 portable handheld probe (Yellow Springs Instrumentation, Yellow Springs, Ohio). The probe was calibrated within 24 hours before measurements were taken and data were downloaded using EcoWatch, a software interface to the YSI. Water temperature was measured with HOBO water temperature Prov 2 data loggers (Onset Computer Corp., Bourne, Massachusetts) that were deployed for the study duration, one per site, attached by zip-tie to the handle of one silo. Temperature data were downloaded monthly from the HOBO data logger by shuttle and HOBOware Pro Software (version 3.2.1).

Analytical chemistry

Composite whole body mussel samples from all study sites and the AWCC Controls were analyzed for organochlorine and organophosphate pesticides, total polychlorinated benzenes, total petroleum hydrocarbons, and metals (including arsenic, mercury, and selenium) through the Service's Analytical Control Facility contract laboratories. Inorganic analyses of the control mussels were conducted at Texas Environmental Research Laboratory at Texas A&M University, College Station, Texas. Organic analyses of the control mussels were conducted at GPL (*dba* Centauri Labs) Frederick, Maryland.

End of the monitoring period body- burden analyses for all mussels (Sites 1, 2, 3 and AWCC Control Site) were conducted. TERL conducted the inorganic analyses. Geochemical and Environmental Research Group at Texas A&M University conducted organic analysis of the AWCC Control Site mussels and Mississippi State Chemical Laboratory analyzed the NFHR study site mussels for all organic analyses, including the organophosphate pesticides.

Procedural blanks, standard reference material samples, duplicates and spiked recovery samples were run in accordance with the Service's ACF contract requirements. Data quality assurance (QA) and quality control (QC) review was conducted by the Service's Analytical Control Facility prior to release of the data to the field station. All methods and QA/QC results are maintained at the Service's ACF and Virginia Field Office.

Live mussel whole body tissue was harvested using chemically clean stainless steel forceps into certified chemically clean glass for all analyses, samples were weighed, then placed immediately in a freezer where they were held frozen until overnight shipment on ice to the laboratories.

Food analysis
Beginning April 15, 2009, and continuing on June 15, August 15 and October 15, five discreet 1-liter water samples were collected in chemically clean high density polypropylene bottles at each of the silos at each study site in the NFHR and at the AWCC Control site. Food availability, estimated as ash-free dry weight of organic matter, was evaluated to determine if food was a growth limiting factor. On the date of collection, water samples were transported in coolers on ice to White Sulphur Springs National Fish Hatchery (WSSNFH). This analysis allowed a partitioning of the effect of food variability on mussel growth among sites such that the effect on growth of other stressors could be assessed. Water samples were chilled and processed immediately at WSSNFH or frozen and later thawed and processed. Following Kreeger (1995), weighing pans and filters were dried at 60 degrees C and weighed, water samples filtered, filters, pans and samples dried again for 24 hours at 60 degrees, and then ashed at 450 degrees C for 4 hours and weighed immediately thereafter.

Data Analysis

Mussel growth
All statistical analyses for this EC Special Study were conducted using Minitab 14 software (Minitab Inc., State College, Pennsylvania). Mean mussel growth by silo, measured by calculating mussel shell length at initial in-stream deployment (Time = 1) and end of deployment (Time = 2), is shown in Figure 1. One-way Analysis of Variance (ANOVA) followed by Tukey's Range Test for multiple comparisons was used to test for statistical differences in mean mussel length among silos within a site and among sites. The same ANOVA methods were used to test for differences in mean food among silos within and among sites and water temperature among sites.

Histopathology Evaluation
In November 2009 at the end of the in-stream monitoring period, nine rainbow mussels, three (n = 3) from each study site, were selected for histopathological evaluation. Mussel tissue from each of the nine specimens was harvested in the lab within three hours of mussels being pulled from the river. Harvested tissues were immediately preserved in Bouin's fixative, and transported at a later date to the FMCC. At FMCC, tissues were processed through a series of progressive concentration of alcohol and xylene and paraffin embedded (Bancroft and Gamble 2002). Tissue sections (two 5 µm sections from each mussel) were cut on a rotary microtome and adhered to microscope slides. Tissues were stained with hematoxylin and eosin for microscopical evaluations using light microscopy (Olympus BX 41 light microscope, Olympus America, Incorporated, Center Valley, Pennsylvania).

Histological evaluations determined fractions of gonads with reproductive acini containing mature and/or developing gametes, acini containing resorbing gametes, gill filament termini with degraded cilia and/or epithelia, and digestive gland diverticula cells containing vacuolated or regressed cytoplasm. The presence of lipofuscin in cells of kidney diverticula was evaluated qualitatively. Histological evaluation included observations of trematode presence/absence in the mussel tissue. Data were obtained by evaluating histological sections by light microscopy using point-counting (Chalkey 1943). Tissues were assessed for the histological dependent variables by recording whether the types of tissues described for the variables visually occurred

under the ocular dots (0 = absence and 1 = presence). Fifty blind histological observations were recorded for each variable from each histological section for statistical analyses. Blind histological observations remove the possibility of evaluator bias for personal knowledge of specific study site conditions or mussel population trends at study sites. The non-parameteric, one way ANOVA by ranks Kruskal-Wallis Test was used to test differences in median values of means of the histological dependent variables.

III. RESULTS / DISCUSSION

Growth and mortality.
Over the course of the monitoring period, among silos within each site there was no difference in mean length. Within study sites, one exception is Silo 10 at Site 3, which had smaller mean mussel length than counterpart silos at the end of deployment. Mussels at the AWCC Control Site did not grow as well as those at the study sites and a statistical difference in growth was observed for AWCC Control Site silo 15 and AWCC Control Site silo16, which both had smaller mean length than AWCC Control Site silos 13 and 14.

In comparisons of mussels pooled by site (Figure 2), there was no difference between Sites 1 and 2, and mean mussel length at Site 1 was greater than that at Site 3 ($p < 0.001$). Mussels at all three NFHR sites grew longer than the controls ($p < 0.001$). This is supported by the fact that differences were not statistically significant in the initial pooled mean length values by site (Site 1: 16.16 mm, Site 2: 16.39 mm, Site 3: 16.58 mm, and AWCC Controls: 16.78 mm). Relative to all sites, mean mussel length at the control site was greatest at the beginning and lowest at the end of deployment.

Among all sites mussel mortality was low. At Sites 1, 2, 3, and the AWCC control 5, 8, 3, and 11 individuals died, respectively. In general mortality was gradual, with the loss of zero to two individuals at a site per visit. However, between September and late October 2009, 10 individuals in one chamber held at the AWCC control site died, likely because the chamber became clogged with sediments on both ends.

Food analysis
Per sampling events in April, June and August, there was no difference in food among silos by site or among sites. There were two statistical temporal differences in food by site, AWCC Control Site in April and the NFHR Site 2 in June. This may be due to water temperature differences, thus food productivity differences, over time. There was no difference ($p > 0.05$) in available food among sites for April, June and August sampling events, thus differences in available food between sites for that time period did not affect growth between sites. Due to errors in either data recording during the weighing of food or the procedure, only the water samples collected to assess for food availability for the months of April, June and August could be statistically analyzed.

In-stream temperature
Among Sites 1, 2, and 3, temperature means were not statistically different. However, temperature maxima were greatest in the summer months at NFHR Site 1 (Figure 4), which may explain the slight, although statistically significant, difference in growth relative to NFHR Site 3.

Temperature data from AWCC control site could not be obtained due to software errors and are pending retrieval from the logger manufacturer.

Figure 2. Length distributions, all chambers

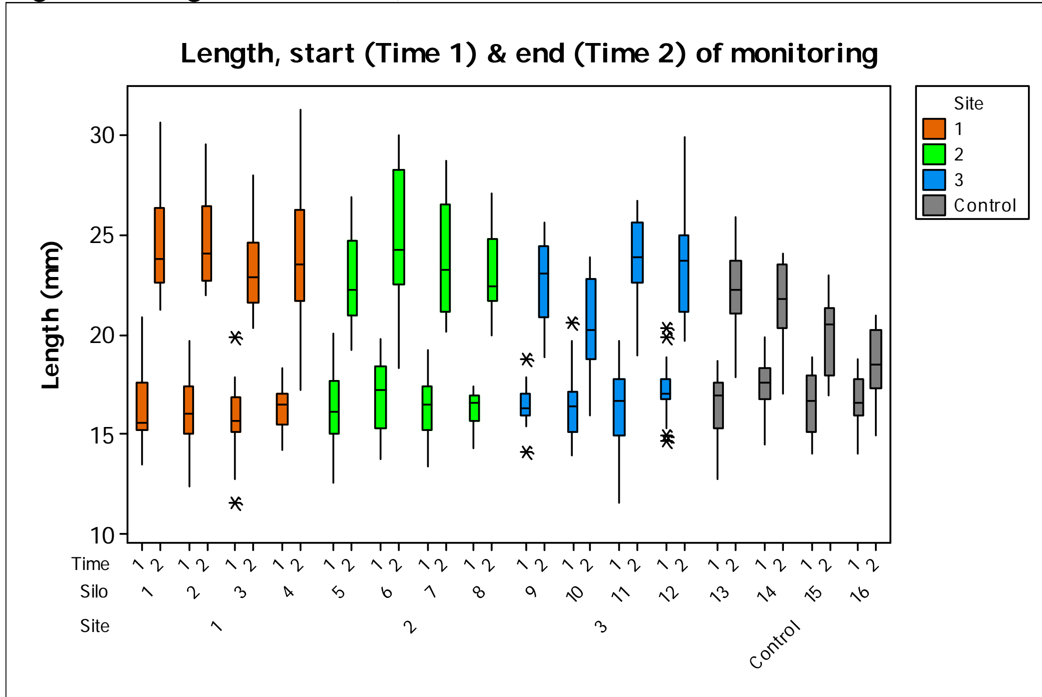


Figure 3. Length distributions pooled by site

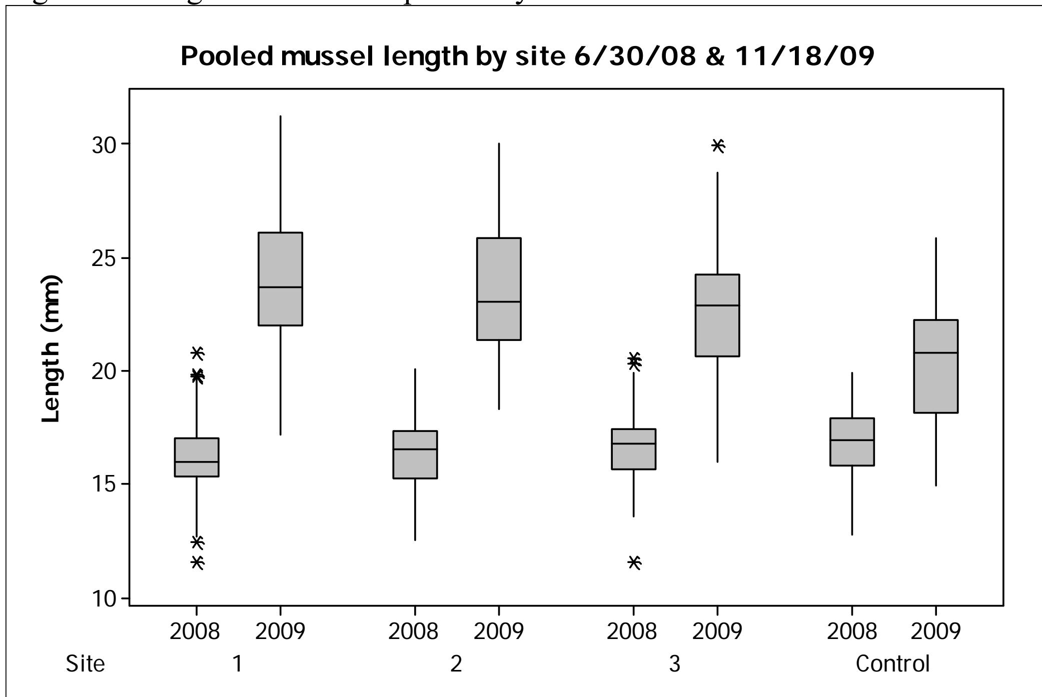
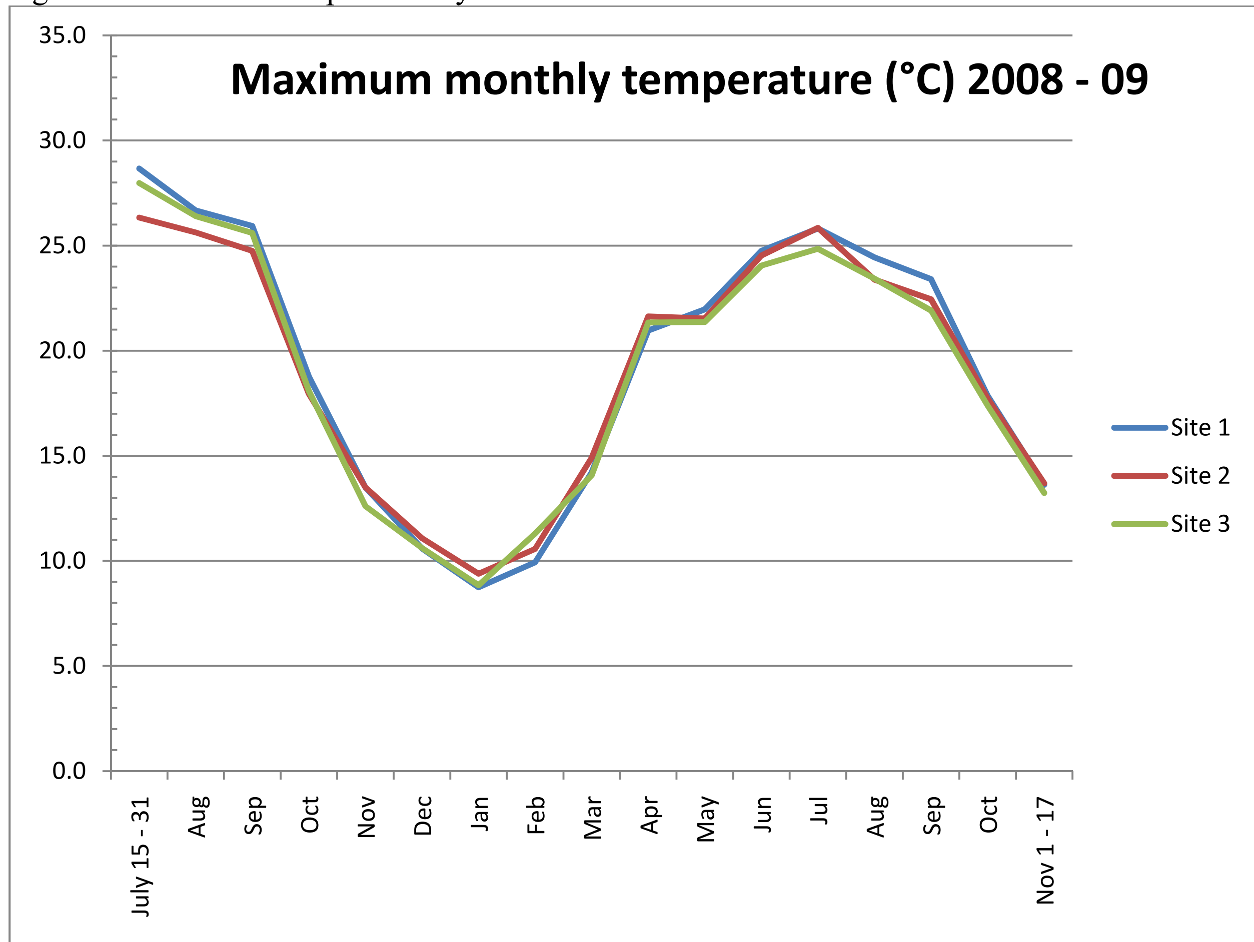


Figure 4. Maximum temperature by site



Chemistry

Organics

No organic compounds were detected in the control mussels at the beginning of the study. At the end of the study, the AWCC Control mussel sample showed total petroleum hydrocarbons present at 32 micrograms per gram (µg/g). Also, 1,2,3,4 – tetrachlorobenzene was measured at 0.00025 µg/g and detection confirmed. Mussels can bioaccumulate organic compounds from water (across their gills) and by pedal feeding in sediments. The bioaccumulation that occurs may manifest in sublethal effects, such as change in filtration or ventilation or a change in reproductive health, or histopathology. Determining that observed adverse effects are due to a particular contaminant can be difficult as effects may be confounded by other stressors that may also cause adverse effects. The fact that the AWCC Control Site mussels grew less than the *in situ* study site mussels may be related to a low level stress from the organic body-burden. To confirm these body burden results observed from one composite sample and in an attempt to isolate a potential source, more testing with mussels, water, and sediment at the AWCC is underway. In addition, n-heptacosane and n-hexacosane were reported in the AWCC Control Site mussels. Duplicate analyses revealed a 10 – 20% difference between initial and duplicate analysis. Spike recoveries, while within acceptable ranges, were both over-recovered (n-

heptacosane) and under-recovered (n-hexacosane). No other similar compounds were reported in the AWCC Control sample, a source is unknown, and confirmatory sampling is underway.

Diazinon was detected at 0.02 µg/g in the mussels from NFHR Site 2. QA/QC review confirms measurement. While published research on the effects of diazinon on freshwater mussels is limited, Conners and Black (2004) found diazinon exerted genotoxic effects at concentrations below the no-observed-effect concentrations established during toxicity tests. Diazinon is a restricted use organophosphate insecticide currently registered for use on a number of fruits, vegetables, nuts, and ornamentals grown outdoors in nurseries, and in cattle ear tags. Residential use is no longer approved. U.S. Environmental Protection Agency (2008) states "Diazinon is very toxic to aquatic invertebrates, and the screening level assessment indicates potential risks to aquatic invertebrates from all uses." While the mussels at NFHR Site 2 showed no effects to growth or evidence of histological abnormality, DNA damage cannot be discounted as a potential stressor. No other organics were detected at biologically significant levels.

Inorganics

Naturally occurring elements, metals, were detected in the AWCC Control Site mussels at the beginning of the study when the mussels were approximately 11 months of age; however, none of the concentrations were biologically significant. At the end of the monitoring period, all body-burdens had decreased in the AWCC Control Site mussels over time. This would be attributed to the fact that metals availability in the water column was constant over time, mussel intake and elimination reach a steady state, and as the mussels grew the initial body burden became dilute in an increased body mass. AWCC Control Site body-burdens of metals were not biologically significant.

Uptake of metals by freshwater mussels will depend on the characteristics of the metals, the water column and sediment concentrations of the metal, the metal uptake rate, site of interaction, and will vary among mussel species (Naimo 1995; Thorsen et al. 2007). Some metals such as zinc, copper and calcium are essential elements for biological processes. At low concentrations these are not toxic, and freshwater mussels are better able to regulate uptake, which becomes important at higher concentrations. Similarly, mussels are not typically regulating non-essential elements, and thus at high concentrations, toxicity may occur as mussels are unable to regulate the uptake and elimination. Naimo (1995) asserts that since freshwater mussels in natural settings are exposed to mixtures of metals, assessment of the effects of multiple metal exposure cannot be based on laboratory test results with single metal exposure. Measures such as growth and histological evaluation may be among the most useful tools to assess interactive effects of contaminants.

Metals body-burdens measured in the Study Site mussels reflect the normal background ranges and composition of metals in freshwater sediments (NOAA SQuiRT 2008) and USEPA Freshwater sediment benchmark screening values available at: http://www.epa.gov/reg3hwmd/risk/eco/btag/sbv/fwsed/screenbench.htm#table. A review of 30 years of Virginia Department of Environmental Quality water quality and sediment data for NFHRM 97.6 (above Study Site 2 and below reference Study Site 3) and NFHRM 89 show that water column metals concentrations reflect the range of metals sediment concentrations; the metals body burden levels

in the freshwater mussels reflect background conditions. Ambient water quality and sediment data for NFHR is available at http://www.deq.virginia.gov/water/mon_data_retrieval_app.html.

Both methylmercury and inorganic mercury were measured *in situ* and in the AWCC Control Site mussels. Among all sites, mercury body-burdens did not differ. Methylmercury body-burdens for mussels held *in situ* among study sites did not differ and *in situ* body-burdens were nearly twice that of the AWCC Control Site mussels. Sediment conditions in the NFHR would allow for mercury methylation, while there is little sediment in the AWCC Control Site holding tanks and the mussels in silo chambers at the AWCC Control Site were not burrowed in the limited, available sediments. The metals body-burdens were not at biologically significant levels; this is supported by growth and tissue condition exhibited by mussels held *in situ* at NFHR Sites 1, 2, and 3.

Histological evaluation

Histological observations of the nine mussels showed no abnormalities and no significant differences were detected among tissue from any of the collection sites, including fractions of gonads with reproductive acini containing mature and/or developing gametes, acini containing resorbing gametes, gill filament termini with degraded cilia or epithelia, and digestive gland diverticula cells containing vacuolated or regressed cytoplasm ($p > 0.05$).

No trematodes were observed in viscera of any of the mussels evaluated. While the histological observations were limited in number ($N = 9$), the absence of trematodes in the mussels held *in-situ* for 14 months correlates with more recent observations in the NFHR that indicate the mussel die-off may be over. In 2006, Ostby and Neves observed 8 species and 391 mussels in a 100 meter reach near Study Site 3. The histological evaluations that detected no trematodes and no tissue abnormalities in mussels held at the Site 1 and Site 2 indicate these reaches may now be suitable for maintaining histological integrity and gametogenesis in freshwater mussels. Henley (2003) investigated the observed mussel die-off at Site 1 using histopathology and observed some individual mussels so infested with trematodes that mussel gender could not be determined. The lifecycle of trematodes involves a mollusk as the first host and a final vertebrate host such as fishes, birds, turtles, amphibians or mammals. While there have been some limited actions to fence cattle out of the NFHR, it has not resulted in noticeable reductions of cattle in stream, and it is presumed that other trematode lifecycle host availability has remained constant over time. The die-off may have waned due to presence of fewer trematodes because of the effect of the initial die-off leaving fewer mussels available to support the trematode lifecycle.

Henley (2011) provides a full accounting of the methods and results of the histological work conducted for this study (Attachment 1).

IV. CONCLUSIONS

There was no biologically relevant, statistically significant difference in available food or in-stream temperature among sites for each sampling event, these variables did not affect growth differences between sites. Mussel growth appears to be similar among sites. Histological evaluations detected no trematodes and no tissue abnormalities in mussels held at the NFHR

Study Sites 1, 2 and 3. NFHR Sites 1 and 2, reaches at which mussels (Henley 2005) were most infested with trematodes in the late - 1990's and early 2000's, now appear to be suitable for maintaining histological integrity and gametogenesis in freshwater mussels. Sample size was small and, therefore, to increase certainty regarding the suitability of these sites for translocation or augmentation of the native mussel fauna, histological evaluation could be repeated with larger sample size.

To address the finding of insecticide in mussels from NFHR Study Site 2, we recommend the following:

1) Sampling of the native mussel fauna, of several species, for analysis of pesticides (organophosphates).
2) Sampling of sediments at that site for pesticide analysis.
3) Work with the local agricultural extension agent to a) identify the pesticides sold and used in the localized watershed; b) work with landowners to understand the sensitivity of the fauna to land and agricultural management practices; and c) initiate and conduct a mussel outreach event to be held in the upper NFHR watershed to raise awareness about the fauna.

To address the AWCC Control Site mussels with a measurable total petroleum hydrocarbon body-burden, an unexpected finding in the control mussel analyses, an investigation is underway at the facility that includes collecting and analyzing tissue, water, and sediment (holding pond) samples for confirmation and possible isolation of a source.

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Final Report

Histological Evaluations of Organ Tissues of Rainbow Mussels (*Villosa iris*) Deployed and Subsequently Collected from Selected Sites on the Upper North Fork Holston River, Virginia

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Abstract

Tissues of rainbow mussels (*Villosa iris*) from three sites on the North Fork Holston River (NFHR) upstream of Saltville, Virginia, were histologically evaluated to determine abnormalities in organs. Organs evaluated included gills, digestive glands, and gonads, and incidences of trematode infections also were noted. Mussels were held within a screened polyvinylchloride sleeve anchored within a cement “silo” at three NFHR sites for 14 months. At the end of the study, three mussels were collected from the silos at each of the three NFHR sites. The organ tissues were paraffin embedded, cut with a rotary microtome, stained with hematoxylin and eosin, and histologically evaluated using light microscopy. Histological evaluations determined fractions of gonads with reproductive acini containing mature and/or developing gametes, acini containing resorbing gametes, gill filament termini with degraded cilia and/or epithelia, and digestive gland diverticula cells containing vacuolated or regressed cytoplasm. Kidneys of mussels were not revealed in many cuts of the mussels, therefore, the presence of lipofuscin in cells of kidney diverticula were evaluated qualitatively.

The results showed that the histological variables were not significantly different among mussels from the NFHR sites ($p > 0.05$). All mussels contained mature and/or developing gametes. All females showed resorbing oocytes and males showed residual mature sperm in their reproductive acini. All mussels showed low fractions of digestive diverticula cell with vacuolated and/or reduced cytoplasm and gill filaments with reduced cilia and/or epithelia. The exceptions were one female collected from NFHR Mile (NFHRM) 98.9, three that showed a relatively high fraction of digestive cells with reduced cytoplasm, and another female collected from NFHRM 88.8 with a very high fraction of gill filaments with reduced epithelia. Evaluations of female and male gonads showed that they had completed the normal oogenic cycle for the season, and were resorbing residual spermatogenic and oogenic materials in their acini. Higher fractions of reduced cytoplasm in digestive cells and epithelia of gill filaments were probably due to stress related to energetic requirements of gametogenesis. Histological sections that contained kidney tissue showed low abundances of lipofuscin, and there were no apparent differences among mussels from the three sites. Evaluations of the histological sections showed that none of the mussels was infected with trematodes.

Results of the histological evaluations show that tissues from mussels from all sites were comparable. Good condition of the mussels at the NFHR sites was substantiated by normal progression of gametogenesis, and absences of tissue abnormalities in gills, digestive glands, gonads, and kidneys.

Introduction

Historically, the North Fork Holston River (NFHR) upstream of Saltville, Virginia contained a diverse mussel fauna with 21 species; however, in surveys conducted during 2000-2004, only 13 species were documented (Jones and Neves 2007). Recently, unexplained mussel die-offs have occurred at multiple sites on the upper NFHR (Jones and Neves 2007), and among dead mussels were the federally listed endangered shiny pigtoe (*Fusconaia cor*) and a federal candidate species, slabside pealymussel (*Lexingtonia dolabelloides*). A contributing factor partially explaining these mortality events was thought to be trematode infestations in local mussels. Other anthropogenic activities also may be causative. Henley and Neves (2003) observed a mean incidence of 26% trematode infestations in rainbow mussels and slabside pealymussel at nine sites on the upper NFHR and specifically a 50% trematode infestation rate at NFHRM 88.7, which is Site 1 in the Special Study. Percentages of incidence of infection ranged from 0% to 100% at the Henley and Neves (2003) sites.

This report presents the histological evaluation findings and is one component of a three year U.S. Fish and Wildlife Service (Service), Division of Environmental Quality Special Study. In June 2008, 18 month-old native rainbow mussels, captive-reared at the VA Tech Freshwater Mollusk Conservation Center (FMCC), were deployed in Barnhart silos at three sites in the NFHR to assess the effects of agricultural related non-point source discharges, and other anthropogenic-related stressors, on federally listed, imperiled mussels in the NFHR. The possibility exists of adverse effects of pesticides to freshwater mussels. The Special Study investigation measured instream mussel exposure to contaminants and evaluated effects to sublethal endpoints (growth and histology). Mussel tissues were analyzed for body-burdens of pesticides, heavy metals, and other contaminants. This study is among the first to use native mussels placed *in-situ* in the NFHR to investigate effects. Two sites (Site 1: NFHRM 88.8 and Site 2: NFHRM 95.3) were located within the river reach where significant mussel die-off had occurred and bracketed agricultural lands (crops and pasture), and a reference site (Site 3: NFHRM 98.9) was located upstream of all discharges except for non-point source runoff from pasturing.

The objectives of the histological evaluation study were to 1) evaluate prepared organ tissues, including gills, digestive glands, gonads, and kidneys, to determine abnormalities in these mussel tissues; 2) determine incidences of trematode infestations in the mussels; and 3) statistically compare quantitative data from these evaluations among the study sites.

Histological evaluations have been used to determine the effects of contaminants on bivalve tissues, including digestive glands, gills, gonads, and kidneys (Bayne *et al*. 1981, Seiler and Morse 1988, and Au 2004). The quality and characteristics of cells in these organs have served as biomarkers for assessments of environmental contaminant effects. Because digestive glands, gills, and kidneys of bivalves are the sites of absorption, sequestration, accumulation, and excretion of contaminants, cellular alterations can occur in these organs (Seiler and Morse 1988, Domouhtsidou and Dimitruadis 2000). The digestive gland of bivalves is anatomically connected to the stomach by tubules (Owen 1973), and is responsible for intra- and extra-cellular digestion, nutrient absorption, lipid and glycogen storage, and contaminant detoxification (Loboda-Cunha 2000, Petrović et al. 2001). These functions occur in blind-end tubules called diverticula whose epithelial layer consists of secretory (basophilic) and digestive cells (Owen

1970, Lobo-da-Cunha 1999 and 2000). The digestive epithelial cells have been shown to be sensitive indicators of the effects of contaminants, including crude oil (Lowe et al. 1981, Au 2004, Usheva et al. 2006). Cellular alteration in digestive tubules due to contaminants includes thinning of epithelial layers due to reduction of cytoplasm (Lowe et al. 1981, Usheva et al. 2006).

Gills (ctenidia) of freshwater mussels are paired ciliated organs that are involved in gas exchange, osmoregulation, and food transport; therefore, mussel gills are larger than required for fulfillment of metabolic oxygen requirements alone (McMahon and Bogan 2001). As an important uptake organ, mussel gills can be sites of pollutant accumulation (Gómez-Mendikute et al. 2005). Observed histological alterations in mussel gills that have been linked to contaminant exposures are fusion of gill filaments, decreased height, and loss of cilia, inflammation, necrosis, sloughing of the epithelial layer, and increased mucus production due to proliferation of mucus vacuoles (Domouhtsidou and Dimitruadis 2000, Lajtner et al. 2003, Gómez-Mendikute *et al*. 2005, Supanopas et al. 2005).

In adult mussels, gametes develop through the mitotic-meiotic pathway from germ cells within acini (Henley *et al*. 2007). A time-series of histologically-based observations reveals that gametogenesis in unionoid freshwater mussels is a cyclic process that usually occurs annually (McMahon and Bogen, 2001). The gametogenic cycle includes development and maturation of gametes, spawning, and subsequent resorption of residual gametes (Kennedy and Battle 1964, Pipe 1987, Dorange and Le Pennec 1989, Barber 1996). Although post-spawning resorption of oocytes is a normal element of the oogenic cycle, untimely resorption of oocytes has been observed due to stress and exposure to contaminants (Bayne and Thompson 1970, Bayne et al. 1981, Tay et al. 2003). In extreme cases of contaminant exposures, atrophy of acini also can occur (Tay et al. 2003).

The occurrence of lipofuscin in cells of the digestive glands, gills, and kidneys can result from general physiological oxidative stress and contaminant exposure (Domouhtsidou and Dimitruadis 2000, Riveros et al. 2002, Gómez-Mendikute et al. 2005, Seehafer and Pearce 2006, Usheva et al. 2006). Although the occurrence of intracellular lipofuscin granules is age-related, these granules also are a means for compartmentalizing contaminants by combining them with insoluble lipid peroxidation byproducts (Lomovsdky et al. 2002, Riveros et al. 2002, Dimitriadis et al. 2004, Seehafer and Pearce 2006). The abundance of lipofuscin granules can be related to the degree of contaminant exposure (Riveros et al. 2002, Kagley et al. 2003).

The kidney is responsible for ultrafiltration of hemolymph, ion exchange, and excretion in mussels (Dietz et al. 2000, Fahrner and Haszprunar 2002). The kidney in unionid mussels is composed of convoluted diverticula and tall columnar cells with microvilli (Henley et al. 2007). A notable function of the kidney concerning contaminant detoxification is excretion of lipofuscin granules by exocytosis; and the abundance of tertiary lysosomes (containing lipofuscin) in the kidney has been related to contaminant exposure (Doyle et al. 1978, Seiler and Morse 1988, Seehafer and Pearce 2006).

Methods

Mussels were deployed at three sites in the upper NFHR for 14 months (June 2008 - November 2009). Two sites (Site 1: NFHRM 88.8 and Site 2: NFHRM 95.3) were located within the river reach where significant mussel die-off had occurred and bracketed agricultural lands (crops and pasture), and a reference site (Site 3: NFHRM 98.9) was located upstream of all discharges except for non-point source runoff from pasturing. Silos were visited monthly to clear sediments from the silo chambers, ensure mussels were alive and that the silos were still in place. Using calipers, mussels were measured for growth four times per year (April, June, August and October). In November 2009, all surviving mussels were harvested for histological evaluation, measurement of uptake of heavy metals, pesticides, PAHs, and other organics.

Three rainbow mussels (n = 3) were collected from silos at three NFHR sites. After collections of mussels, their tissues were immediately preserved in Bouin's fixative, and transported from the field to FMCC. At FMCC, tissues were processed through a series of progressive concentration of alcohol and xylene and paraffin embedded (Bancroft and Gamble 2002). Tissue sections (two-5 µm sections from each mussel) were cut on a rotary microtome and adhered to microscope slides. Tissues were stained with hematoxylin and eosin for microscopical evaluations using light microscopy (Olympus BX 41 light microscope, Olympus America, Incorporated, Center Valley, Pennsylvania).

Histological evaluations determined fractions of gonads with reproductive acini containing mature and/or developing gametes, acini containing resorbing gametes, gill filament termini with degraded cilia and/or epithelia, and digestive gland diverticula cells containing vacuolated or regressed cytoplasm. Because kidneys of mussels were not revealed in many cuts of the mussels, the presence of lipofuscin in cells of kidney diverticula were evaluated qualitatively. These data were obtained by evaluating histological sections by light microscopy using point-counting (Chalkey 1943). Six dots were placed on one of the ocular lenses of the microscope, and the stage of the microscope was randomly positioned in two dimensions. Tissues were assessed for the histological dependent variables by recording whether the types of tissues described for the variables visually occurred under the ocular dots (0 = absence and 1 = presence). Fifty observations were recorded for each variable from each histological section for statistical analyses. All histological evaluations were conducted blindly, so that the evaluator did not know the NFHR site of mussel collection.

At all three sites, the three mussels collected consisted of one female and two males. Therefore, the use of statistical procedures when using gender as a statistical factor was questionable. Because only nine total mussels (three per each of the three sites) were used in the statistical comparisons, the non-parameteric, one way analysis of variance by ranks Kruskal-Wallis test was used to determine differences in median values of means of the histological dependent variables.

Results and Discussion

No significant differences were detected among tissue from any of the collection sites, including fractions of gonads with reproductive acini containing mature and/or developing gametes, acini containing resorbing gametes, gill filament termini with degraded cilia or epithelia, and digestive gland diverticula cells containing vacuolated or regressed cytoplasm ($p > 0.05$). No trematodes were observed in viscera of any of the mussels evaluated. Mean fractions of digestive gland cells with vacuolation and/or reduced cytoplasm ranged from 0.23 to 0.31, with overall appearance of the digestive glands in the mussels showing healthy tissues (Table 1, Fig. 1A). One female from NFHR Site 2 showed a higher (not significant) fraction of degraded cytoplasm in digestive cells (Fig. 1B). Mean fractions of gill filaments with reduced cilia and/or epithelia ranged from 0.07 to 0.24, but the means do not represent the data (Table 1). Almost all of the mussels at all of the sites showed low fractions of gill filaments with reduced cilia and/or epithelia (Fig. 1C), except one female from NFHR Site 1 that had 72% of filaments with reduced cilia and/or epithelia (Fig.1D). The few sections that contained kidney tissues did not show abundances of lipofuscin in the cells of the kidney diverticula (Fig. 1E).

At all sites, gonads of all mussels contained mature and/or developing gametes (Table 1). Mean fractions of gametogenic acini containing resorbing gametes ranged from 0.33 to 0.39, but these means do not represent the data (Table 1). Whereas almost none of the males showed resorbing gametes, almost all gonads of the females contained resorbing oocytes (Table 1, Figs. 1F and 2A). It was apparent that both males and female mussels from all collection locations were in a post-spawn state with residual, mature gametes in their acini (Figs. 1F and 2A). The post-spawn condition was indicated by scant abundances of mature and developing gametes in acini, and few acini in somewhat disorganized connective tissues (Figs. 1F and 2A).

Histological evaluations of mussel tissues have been used to assess sites for contaminants (Bayne et al. 1981, Seiler and Morse 1988, and Au 2004). Histological variables have not been used to determine suitability of sites for release or translocation of freshwater mussels, but the absence of tissue abnormalities and trematodes in organs of mussels held at the NFHR study sites indicates that conditions in the river were suitable for maintaining normal histological integrity of the mussels. Importantly, the results of this study showed that mussels completed the gametogenic cycle while held in silos at the NFHR sites.

Conclusions

The histological evaluation detecting no tissue abnormalities in mussels held at the NFHR study sites indicates these sites likely are suitable for maintaining histological integrity and gametogenesis in freshwater mussels. Sample size was small and, therefore, to increase certainty regarding the suitability of these sites for translocation or introduction of native mussels, this work could be repeated with larger sample size.

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Table 1. Means (standard deviations) of fractions of reproductive acini containing mature and/or developing gametes (FAMD), acini containing resorbing gametes (FAR), digestive gland cells showing vacuolated and/or regression of cytoplasm (FDGVRC), gill filament termini without cilia and/or regressed epithelia (FGFCRE), and viscera containing trematode sporocytes. ***n*** = 3 mussels per NFHR site; at all three sites, one female and two males were serendipitously collected.

	Dependent Variables									
	FAMD		**FAR**		**FDGVRC**		**FGFCRE**		**FTREMS**	
Site	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Site 1 (NFHRM 88.8)	1.00 (0.00)	1.00 (0.00)	0.80 (0.40)	0.19 (0.39)	0.18 (0.39)	0.26 (0.44)	0.72 (0.45)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Site 1 Grand Mean	1.00 (0.00)		0.39 (0.49)		0.23 (0.42)		0.24 (0.43)		0.00 (0.00)	
Site 2 (NFHRM 95.3)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	0.00 (0.00)	0.24 (0.43)	0.35 (0.48)	0.00 (0.00)	0.11 (0.31)	0.00 (0.00)	0.00 (0.00)
Site 2 Grand Mean	1.00 (0.00)		0.33 (0.47)		0.31 (0.47)		0.07 (0.26)		0.00 (0.00)	
Site 3 (NFHRM 98.9)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	0.00 (0.00)	0.56 (0.50)	0.14 (0.35)	0.00 (0.00)	0.10 (0.30)	0.00 (0.00)	0.00 (0.00)
Site 3 Grand Mean	1.00 (0.00)		0.33 (0.47)		0.28 (0.45)		0.07 (0.25)		0.00 (0.00)	

Figure 1. Organ tissues of mussels held at NFHR locations. A. Normal digestive gland tissue with darkly stained basophilic (bc) and digestive (dc) cells in diverticula of mussel held at NFHRM 95.3 (Site 2). Both types of cells are not showing reduced cytoplasm. B. Abnormal digestive gland tissue with darkly stained basophilic (bc) and digestive (dc) cells in diverticula of mussel held at NFHRM 98.9 (Site 3). Note reduced and vacuolated cytoplasm of both types of cells. C. Normal gill filament tissue from mussel held at NFHRM 88.8 (Site 1). Note abundances of frontal cilia (fc) and epithelia (e). D. Abnormal gill filament tissue from mussel held at NFHRM 88.8 (Site 1). Note abundances of latero cilia (fc) and reduced epithelia (e). E. Kidney diverticula from mussel held at NFHRM 95.3 (Site 2). Note scant abundance of lipofuscin (bold arrow). F. Spermatogenic acini from mussel held at NFHRM 88.8 (Site 1). Note residual luminal sperm in acini. Spermatocytes contained in reduced acinar accessory cells (aac), and acinar walls (aw) thin and reduced. Stained with hematoxylin and eosin. Bars = 10 µm.

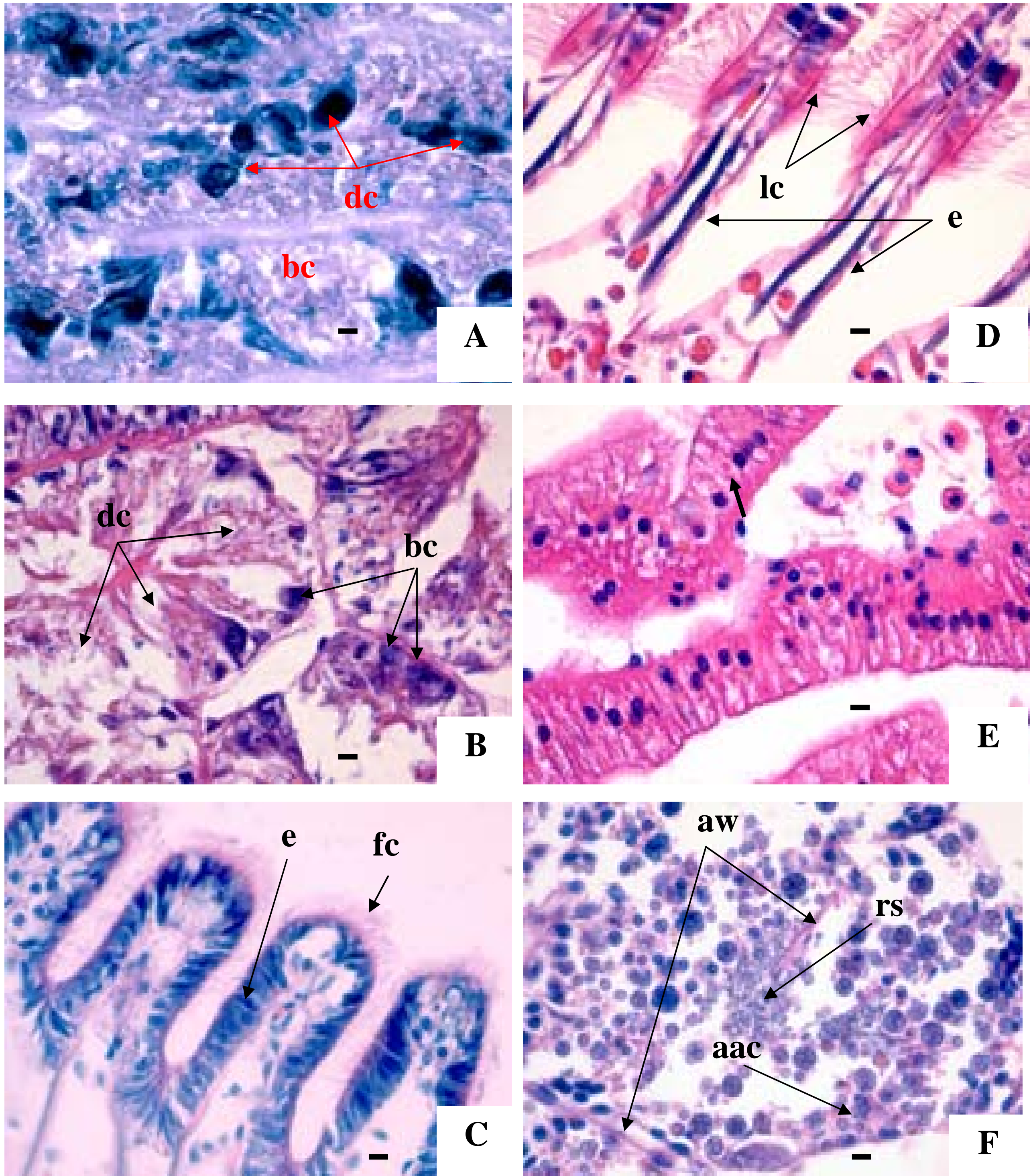

Figure 2. A. Residual after spawning mature, luminal resorbing oocytes (rmo) in gonad tissues from mussel held at NFHRM 88.8 (Site 1). Note resorbing vitellogenic oocytes (rvo) within very granular acinar accessory cells (acc) and reduced and thin acinar wall (aw). Stained with hematoxylin and eosin. Bar = 10 µm.