Molecular and Analytical Approaches to Evaluate Chromium Contamination of Estuarine Systems in Hampton Roads, Virginia

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Abstract: Determining the water quality of estuarine ecosystems is difficult because of the environment's variable properties. Hexavalent chromium is a toxic metal found in estuarine ecosystems due to pollution from industrial surroundings, and methods are needed to determine biotic responses to chromium contamination. It is proposed that expression of the fatty-acid binding protein (FABP) gene in (*Fundulus heteroclitus*), a common estuarine inhabitant, and Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) analytical techniques can be used as indicators of hexavalent chromium contamination. Using reverse transcriptase polymerase chain reaction (RT-PCR), FABP gene expression was analyzed to see if expression or non-expression occurred after contamination with hexavalent chromium. Compared to the more complex real-time PCR procedure that has previously been used to evaluate FABP gene expression, RT-PCR serves as a user-friendly water quality assessment procedure that is manageable, requires less training, can be performed more quickly, and at a lesser cost. ICP-OES was used to confirm the presence of chromium at experimental sites. Low levels of chromium contamination were detected across Hampton Roads, with significant differences between sites. FABP gene expression was variable across sites, suggesting further refinement of the method is needed to use gene expression as an indicator of contamination.

Key words: Hexavalent chromium, FABP, *Fundulus heteroclitus*, RT-PCR, ICP-OES

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Estuarine habitats are semi-enclosed, brackish bodies of water created by the mixture of freshwater from rivers and streams with high salinity water from nearby oceans (Pritchard 1967). The Chesapeake Bay is the largest estuary on the east coast of the United States. Production of native flora and fauna in the Chesapeake Bay has declined over the last several decades, most notably among groups important on an ecosystem level such as seagrasses and oysters, due to anthropogenic stressors that include eutrophication, sedimentation, and contamination with a variety of point and nonpoint source pollutants (Boesch 2006). The human population along the coast in the Northeast and mid-Atlantic regions of the United States has increased considerably over the years and is projected to grow more in the future (Ruark 2010), which is likely to further increase anthropogenic stressors on the Chesapeake Bay. One of these stressors is the input of heavy metals such as mercury, cadmium, lead, and chromium. Anthropogenic sources of these metals include mining, smelting, processes associated with generation of electricity, deposition of aerosols from automobile emissions, and use of antifouling compounds on marine vessels (Kennish 1997). Many metals enter Chesapeake Bay watersheds adsorbed to sediments or rapidly adsorb to suspended particles upon entering the system. In the Chesapeake Bay, trapping efficiency of suspended material is 98 ± 2%, indicating that only a minimal amount of anthropogenic metals are removed to coastal waters (Sinex and Wright 1988). Resuspension of contaminated sediments then contributes to the overall contaminant burden within the system (Kennish 1997). The influence of anthropogenic activities on sediment contamination can be highly localized, with the highest correlation between land use patterns and sediment contamination in Chesapeake Bay watersheds seen at a scale of ~10 km (Comeleo et al. 1996).

Because of the brackish, highly variable nature of estuarine systems, assessing water quality in these environments can be difficult and time-consuming. More so than in most freshwater and marine systems, water quality in estuaries varies temporally and spatially as a result of tidal mixing, gradients in salinity and other physical factors, and variation in freshwater inflow and associated watershed inputs (Hopkinson and Vallino 1995, Dauer et al. 2000). Many resource managers have found that biotic indices are a reliable approach for assessing water quality and their use has increased

(Deegan et al. 1997). Biotic indices may consider the response of a community (in terms of presence or absence of sensitive species) or the response of a single indicator species. The use of fish can be a dependable method for assessing water quality because certain species are abundant within estuarine systems throughout most of the year and can be easily caught and sustained within a laboratory setting for experimentation (Cossins and Crawford 2005).

In this study, mummichogs (*Fundulus heteroclitus*), killifish readily found in estuarine habitats along the Atlantic coast (Adams et al. 2006), were selected as the biotic indicator. They are non-migratory, have a small home range, and accomplish their complete life cycle in estuarine habitats (Abraham 1985, Maples and Bain 2004). Mummichogs are frequently abundant in polluted environments (Jung et al. 2009), and they display phenotypic variation that is reflected in population genetic patterns when exposed to contaminants (Nacci et al. 2010).

Differential gene expressions in mummichogs have been extensively used as a method of determining water quality of estuarine systems. Exposure to hexavalent chromium, for example, caused differential expression in 16 genes in mummichog larvae and 10 genes in adults, most of which were involved with energy metabolism and growth (Roling et al. 2006). Exposure of mummichog to organic toxins such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) has also been shown to affect expression of genes associated with hormone production and homeostasis and enzymatic pathways responsible for xenobiotic metabolism (Dong et al. 2008, Nacci et al. 2010). Overall levels of nuclear and mitochondrial DNA damage also appear to provide a sensitive indicator of exposure of mummichog to a variety of pollutants (Jung et al. 2009). Given the similarity between physiological and developmental pathways in fishes and higher vertebrates, effects on mummichog are likely to be a good indicator of the effects that pollution and contamination will have on humans, as well.

Chromium is a pollutant frequently found in estuarine ecosystems. It is a heavy metal that enters estuaries through anthropogenic activities such as mining, smelting, and chrome plating (Roling et al. 2006). Chromium is commonly found in two valence states: as trivalent chromium and the more toxic, hexavalent chromium. Trivalent chromium has been known to have benefits in the environment and acts as a nutrient (Bagchi et al. 2001). Hexavalent chromium is frequently used in the paint, steel, chrome, and metal and wood treatment and finishing industries. In contrast to trivalent chromium, hexavalent chromium can be easily absorbed by organisms and causes imbalances and disturbances in the redox potential and states of their cells (Roling et al. 2006). An imbalance in a cell's redox state causes the cell to be unable to cleanse and decontaminate toxins which can ultimately lead to death. Hexavalent chromium also has the potential to severely damage an organism's DNA, modify gene expression, cause cell death, and is considered carcinogenic to humans and other organisms (Bagchi et al. 2001, Rolling et al. 2006, Mahmood et al. 2008).

We investigated molecular and analytical techniques to determine the presence and quantity of hexavalent chromium in estuaries of Hampton Roads, Virginia. We focused on assessing expression of the fatty-acid binding protein (FABP) gene using reverse transcriptase polymerase chain reaction (RT-PCR), a qualitative method that uses agarose gel electrophoresis and ethidium bromide staining to detect gene expression. The method provides a binary result for gene expression of a band on the agarose gel that is either expressed (band is present) or not expressed (band is absent) (Promega 2011). The FABP gene was targeted because it is found in the liver of mummichog and facilitates lipid metabolism in the liver (Bain 2002). FABPs have also been used in genetic and chemical research because of their responses to contaminants that alter lipid metabolism and immune function (Bain 2002). The expression or lack of expression of FABPs can be changed by the introduction of a foreign substance or contaminant into an organism's environment (Bain 2002). FABP has been widely used as an indicator of differential gene expression of mummichogs based upon their exposure to contaminants in their environment, such as heavy metals and endocrine disruptors (Maples and Bain 2004). Whereas previous research used methods to quantify changes in gene expression, the objective of our study was to determine if RT-PCR was effective at identifying areas of chromium contamination, which would be a simpler technique for water resource managers to implement.

Methods

Study Sites

The study was conducted at four sites in the Hampton Roads region of the Chesapeake Bay (Figure 1). Hoffler Creek, a second-order tributary of the James River, is located on the property of Hoffler Creek Wildlife Preserve in Portsmouth, Virginia. Its status as a wildlife preserve and geographic isolation from any known or suspected regions of contamination led us to hypothesize that the mummichog from this site could serve as uncontaminated controls for this experiment. The upstream land use is predominantly residential, although the immediate land use is forested at the Hoffler Creek Wildlife Preserve, which provides some buffer for overland nonpoint source pollution. Wayne Creek is located in Norfolk, Virginia. It is a third-order, tidal tributary of the Lafayette River. The upstream land use is predominantly residential and sec-

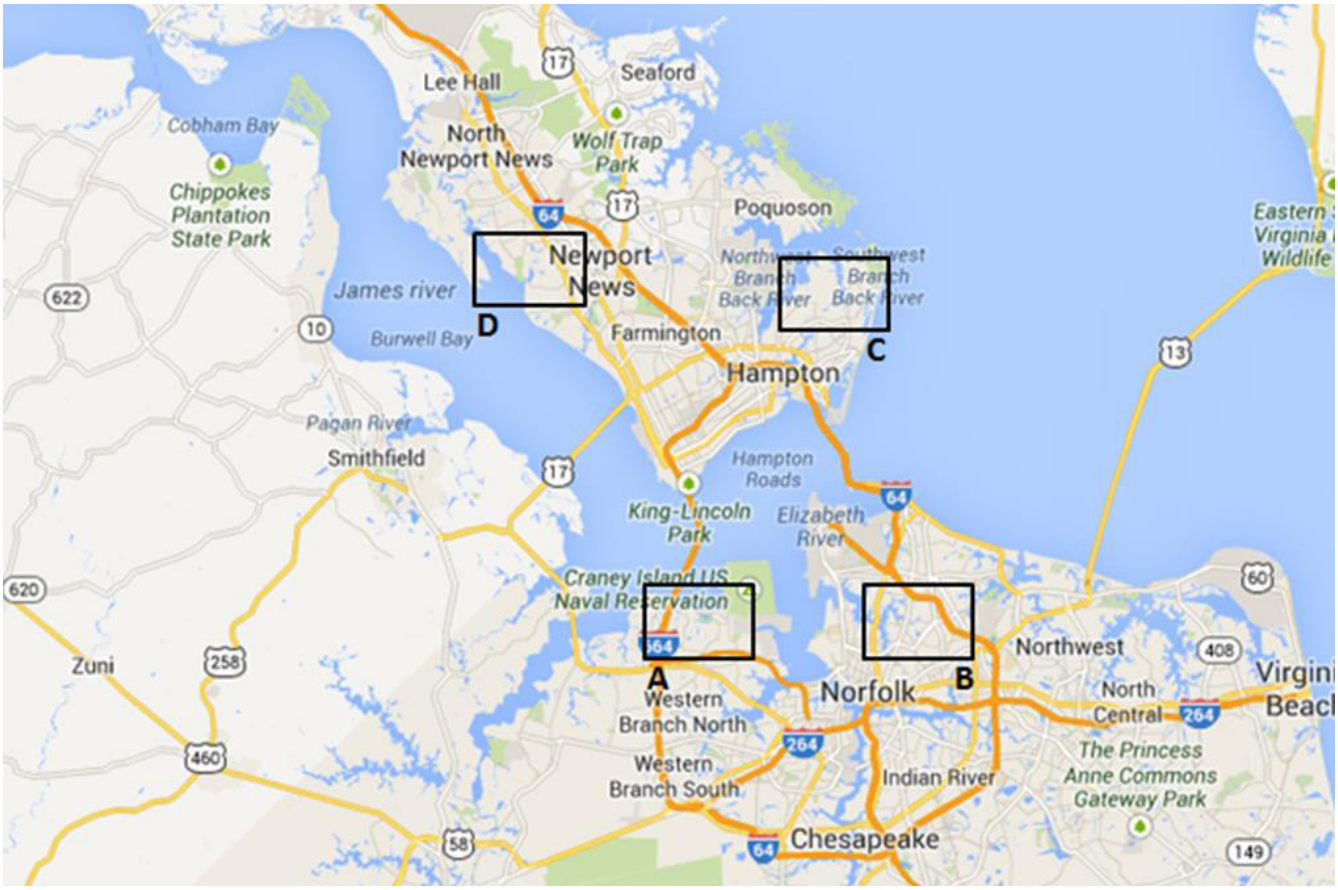

Figure 1. View of (A) Hoffler Creek Wildlife Preserve, Portsmouth, (B) Wayne Creek, Norfolk, (C) Back River, Hampton, and (D) Deep Creek, Newport News, in relation to Hampton Roads, Virginia.

ondarily industrial. Back River is an unnamed first-order tributary of the Southwest Branch of the Back River located in Hampton, Virginia, and the upstream land use is predominantly residential. Deep Creek is a second-order tributary that eventually flows into the James River and is located in Newport News, Virginia. The upstream land use is primarily residential, but secondarily industrial.

Exposure of Hoffler Creek Fish to Cr (VI)

Fish Collection, Acclimation, and Exposure. Male mummichogs were collected from Hoffler Creek in spring 2011 and 2012 using minnow traps. Males were targeted because they are most easily identified and the testes could be an additional target for analysis of gene expression by water quality managers. Once the fish were collected, they were allowed time to acclimate to the laboratory environment for a minimum of two months in order to control for mortality due to capture, transport, and transition to laboratory conditions. Fish were held in a 113.6-L tank with an 18-ppt saltwater solution (Instant Ocean Sea Salt, Instant Ocean, Spectrum Brands Inc.) at 20–25 C. The fish were fed daily using commercial fish food (TetraMin Plus Tropical Flakes, Tetra, Spectrum Brands, Inc.). Once fish were acclimated, they were assigned to a control group or to an experimental group exposed to hexavalent chromium (Cr(VI)).

Two mummichogs were removed from the acclimation tank and placed into a smaller, 4-L tank. Earlier experiments indicated that no more than two fish could survive when placed into the 4-L tanks. The fish were exposed to Cr(VI) in the form of potassium chromate (K_2CrO_4) at a concentration of 0.3347 ppm for 7 days with a complete water change at 84 h (3.5 days). Two additional mummichogs were removed from the acclimation tank and placed into a smaller, 4-L tank as the control. These fish also remained in the smaller tank for 7 days with a complete water change at 84 h. Control and experimental fish were fed commercial fish food daily at a rate sufficient to allow for satiation of the fish without excessive fouling of the water. At the end of the 7-day period, the liver of each fish was dissected and immediately preserved in RNA*later* (Invitrogen) solution for 24 hours in order to preserve the RNA in the specimen for later manipulation; after 24 hours, the preserved livers in the RNA*later* (Invitrogen) solution were stored in a –80 C freezer until RNA extraction.

RNA Extraction and Reverse Transcription. Preserved livers in the RNA*later* (Invitrogen) solution were removed from the –80 C freezer and thawed on ice in order to begin the RNA extraction. RNA extraction was performed following the RNeasy Mini Kit, Part 1 (Qiagen, Valencia, California). Extracted RNA was stored at –80 C until reverse transcription was performed (RNeasy Mini Kit, Part 1, Qiagen).

Reverse transcription (RT) was performed using Omniscript Reverse Transcription Kit (Qiagen, Valencia, California). The RNA and RNase-free water solution from the RNA extraction were thawed on ice, incubated at 65 C for 5 min, and placed immediately on ice. A master mix solution was created using the following components: 2 µl 10x Buffer RT, 2 µl dinucleotide triphosphate (dNTP) mix, 2 µl Oligo dT primer, 1 µl Omniscript Reverse Transcriptase, 8 µl RNase-free water, and 5 µl Template RNA; total volume was 20 µl (Omniscript® Reverse Transcription Kit, Qiagen, with components altered to suit this experiment). The reverse transcription product was stored at –20 C to be used for PCR.

RT-PCR Amplification and Fragment Analysis. RT-PCR products were divided in half so that the samples could be analyzed using both β-actin and FABP primers. β-actin primers were used to test for the presence of RNA in the sample because the β-actin gene is ubiquitously expressed. PCR master mixes contained: 1 µl template cDNA, 0.5 µl each of forward and reverse primers, and 18 µl of PCR Supermix (Invitrogen). The primer sequences were as follows: FABP forward and reverse primers 5'-CTCTGCTGCA AACATGGTCG-3' and 3'-CGGTCAGCAGTAACAATAATAC-5' respectively (Maples and Bain 2004), and β-actin forward and reverse primers 5'-CTTGCGGAATCCACGAGACC-3' and 3'-CCA GGGCTGTGATCTCCTTCT -5', respectively (Rhee et al. 2009).

FABP amplifications were performed using a thermal cycler under the following conditions: initial denaturation at 95 C for 5 min, followed by 40 cycles of denaturation at 95 C for 30 sec, annealing at 51 C for 30 sec, and extension at 72 C for 30 sec, with a final extension at 72 C for 7 min (Maples and Bain 2004). The expected product size was 341 bp (Maples and Bain 2004). β-actin amplifications were performed using a thermal cycler under the following conditions: initial denaturation step of 94 C for 5 min, 30 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 1 min (30 sec more than reference), and an extension at 72 C for 30 sec, and final extension at 72 C for 7 min (Rhee et al. 2009). The expected product size was 148 bp (Rhee et al. 2009).

RT-PCR fragments were analyzed using 1% agarose gel electrophoresis in a 1X TBE buffer. Loading dye (4 µl) was added to each 20 µl RT-PCR reaction; a total volume of 24 µl was injected into each well. Gel electrophoresis was performed at 125 volts for 25 min or until the loading dye traveled down 3/4 of the gel. The gel was visualized using a digital imaging system (Gel Logic 200 Imaging System, Kodak).

RT-PCR results were interpreted based on the presence or absence of a band of expected size in the electrophoresis lane where each RT-PCR product was injected. RT-PCR products are negatively charged and migrate toward the positive end of the gel box at different speeds depending on the number of base pairs (bp) in the given product. The location where the product stops migrating, which shows up as a band in the lane where the product was injected, indicates the size or number of bp in the product. Therefore, lanes with a band present at the location (or bp size) expected for the FABP gene product indicate that the gene was expressed, whereas lanes without a band present at that location lack FABP gene expression. Multiple lanes are present on each electrophoresis gel, so the lanes were labeled sequentially to allow for interpretation of the results.

Examination of Wild Mummichogs for Cr(VI) Exposure

Male mummichogs were collected from a single site in Wayne Creek ($n = 5$), Back River ($n = 6$), and Deep Creek ($n = 6$) using minnow traps as described above. These fish were transported back to the lab in water from their respective sites; they were then dissected, and livers were immediately preserved in RNA*later* (Invitrogen) solution as described above. Liver tissue from each fish was tested for FABP expression using the methods described above.

Testing Water Samples for Presence of Cr(VI)

Five 50-mL water samples were collected at Wayne Creek and six 50-mL water samples were collected at each of the other study areas. Replicate water samples were collected in 50-mL Falcon tubes (BD Biosciences, San Jose, California) from the water surface at a single site along the shoreline of each water body corresponding to the location of fish collection. Five samples, rather than six, were collected from Wayne Creek due to technical difficulties in the field. After collection, the samples were frozen at –20 C until analyzed using ICP-OES.

Standards of known chromium concentrations were created using a serial dilution of a 1-g L^{-1} K_2CrO_4 stock solution in distilled, deionized water. Chromium concentrations were measured using the Varian 720-ES ICP-OES (Varian, Inc.) with an autosampler in conjunction with ICP Expert II (Agilent Technologies) software. A total of five standards were used to calibrate the instrument for chromium analysis (0.5355 ppm, 0.2678 ppm, 0.0268 ppm, 0.0027 ppm, and 0.0003 ppm Cr(VI)). Radio frequency wavelength 276.653 nm was chosen for chromium concentration readings ($R^2 = 0.9999$, $y = 17672x + 22.253$) (Agilent Technologies).

Microsoft Excel was used to conduct an ANOVA statistical test to compare the concentration of chromium present in the water samples from each of the four collection sites. ANOVAs were considered significant if $P < 0.05$ and a Tukey test was then performed to determine which sites had significantly different chromium concentrations.

Results

FABP Expression in *Mummichogs*

Hoffler Creek. In analysis of Hoffler Creek fish, lanes 2 and 3 contained RNA extracted from livers of control fish that were not contaminated with Cr(VI) in the lab. Lanes 4 and 5 contained RNA extracted from livers that were contaminated with 0.3347 ppm Cr(VI). No band was present in lane 2 (indicating no FABP gene expression) and bands were present in lanes 3, 4, and 5 (indicating the FABP gene was expressed). The expressed bands in lanes 3, 4, and 5 were not 341 base pairs (bp), as originally expected based on the relevant literature (Maples and Bain 2004), but instead 523 bp (Figure 2). Using the National Center for Biotechnology Information database, the sequence for the mummichog FABP gene was analyzed using BLAST (www.ncbi.nlm.nih.gov), and the primer sequences utilized for amplification. It was determined that 523 bp was the correct product size that would result from using the FABP primers.

Wayne Creek. Of the five fish collected from Wayne Creek, three fish showed expression of the FABP gene (bands present at 523 bp in lanes 3, 4, and 6; Figure 3) while two fish did not (bands absent in lanes 2 and 5; Figure 3). Lanes 12–16 were used to test for the presence of RNA in the sample from each fish by testing for expression of the β-actin gene, which is ubiquitously and constitutively expressed. The presence of a band at 148 bp (the expected size for the β-actin expression product; Rhee et al. 2009) in lanes 12–16 (Figure 3) shows that RNA was present in all samples. Therefore, the lack of bands in lanes 2 and 5 indicate a lack of FABP gene expression, rather than a lack of RNA in the sample as might occur due to technician error.

Back River. All six fish collected from Back River showed expression of the FABP gene (bands present at 523 bp in lanes 2–7; Figure 4). One sample did not show expression of β-actin (absence of a band in lane 15; Figure 4), indicating technician error in processing the β-actin product for that fish; however, the presence of a product in lane 5 (using FABP primers) indicates the presence of RNA in the sample (Figure 4).

Deep Creek. Three fish collected from Deep Creek showed expression of the FABP gene (bands present at 523 bp in lanes 5–7;

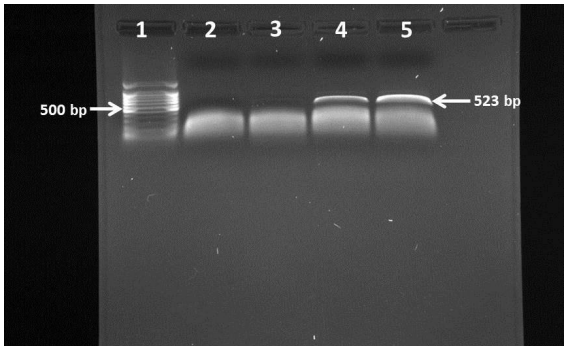

Figure 2. Hoffler Creek: Representative reverse transcriptase PCR agarose gel for FABP gene expression. Control mummichogs were not contaminated with Cr(VI), contaminated mummichogs were contaminated with 0.3347 ppm Cr(VI). Lane1:100bp DNA ladder. Lane 2: Control mummichog liver- not expressed. Lane 3: Control mummichog liver- lightly expressed at 523 bp. Lane 4: Contaminated mummichog liver- expressed at 523 bp. Lane 5: Contaminated mummichog liver- expressed at 523 bp.

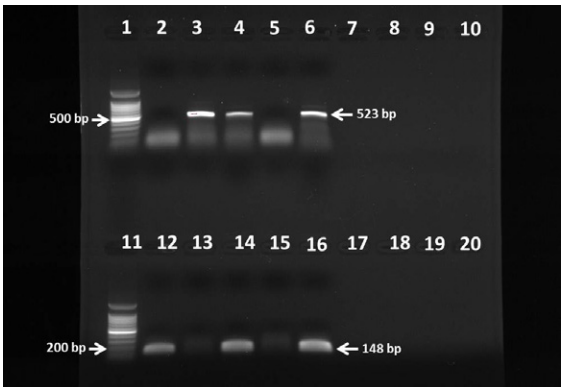

Figure 3. Wayne Creek: Representative reverse transcriptase PCR gel for FABP and β-actin gene expression. First tier of lanes represent amplified RT-PCR products with FABP primers, second tier of lanes represent amplified RT-PCR products with β-actin primers. Lanes 1 and 11: 100bp DNA ladder. Lanes 2 and 5: Mummichog liver- not expressed. Lanes 3, 4 and 6: Mummichog liver-expressed. Lanes 12, 14, and 16: Mummichog liver- expressed. Lane 13 and 15: Mummichog liver- lightly expressed.

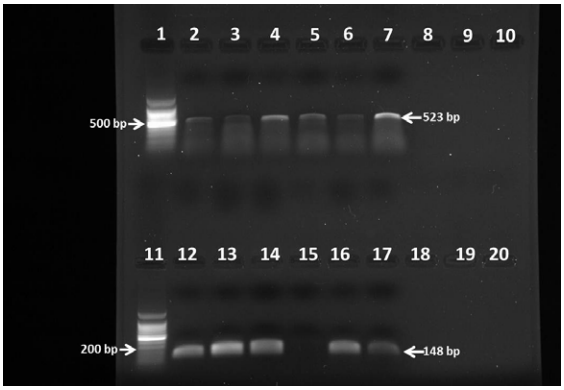

Figure 4. Back River: Representative reverse transcriptase PCR gel for FABP and β-actin gene expression. First tier of lanes represent amplified RT-PCR products with FABP primers, second tier of lanes represent amplified RT-PCR products with β-actin primers. Lanes 1 and 11: 100bp DNA ladder. Lanes 2–7: Mummichog liver- expressed. Lanes 12, 13, 14, 16, and 17: Mummichog liver- expressed. Lane 15: Mummichog liver- not expressed.

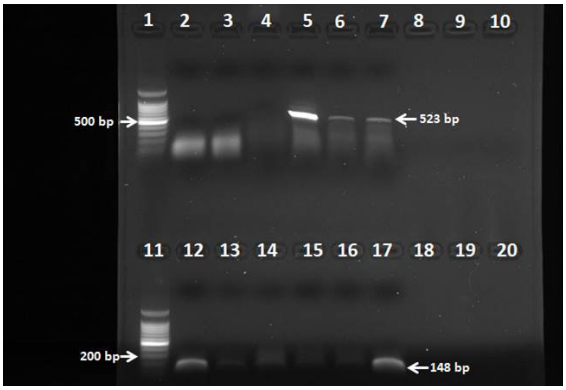

Figure 5. Deep Creek: Representative reverse transcriptase PCR gel for FABP and β-actin gene expression. First tier of lanes represent amplified RT-PCR products with FABP primers, second tier of lanes represent amplified RT-PCR products with β-actin primers. Lane 1 and lane 11:100bp DNA ladder. Lanes 2–4: Mummichog liver- not expressed. Lanes 5–7: Mummchog liver-expressed. Lanes 12–17: Mummichog liver- expressed.

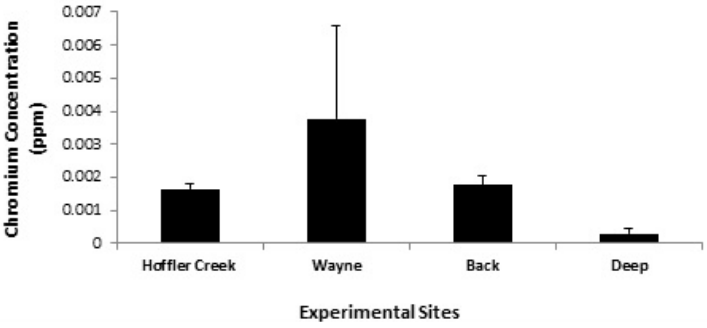

Figure 6. Mean and 1 SD (error bars) hexavalent chromium concentrations (ppm) at the four study sites.

Table 1. Summary of agarose gel results. Number of mummichogs that expressed FABP and β-actin genes compared to total.

Location	FABP expressed	β-actin expressed
Hoffler Creek	3/4	N/A
Wayne Creek	3/5	5/5
Back River	6/6	5/6
Deep Creek	3/6	6/6

Figure 5) whereas three fish did not (absence of a band in lanes 2–4; Figure 5). All samples showed expression of β-actin (presence of bands at 148 bp in lanes 12–17; Figure 5), demonstating that the lack of bands in lanes 2–4 indicate a lack of FABP gene expression, rather than a lack of RNA in the sample.

Direct Detection of Chromium in Water Samples

Water samples from the four experimental sites were analyzed using ICP-OES to determine the concentration of chromium present at each experimental site. The average total chromium concentration at Hoffler Creek was 0.0017 ppm (SD = 0.0002, $n = 6$), Wayne Creek was 0.0038 ppm (SD = 0.0028, $n = 5$), Back River was 0.0018 ppm (SD = 0.0003, $n = 6$), and Deep Creek was 0.0003 ppm (SD = 0.0002, $n = 6$) (Figure 6). Wayne Creek had a greater concentration of chromium than Deep Creek; concentrations in the Back River and Hoffler Creek were similar to each other and to those in both Wayne Creek and Deep Creek ($F = 5.324$, df = 19, $P = 0.0078$).

Discussion

Overall results on FABP gene expression in mummichog using RT-PCR techniques were highly variable. Initially, it was hypothesized that mummichogs collected from Hoffler Creek would serve as uncontaminated controls to compare to fish contaminated in the lab. However, one of the two control fish from Hoffler Creek showed gene expression for FABP. This result led to the hypothesis that Hoffler Creek may have been contaminated with Cr(VI) and suggested the need to test the site for chromium contamination to determine whether the mummichogs were previously exposed to chromium. In this case, these fish would obviously not have offered a valid control. This hypothesis was supported using ICP-OES analysis, which confirmed the presence of chromium in the water taken from Hoffler Creek. Initially, we planned to test additional control and laboratory contaminated fish from Hoffler Creek, hence our small beginning sample size. The lack of an uncontaminated control led us to modify our study objectives to consider the relationship between the presence of chromium and FABP expression, as detected by RT-PCR, in wild caught fish from additional sites in southeastern Virginia.

Analysis with ICP-OES showed the presence of chromium at the three sites other than Hoffler Creek, sampled, with the highest concentration at Wayne Creek. Hence fish from all of these sites were likely exposed to chromium contamination in the wild and thus should have shown measureable levels of FABP expression (Maples and Bain 2004, Roling et al. 2006). However, only some contaminated fish showed expression for the FABP transcript, while others did not show expression (Table 1). The variability of results could be due to differences in genetic composition of the mummichogs collected. Thus, all fish in the same population will not react the same to perturbations and disturbances. Some mummichogs may be more sensitive to the change in environment, while others may have a higher tolerance, resulting in individual differences in FABP expression.

In addition, Roling et al. (2006) found that some genes of adult mummichogs were activated when exposed to 2.5 mg L^{-1} of Cr(VI) but not when exposed to 3.0 mg L^{-1} Cr(VI). This suggests that this species may have a method of activating different genes under various stressors in order to survive, which could be a contributing factor to their hardiness. The fish that are exposed to a lower concentration of Cr(VI) activate or deactivate certain genes as a means of tolerance, but when higher concentrations are introduced, other genes are activated and deactivated as a means of survival (Roling et al. 2006). The fish in our study showed variable results when exposed to levels of Cr(VI) present in their environment. Some contaminated fish expressed detectable levels of FABP, while others did not. Because these fish were not genetically identical, some may have activated the FABP as a means of tolerance, while others did not.

Additional variability may stem from the use of RT-PCR in this study. Previous studies used quantitative real-time PCR to assess FABP expression in mummichog (Maples and Bain 2004, Roling et al. 2006). Real-time PCR quantitatively measures a change in gene expression, rather than providing a binary (expressed/not expressed) result as standard RT-PCR does, and is substantially more expensive to perform. With RT-PCR, even if a band of gene expression is not visible on the gel, a very small amount may be present; this can only be determined using real-time PCR. The contaminated mummichogs that did not visibly show expression in this study may have actually expressed FABP at levels undetectable using standard RT-PCR methods.

Overall, the four experimental sites in the Hampton Roads area of the Chesapeake Bay were contaminated with chromium at levels that have an effect on expression of the FABP gene in mummichogs. The use of RT-PCR to assess gene expression in this species as a bioindicator of the health of estuarine systems is a valid approach when used in conjunction with ICP-OES analysis of the water bodies where fish were collected. However, not all of the mumichogs sampled showed gene expression for the FABP gene. In the future, other genes should be examined to determine the effect of chromium concentration on gene expression. Further studies could also be conducted using a larger sample size of mummichogs to determine whether the percentage of fish expressing the FABP gene is related to the increasing concentration of chromium per site.

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