CAUSE FOR CONCERN: BIOLOGICAL IMPLICATIONS OF HEAVY METAL CONTAMINATION IN KAZAKHSTAN’S SYR DARYA RIVER

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CAUSE FOR CONCERN: BIOLOGICAL IMPLICATIONS OF HEAVY METAL CONTAMINATION IN KAZAKHSTAN’S SYR DARYA RIVER

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ABSTRACT

The Syr Darya, one of the largest rivers in southern Kazakhstan, is a major source of freshwater feeding the Aral Sea. In the 1950s, water was diverted from the Syr Darya to support agricultural production leading to the drying of the Aral Sea, which has been characterized as one of the worst environmental catastrophes in modern day history. Mismanagement of these diverted waters has paved the way for potential surface water contamination in the Aral Sea Basin. While efforts to revive the Aral Sea are underway, few investigations have sought to assess the impacts of potential heavy metal contamination in the Syr Darya Watershed. As such, the goal of this study was to assess the presence and biological effects of heavy metal contaminants in the Syr Darya. This was accomplished by collecting water and sediment samples from five sites and roach (Rutilus rutilus) samples from three sites along the Syr Darya. Water, sediment, and roach muscle tissue samples were analyzed for a suite of contaminants, while roach liver, brain, gonad, and gill tissues were analyzed for the expression of genes considered to be biomarkers of heavy metal exposures (e.g., metallothionein and superoxide dismutase). Water and fish muscle tissue analysis revealed the presence of multiple heavy metals above local regulatory limits. Roach fish from two of the three sites experienced alterations in the expression of genes considered biomarkers of contaminant exposure, suggesting that chemical loads at some of the sites in the Syr Darya were sufficient to induce biological effects. Data collected as part of this study will be utilized to complete an ecological risk assessment of the Syr Darya River basin.
ACKNOWLEDGEMENTS

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INTRODUCTION

Originating in the Tian Shan Mountains of Kyrgyzstan and Uzbekistan, the Syr Darya drains a 402,760 km² watershed that extends into Tajikistan and Kazakhstan (Savoskul, 2003). The Syr Darya watershed has been subject to a host of environmental problems following what is considered one of the worst environmental disasters in modern history, the drying of the Aral Sea (Glantz, 1999; Small and Bunce, 2003; Micklin, 2007). Extensive irrigation in the 1950s required the diversion of water from the Syr and Amu Darya rivers, each of which flow into the Aral Sea, to supplement increasing levels of domestic cotton production (Micklin, 1991; see review Small and Bunce, 2003). The mismanagement of these diverted waters facilitated the drying of the Aral Sea, led to the disappearance of commercial fisheries, and paved the way for potential surface water contamination within the Aral Sea Basin (Micklin, 1991; see review Small and Bunce, 2003; Savoskul, 2003).

Heavy metal contamination in the Aral Sea Basin is of particular concern given that heavy metal concentrations exceeding World Health Organization (WHO) drinking water guidelines have been observed in ground and surface waters (Kadryzhanov et al., 2005; Froebrich et al., 2006; Friedrich, 2009). For example, high concentrations of uranium have been found in the Amu Darya and parts of the Syr Darya river basin (Kadryzhanov et al., 2005; Froebrich et al., 2006; Friedrich, 2009). Potential sources of surface water heavy metal contamination in the Syr Darya watershed include runoff from agricultural lands, uranium mines and tailings, and radioactive waste storage sites (Micklin, 1991; Fyodorov and Kayukov, 2002; Kadryzhanov et al., 2005; Abdelouas, 2006; Friedrich, 2009).

From a biological perspective, the presence of heavy metals may pose hazards to fish and other aquatic organisms inhabiting the Syr Darya River. Heavy metals are known to cause a
range of adverse biological effects including DNA damage, carcinogenesis, and apoptosis (Wang et al., 2001; see review Tchounwou et al., 2012). In addition, heavy metals may induce the production of harmful reactive oxygen species in fish and thus induce oxidative stress (Ahmad et al., 2000; Song et al., 2012). Many studies in fish exposed to heavy metals have observed the increased expression of genes thought to protect fish from heavy metal exposures (e.g. metallothionein, superoxide dismutase, and catalase), suggesting the utility of these genes as biomarkers in screening for the exposure and effects of heavy metals (Schlenk et al., 1999; Quiros et al., 2007; Woo et al. 2009; Espinoza et al., 2012).

Despite the known ecological impairment of the Aral Sea Basin and associated watersheds, information regarding heavy metal contamination and its impacts on resident aquatic organisms within the Syr Darya River remains scarce. As such, this study sought to conduct an ecotoxicological assessment of several sites along the Syr Darya River. The specific objectives of this study were to: 1) assess the presence of heavy metals in the Syr Darya River and 2) evaluate the biomarker responses of wild-caught fish from a subset of sampling sites. An integrated approach featuring water grab samples, sediment grab samples, and muscle tissue from wild-caught roach (Rutilus rutilus) was utilized to determine the presence of 14 heavy metals at five sampling sites along a 250-km stretch of the Syr Darya. The biomarker responses of roach captured from three sampling sites were evaluated using a gene expression approach in an effort to gain insight regarding heavy metal exposure history and potential biological effects.
MATERIALS AND METHODS

Site Selection and Sampling Methods. Permission for collection of native fish was secured in advance from the South Kazakhstan Natural Resources and Environmental Management. The most accessible locations along a stretch of the Syr Darya from where it enters Kazakhstan to a point upstream from the Arys river south of Turkistan, KZ (Fig. 1) were identified for sampling, monitoring and collection of fish tissues. A satellite navigation device (Garmin GPS 12XL) was used to determine geographic coordinates for sampling sites (Fig. 1). General water quality parameters (pH, temperature, turbidity, dissolved oxygen) were measured at the time of sample collection using a Hach field probe. Water grab samples and bottom sediment grab samples were collected at all 6 locations and roach fish were collected near the Zhetisay, Shardara, and Koksaray Bridges, indicated as sites 1, 2, and 5, respectively (Fig. 1), between June 6-8, 2015.
Water samples taken for heavy metal analysis were preserved in nitric acid. Sediment samples (approximately 200 grams) were collected in 250 mL amber jars, held on ice and then transferred to a freezer for subsequent extraction and analysis as described below.

**Fish Collection and Processing.** Roach were captured at each site using a combination of gill net or seine. Fish were sacrificed using a lethal dose (0.3 g/L) of buffered tricaine mesylate (MS-222). Total body weight was recorded, and length was recorded to the nearest millimeter using a ruler. Condition factor was calculated as \[
\frac{\text{body mass (g)}}{\text{total length (mm)}^3} \times 100
\]. Upon dissection, liver and gonad tissue were weighed for the determination of gonadosomatic index (GSI) and hepatosomatic index (HSI). GSI and HSI were calculated as
[(tissue mass (g)/body mass (g)) x 100]. Liver, gonad, brain and gill tissue were placed into separate 2 mL microtubes pre-filled with 1 mL of RNAlater Stabilization Solution (Ambion, Thermo Fisher Scientific, Wilmington, DE) for subsequent gene expression analysis. Microtubes were then immediately placed into a cooler with a small ice pack until transferred to Texas Christian University where they were stored at -80°C until processing. Muscle tissue was collected for heavy metals analysis by taking a fillet from each fish. Muscle fillets were placed into glass test tubes and kept on ice until transferred to Al-Farabi Kazakh National University for heavy metal analysis.

**Gene Expression Analysis.** Methods for gene expression analysis are outlined in Jeffries et al. (2014). All tissues were homogenized using a QSonica tissue sonicator (QSonica, Farmingdale, NY), and total RNA was extracted from each sample using the Maxwell 16 LEV simply RNA Purification Kit (Promega, Madison, WI) per manufacturer protocol. Using the NanoDrop 1000 (ThermoScientific, Wilmington, DE), total RNA was quantified and checked for purity. All samples had 260/280 nm ratios ≥ 2.04. A subset of samples from each batch of RNA extractions was randomly selected to ensure RNA quality using the Experion RNA StdSens analysis kit (BioRad, Hercules, CA). First-strand cDNA was synthesized using the iScript cDNA synthesis kit (BioRad, Hercules, CA) per manufacturer protocol. Each 10 μL synthesis reaction contained the following: 2 μL of 5x iScript reaction mix, 0.5 μL iScript reverse transcriptase and 0.1 μg of total RNA diluted into 7.5 μL of nuclease free water. Reactions were performed using a TC100 thermal cycler (BioRad, Hercules, CA) with a thermal cycling program of 5 min at 25 °C followed by 30 min at 42 °C and 5 min at 85 °C.

All qPCR reactions were performed in triplicate using a CFX Connect real-time PCR detection system managed by CFX Manage Software version 3.0 (BioRad, Hercules, CA). Each
10 μL qPCR reaction contained the following: 5 μL of SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA), 0.3 μL primer, 4.3 μL of nuclease free water and 0.4 μL of cDNA. The thermal cycling program consisted of an activation step (95 °C, 30 sec) followed by 40 cycles of denaturing (95 °C, 10 sec) and annealing (primer specific temperature, 15 sec). A final melting curve was generated at the end of each program to assess amplicon specificity. The standard curve method was used to quantify the expression of each target gene and expression was normalized to that of a reference gene, ribosomal protein L8 (l8). For liver, gonad and brain, there were no differences in l8 expression between groups (ANOVA, all p values ≥ 0.07).

Target genes analyzed included heat shock protein 70 (hsp70), Cu/Zn superoxide dismutase (sod), catalase (cat), glutathione s-transferase mu (gst), and metallothionein (met). These genes were selected given that they are commonly used as biomarkers in aquatic toxicology. Primer sequences not found in the literature were designed using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and optimal annealing temperature was determined by selecting the greatest amplicon yield from a series of identical qPCR reactions conducted across a thermal gradient.

**Statistical analysis.** All biological data were analyzed via one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparisons test to determine which groups were significantly different from one another. In cases where the assumption of homogeneity of variances was not met, significant differences were determined by way of a Wilcoxon test followed by Steel-Dwass post-hoc multiple comparisons test. All analyses were conducted using the statistical software package, JMP version 11.2.0 (SAS Institute) and α was set at 0.05.
RESULTS

Heavy Metal Analysis of Water, Sediment, and Muscle Tissue. No significant differences were seen in individual or total metal concentrations in water and sediment samples across sites. However, fish from site two had 2.47 and 1.71 fold higher levels copper concentrations in the muscle tissue samples compared to fish from sites one and five, respectively (ANOVA, \( p \) value = 0.051; Table 1). Water grab samples revealed the presence of copper, magnesium, and vanadium at levels above local regulatory limits. Additionally, no differences were observed in individual or total metal concentrations in fish muscle tissue across sites.

<table>
<thead>
<tr>
<th></th>
<th>V</th>
<th>Cr</th>
<th>Mn</th>
<th>Fe</th>
<th>Co</th>
<th>Ni</th>
<th>Cu</th>
<th>Zn</th>
<th>Se</th>
<th>Sr</th>
<th>Cs</th>
<th>Ba</th>
<th>U</th>
<th>Total</th>
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<tr>
<td>Site One</td>
<td>0.10</td>
<td>0.39</td>
<td>1.39</td>
<td>37.30</td>
<td>0.06</td>
<td>0.51</td>
<td>0.65</td>
<td>5.39</td>
<td>0.91</td>
<td>5.40</td>
<td>0.07</td>
<td>1.17</td>
<td>0.01</td>
<td>53.37</td>
</tr>
<tr>
<td>Site One</td>
<td>±0.05</td>
<td>±0.06</td>
<td>±0.37</td>
<td>±12.81</td>
<td>±0.01</td>
<td>±0.20</td>
<td>±0.14</td>
<td>±0.50</td>
<td>±0.14</td>
<td>±0.72</td>
<td>±0.01</td>
<td>±0.27</td>
<td>±0.00</td>
<td></td>
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<tr>
<td>Site Two</td>
<td>0.07</td>
<td>0.47</td>
<td>0.81</td>
<td>33.00</td>
<td>0.06</td>
<td>1.00</td>
<td>1.65</td>
<td>6.12</td>
<td>1.32</td>
<td>3.42</td>
<td>0.07</td>
<td>0.68</td>
<td>0.00</td>
<td>48.67</td>
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<td>Site Two</td>
<td>±0.02</td>
<td>±0.06</td>
<td>±0.10</td>
<td>±5.52</td>
<td>±0.00</td>
<td>±0.31</td>
<td>±0.34</td>
<td>±0.63</td>
<td>±0.02</td>
<td>±0.36</td>
<td>±0.00</td>
<td>±0.08</td>
<td>±0.00</td>
<td></td>
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<tr>
<td>Site Five</td>
<td>ND</td>
<td>0.36</td>
<td>1.06</td>
<td>21.91</td>
<td>0.05</td>
<td>0.26</td>
<td>0.96</td>
<td>8.08</td>
<td>1.07</td>
<td>11.86</td>
<td>0.07</td>
<td>0.87</td>
<td>0.01</td>
<td>46.57</td>
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<tr>
<td>Site Five</td>
<td>±0.03</td>
<td>±0.48</td>
<td>±5.03</td>
<td>±0.00</td>
<td>±0.04</td>
<td>±0.33</td>
<td>±1.15</td>
<td>±0.13</td>
<td>±6.30</td>
<td>±0.01</td>
<td>±0.16</td>
<td>±0.00</td>
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Table 1. Mean metal concentrations ± standard error in fish muscle tissue from sites 1, 2, and 5. ND indicates not detected.

Morphometric Assessment. Significant differences in body mass, length, and condition factor (ANOVA, \( p \) value < 0.01 for each metric; Table 2) were noted between roach from the three sampling locations. Specifically, roach from site five had significantly lower body masses and lengths than those from sites one and two. Fish from sites one and five had significantly lower condition factors than those from site two. Significant differences were also detected in GSI (Wilcoxon, \( p \) value < 0.01; Table 2) and HSI (ANOVA, \( p \) value < 0.01; Table 2), with fish from site two having significantly higher GSI and HSI than fish from sites one and five.
Table 2. Mean mass, length, condition factor, gonadosomatic index (GSI) and liver somatic index (LSI) between sites. Letters indicate statistically significant differences between sites.

<table>
<thead>
<tr>
<th></th>
<th>Mass (g)</th>
<th>Length (mm)</th>
<th>Condition Factor</th>
<th>GSI</th>
<th>LSI</th>
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<tr>
<td>Site One</td>
<td>38.3^A</td>
<td>134.1^A</td>
<td>1.57^B</td>
<td>0.74^B</td>
<td>1.13^B</td>
</tr>
<tr>
<td>Site Two</td>
<td>46.3^A</td>
<td>137.0^A</td>
<td>1.78^A</td>
<td>1.54^A</td>
<td>2.05^A</td>
</tr>
<tr>
<td>Site Five</td>
<td>14.1^B</td>
<td>99.4^B</td>
<td>1.39^B</td>
<td>0.79^B</td>
<td>0.89^B</td>
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**Biomarkers of Heavy Metal Exposures and Sub-lethal Stress Responses.** No significant alterations were noted in liver (ANOVA, $p$ value = 0.10; Fig. 2) or gill (ANOVA, $p$ value = 0.053; Fig. 2) expression of *hsp70*. However, a 1.9 fold difference was detected in gill *hsp70* expression between roach from sites two and five.
However, significant differences were detected in the expression of genes considered biomarkers of oxidative stress (*i.e.* *sod* and *cat*). Fish from sites one and five experienced significantly higher expression of *sod* (Wilcoxon, *p* value < 0.01; Fig. 3) and *cat* (ANOVA, *p* value < 0.01; Fig. 4) in the liver. Additionally, significantly higher levels of gonad *cat* expression were detected in fish from site five compared to fish from site two (Wilcoxon, *p* value < 0.02; Fig. 4). No statistical differences were detected for *sod* and *cat* expression in gill tissue (ANOVA, *p* value > 0.23 in both cases; Fig. 3-4).

Figure 2. The mean relative expression of heat shock protein 70 (*hsp70*) in Syr Darya roach collected from three sampling sites. Error bars represent standard error.
Figure 3. The mean relative expression of superoxide dismutase (*sod*) in liver, gill and gonad of roach from three Syr Darya sites. Error bars represent standard error. Letters above bars indicate statistically significant differences.
No differences were found in \( \text{gst} \) expression in liver (Wilcoxon, \( p \) value = 0.31; data not shown) or the gill (ANOVA, \( p \) value = 0.30; data not shown). While not statistically significant, fish from site two had 3.6 and 13.2 fold higher levels of \( \text{mt} \) expression in the liver (Wilcoxon, \( p \) value = 0.17; Fig. 5) and gill (Wilcoxon, \( p \) value = 0.10; Fig. 5), respectively, when compared to site five. Fish from site two also had 2.6 and 7.1 fold higher expressions of \( \text{mt} \) in the liver and gill, respectively, when compared to site one.
The objectives of this study were to 1) assess the presence of heavy metals in the Syr Darya River and 2) evaluate the biomarker responses of wild-caught fish from a subset of sampling sites. Results from this study suggest that heavy metals are found in the Syr Darya and associated reservoirs above local regulatory limits, and that resident fish species may be

**DISCUSSION**

The objectives of this study were to 1) assess the presence of heavy metals in the Syr Darya River and 2) evaluate the biomarker responses of wild-caught fish from a subset of sampling sites. Results from this study suggest that heavy metals are found in the Syr Darya and associated reservoirs above local regulatory limits, and that resident fish species may be
experiencing site-specific differences in the expression of genes indicative of heavy metal contamination and sub lethal stress responses.

**Heavy Metal Analysis of Water, Sediment, and Muscle Tissue.** Although no differences were seen in metal concentrations across sites for water, sediment, and muscle tissue analysis, the presence of multiple heavy metals above local regulatory limits suggests that fish from some sites along the Syr Darya may be at high risk of exposure to heavy metals. Extreme reductions in the volume of water in the Syr Darya for agricultural use may have increased the likelihood of heavy metal contamination. Additionally, many heavy metals, including vanadium and copper are known to be released at high concentrations from processes associated with uranium tilling and in situ leach mining operations (Abdelouas, 2006). As such, heavy metal contamination from sources near the Syr Darya in conjunction with extreme reductions in the river’s water volume may be creating toxic environments for fish and other organisms inhabiting the Syr Darya. Therefore, there is a need to correlate the current land use with biological and chemical data to assist in the development of efforts aimed at restoring the environmental quality in the Aral Sea basin.

**Morphometric Assessment.** Reductions in morphometric measurements often used as parameters of fish health (*i.e.* mass, length, condition factor, GSI, LSI [Iwama et al., 2012]) were observed in fish from site five, suggesting that fish from site five may be less robust and healthy than fish from sites one and two. Reductions in condition factor, LSI and GSI in fish from site one compared to site two, suggest that fish from site one may also be less healthy than those from site two. The above differences may be the result of site-specific alterations in environmental quality from factors such as nutrient availability, water levels, water and sediment conditions, or the presence of toxic contaminants. In addition, the sampling location for site two
is located in the Shardara Reservoir. As a result, fish taken from a reservoir may be experiencing different environmental conditions, which could be an explanation for the observed differences in morphometric measurements. Furthermore, the sex and age of fish may have differed between sites. Fish were not sexually mature upon collection and could not accurately be sexed based on physical appearance of the gonads.

**Biomarkers of Heavy Metal Exposures and Sub-lethal Stress Responses.** Heat shock protein 70 (hsp70) is induced in response to environmental stressors, including heavy metals (Yamashita et al., 2010). While no significant differences were noted in hepatic hsp70 expression, the 1.9 fold difference in gill expression between fish from sites two and five may suggest that fish at site five are experiencing elevated levels of biological stress. Previous studies have shown that milkfish (Chanos chanos) exposed to concentrations of heavy metals (i.e., Cu, Pb, Zn, Cd, Mn, and Fe) experienced increases in hsp70 expression in gill tissue (Rajeshkumar et al., 2013). Therefore, it is possible that fish in the present study may be up regulating hsp70 expression as a stress response to heavy metal exposures. However, as hsp70 is induced by a variety of environmental stressors, meaning that the observed differences in hsp70 expression may be the result of other differences in environmental quality, such as alterations in temperature and salinity (Tine et al., 2010; Yamashita et al., 2010).

The present study also analyzed the expression of genes indicative of oxidative stress (i.e., sod, cat). Site-specific differences were noted in hepatic expression of sod and cat as well as expression of cat in gill tissue. Woo et al. (2009) found that sod and cat, two enzymes responsible for the detoxification of harmful reactive oxygen species (McCord and Fridovich, 1969; Leibler and Reed, 1997), may be altered in response to heavy metal exposures. Copper and vanadium, two metals found above regulatory limits in this study, are redox active metals and
produce reactive oxygen species through redox cycling (Sevcikova et al., 2011). The presence of these metals at high concentrations may be causing the production of these harmful reactive oxygen species in fish along the Syr Darya and fish may be responding with the upregulation of stress response genes such as sod and cat. However, it is possible that sod and cat expression could be induced by other contaminants, such as organochlorine pesticides, which have been detected in the Aral Sea basin (Micklin, 1991). For example, a study by Jin et al. (2009) revealed an upregulation in liver sod and cat in response to atrazine exposure.

Though previous studies have shown that exposures to heavy metals may induce gst (Kim and Jung, 2016) the present study did not reveal any significant differences in the hepatic expression of gst. As an antioxidant gene, gst may be induced in response to heavy metal exposure to protect fish from oxidative stress, but this study does not present evidence of this in fish from the Syr Darya.

Multiple studies in fish have shown that mt expression can be utilized as a biomarker of exposure to heavy metals (Schlenk et al., 1999; Quiros et al., 2007; Espinoza et al., 2012). Metallothioneins are responsible for sequestering heavy metals to reduce the production of reactive oxygen species, thus playing a protective role in response to heavy metal toxicity (Hamilton and Mehrle, 1986). In the present study, fold differences in mt expression of 2.6 and 3.6 in liver and 7.1 and 13.2 in gill suggest that fish from site two may be inducing mt as a protective mechanism against heavy metal toxicity. Observed fold differences in the concentration of copper in roach muscle tissue may be responsible for the upregulation of mt in individuals from site two. This increased copper concentration may be inducing mt expression, as mt is responsible for sequestering copper in fish tissues (Cousins, 1985, Hamilton and Mehrle, 1986). It should be noted that while mt expression was higher in fish from site two, sod and cat
expression was higher in fish from sites one and five. With no differences were observed in total heavy metal body burden in fish tissue across sites, fish at site two may be overexpressing mt to protect themselves from heavy metal exposure, thus avoiding the oxidative stress that fish from sites one and five may be experiencing. Research has shown the evolution of resistance in invertebrate populations exposed to heavy metals and environmental pollutants, though there is less evidence in fish (Klerks 1987). The overexpression of mt observed in the present study could provide a potential mechanism for the evolution of resistance to heavy metals in fish within the Syr Darya, and thus explain the perceived differences in the health of roach across sites. Further studies aimed at determining the source of variation in morphometric and gene expression data should be completed in order to understand the differences in fish population responses to heavy metal contamination.

CONCLUSION

The results of this study draw several conclusions. First, water, sediment, and muscle tissue analysis have revealed that there is heavy metal contamination along stretches of the Syr Darya. Second, observed differences in morphometric measurements and the expression of genes indicative of sub-lethal stress responses indicates that there are spatial differences in biomarker responses of fish along stretches of the Syr Darya. However, because site two lies within the Shardara Reservoir, some of this variation could be explained by differences in habitat quality between riparian and reservoir environments. Further studies aimed at utilizing sexually differentiated fish should be completed as variation in targeted gene expression could be explained by differences in developmental stages or biological sex. Finally, data collected as a
part of this study may prove useful in completing an ecotoxicological risk assessment of the Syr Darya watershed.
REFERENCES


