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Effects of coal combustion residues on survival, antioxidant potential, and genotoxicity resulting from full-lifecycle exposure of grass shrimp (*Palaemonetes pugio Holthius*)

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Abstract

Coal combustion residues (CCRs), largely derived from coal-fired electrical generation, are rich in numerous trace elements that have the potential to induce sublethal effects including oxidative stress, alterations in antioxidant status and DNA single strand breaks (SSB). CCRs are frequently discharged into natural and man-made aquatic systems. As the effects of CCRs have received relatively little attention in estuarine systems, the estuarine grass shrimp, *Palaemonetes pugio*, was chosen for this study. Grass shrimp were exposed in the laboratory to CCR-enriched sediments and food over a full life cycle. Survival to metamorphosis was significantly reduced in CCR-exposed larvae (17±4 versus 70±13% in the controls) but not in the juveniles or adults. The COMET assay, a general but sensitive assay for genotoxicity, was used to quantify DNA SSB in the adults. Total antioxidant potential was examined to assess the overall antioxidant scavenging capacity of CCR-exposed and non-exposed adult grass shrimp. Grass shrimp exposed to CCR significantly accumulated selenium and cadmium compared to unexposed shrimp, although an inverse relationship was seen for mercury accumulation. Chronic CCR exposure caused DNA SSB in hepatopancreas cells, as evidenced by the significantly increased percent tail DNA, tail moment, and tail length as compared to reference shrimp. However, no significant difference was observed in total antioxidant potential. Our findings suggest that genotoxicity may be an important mode of toxicity of CCR, and that DNA SSB may serve as a useful biomarker of exposure and effect of this very common, complex waste stream.

Keywords: Antioxidant potential; Cadmium; Genotoxicity; Life cycle exposure; Selenium; Coal combustion residues

1. Introduction

A byproduct of coal-fired electricity generation is solid coal combustion residues (CCRs), which are typically enriched in trace elements including, but not limited to, selenium (Se), cadmium (Cd) arsenic (As), chromium (Cr), copper (Cu) and mercury (Hg) (NRC,

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2006). CCRs may contain trace concentrations of organic compounds depending upon facility-specific waste co-management practices and combustion conditions (EPRI, 1997), although organic compounds, if present, are found at much lower concentrations than the trace elements (EPRI, 1987; NRC, 2006). In 2000, the U.S. produced over 1 billion tons of coal, 90% of which was used for power generation (NRC, 2006). The enormous resulting CCR waste stream presents considerable challenges to industry and regulators in developing and implementing efficient and environmentally protective disposal technology (NRC, 2006).

A common disposal method for CCRs is to pump it as a slurry into settling basins, which are designed to capture solids prior to discharge of the surface water into local systems. However, often these basins do not retain all particulate and dissolved materials from CCR, posing the possibility for contamination of local waterways. The trace elements, particularly, Se, Cd, Cu and Arsenic (As) can be bioaccumulated by resident aquatic organisms, potentially causing mortality, teratogenicity or inducing sublethal responses (see Rowe et al., 2002). For example, negative effects of CCR, including increased standard metabolic rate, were observed in a freshwater shrimp, *Palaemonetes paludosus*, when caged in a CCR disposal basin for 8 months (Rowe, 1998). Additionally, increased metabolic costs have been observed in crayfish (*Procambarus acutus*) following exposure to CCR-enriched sediment (Rowe et al., 2001). Hopkins et al. (2004) demonstrated reductions in growth and survival of juvenile benthic fish exposed to CCR sediment and contaminated food. Lemly (2002) reported developmental, histopathological, and teratogenic effects in numerous fish species inhabiting a CCR-contaminated lake, and a decline in abundance and diversity of fish prior to cessation of CCR inputs. Teratogenic effects have also been observed in CCR exposed frogs (Hopkins et al., 2000). Although individual and population level responses demonstrate significant lethal and sublethal effects of CCR, prior to the onset of such responses, changes at the cellular, biochemical or molecular levels would occur. Through the use of biomarkers it may be possible to detect sublethal effects of CCR in exposed organisms, before population-level effects are observed. Only a few studies have detailed sublethal metrics following exposure to CCR (see Ali et al., 2004; Lohner et al., 2001a,b,c). Thus, there is a need to apply suitable biomarkers as 'early warning signals' of exposure to and/or effect of CCR in resident organisms. In addition, biomarkers can provide insights into the mechanisms resulting in CCR toxicity, identify the target sites of toxic insult and also aid in identifying the specific contaminants or groups of contaminants of concern (van der Oost et al., 2003).

Many transition metals mediate the formation of reactive oxygen species (ROS; Prahalad et al., 2000; Valko et al., 2006). The contaminants associated with CCR can, therefore, be an anthropogenically-related source of ROS production (Livingstone, 2001). Although ROS result from normal endogenous processes, excess production of ROS can alter a cell's redox status, result in conditions of oxidative stress and impair lipids, proteins and nucleotides, potentially leading to serious repercussions, such as carcinogenesis (for a review see Valko et al., 2006). To overcome endogenous and anthropogenic elevations of ROS, organisms have developed effective antioxidant systems. Elevated levels of oxidative stress may decrease these cellular pools of antioxidant scavengers and enzymes. This may simply reflect that the oxidative defense systems have functioned normally, and oxidative damage has not necessarily occurred (Doyette et al., 1997; Diaz et al., 2004). Other studies have shown elevated levels of antioxidant enzymes, which may reflect a compensatory response of the organism to conditions of elevated oxidative stress (see Livingstone, 2001; van der Oost et al., 2003). Downs et al. (2001) found increased oxidative stress in *Palaemonetes pugio* exposed to Cd as exhibited by an adaptive increase in GSH concentrations compared to non-exposed shrimp. Similarly, Griffitt et al. (2006) demonstrated an increase in the antioxidant glutathione peroxidase gene in Cd exposed *P. pugio*. Ali et al. (2004) demonstrated that fly ash caused increased lipid peroxidation and induction of antioxidant enzymes in fish. Similarly, in mammals fly ash has been shown to induce oxidative stress and promote ROS formation (Lewis et al., 2003; Van Maanen et al., 1999; Voelkel et al., 2003). Many biomarkers (including those listed above) have been employed routinely to assess the status of an organism's oxidative stress or antioxidant capacity (see van der Oost et al., 2003; Livingstone, 2001). The measurement of total antioxidant potential (AOP) is a recent approach and covers a wide array of antioxidants collectively as opposed to individually, and is advantageous because it is an integrative approach and gives an overall picture of the organism's free radical scavenging capacity (Winston et al., 1998; Regoli and Winston, 1998; Regoli et al., 1999; Downs et al., 2001).

In addition to eliciting conditions of oxidative stress, many of the CCR trace elements have also been shown to be genotoxic (cause DNA damage, e.g. Cr, Cd, Ni and As) and may result in mutagenic or carcinogenic effects (for a review see Valko et al., 2006). Smith-Sonneborn

et al. (1981), Kubitschek and Venta (1979), Li et al. (1983) and Van Maanen et al. (1999) have shown CCR to be mutagenic or genotoxic to a variety of organisms (including *Paramecium tetraurelia*, *Salmonella typhimurium*, Chinese hamster ovarian cells and rat lung epithelial cells). In addition, *in vitro* exposure of calf thymus DNA to CCR has been shown to cause oxidative DNA damage (8-hydroxydeoxyguanosine (8-oxo-dG)) formation; Prahalad et al., 2000). However, there is limited information regarding DNA damage endpoints in aquatic organisms exposed *in vivo* to CCR. The quantification of DNA single strand breaks (SSB) by the COMET assay can be used as a rapid and sensitive indicator of genotoxicant exposure (Mitchelmore and Chipman, 1998; Steinert et al., 1998). DNA SSB are formed as a result of direct genotoxic insult or following failed DNA adduct repair. Previously, the COMET assay has been demonstrated to be a sensitive marker of DNA damage caused by benzo[a]pyrene, chromium VI, hydrogen peroxide, or UV damage in *P. pugio* (Kim et al., 2000; Lee et al., 2000; Hook and Lee, 2004).

P. pugio is an epibenthic species ubiquitous to estuarine and coastal systems in North America from Maine to Texas (Knowlton and Kirby, 1984) where it serves as an important trophic link between benthic, epibenthic, and pelagic communities (Wood, 1967; Poole, 1988). This species is a model organism (APHA, 1985) in many toxicological studies and studies with its congener *P. paludosus* suggest that it may be chronically affected by CCR (Rowe, 1998). We conducted a full life-cycle experiment in which *P. pugio* was exposed to CCR through diet and sediment to examine the chronic, lethal and sublethal effects of CCR on individuals. The extent of DNA SSB in shrimp hepatopancreas, and the total antioxidant potential of shrimp tail tissue were used as indicators of genotoxicity and oxidative stress respectively. We interpreted the biomarkers with respect to the concentrations of accumulated trace elements following exposure.

2. Materials and methods

2.1. Experimental design

This study involved a full life cycle laboratory exposure of *P. pugio*. We used two treatment regimes (four replicates per treatment); one with CCR sediment and CCR-contaminated food, and a reference treatment in which black sand (used to mimic the color of CCR) served as the sediment and food was uncontaminated (see below). Contaminated sediment was derived from a temporary CCR drainage basin at the D-Area Power Facility on the U.S. Department of Energy's Savannah River Site, SC, USA. Contaminated food for juvenile and adult shrimp (crayfish tissue; see below) was collected from drainage basins and a swamp at the D-Area site. Reference crayfish for juveniles and adults was commercially purchased. Food provided to larval grass shrimp was newly hatched brine shrimp (*Artemia* spp.), which were hatched over contaminated sediment or in the absence of sediment.

Experiments were initiated with larval shrimp from stock cultures of multiple gravid adults (to provide genetic diversity) collected from the Patuxent River, MD (salinity ~9–15‰). Three days prior to the predicted peak of hatching, eight 1500 ml beakers were set up with 200 cm^3 of sediment and 20 μm filtered, Patuxent River water. The initial salinity in each beaker was 20‰ (optimal for hatchling survival; Knowlton and Kirby, 1984) by addition of commercial sea salt and was gradually decreased to the ambient salinity of Patuxent river water (~10‰) throughout the duration of the larval stage. Two hundred newly-hatched larvae were placed in each beaker. Larvae were fed newly hatched brine shrimp (*Artemia* spp.) which were hatched either over CCR sediment (contaminated food preparation) or no sediment (reference food). Food was provided *ad libitum*. Salinity, temperature and dissolved oxygen were measured every three days prior to a 50% water change. Survival was monitored daily until metamorphosis and the number of larvae that metamorphosed were counted, and percentage survival reported.

Upon metamorphosis (occurred from day 22 to 35 post hatching) 39 juveniles from each of the replicates ($N=3$ and 4 for CCR and reference respectively) were used to start the juvenile stage of the experiment, with the same replicates carried over from the larval stage. Thirty nine juveniles were used because this was the minimum number per replicate that survived the larval exposures. Juveniles were placed in 57-L, flow-through tanks containing filtered (20 μm) Patuxent River water of ambient salinity, and 1000 cm^3 of sediment (approximately 3 mm depth). Salinity, temperature, and dissolved oxygen were monitored weekly. Juveniles were fed ad libitum ground, dried, shell-free tissues from crayfish (*Procambarus* sp.) collected from drainage basins and a swamp at the D-Area site (CCR treatment contaminated food) or purchased from a supplier to the retail market (reference food). Sediment was rarely observed in the gut of the crayfish, and thus presumably very little of the food source to be provided to the shrimp consisted of the CCR itself. After 20 weeks of exposure, four non-gravid individuals were randomly removed from each tank for each of the bioassays.

2.2. Trace element analysis

Whole body tissue samples (ten pooled shrimp) were collected from each replicate tank at the termination of the experiment. Two initial samples of each sediment type (reference and CCR) were taken at the beginning of the experiment. At the conclusion of the experiment a single sediment sample was taken from each replicate. Samples of the *Artemia* spp. and crayfish food sources were also collected for trace element analyses. Shrimp tissue and food samples (*Artemia* spp. and crayfish) were freeze-dried and ground with a mortar and pestle. Sediment samples were oven-dried at 60 °C. Water samples were collected from each replicate at the end of the larval stage prior to a water change, and at the conclusion of the experiment from the flow-through tanks. Water samples were filtered through a 0.45 μm filter and acidified with 10% ultrex nitric acid.

Trace elements of primary interest in this study were Se, Cd, Pb, Cu, As, V, Ni, Hg, Sr, and Fe as these elements are the most often elevated in environmental matrices in most sites characterized to date (Prahalad et al., 2000; Rowe et al., 2002; NRC, 2006). Samples were acid digested following EPA Method 200.3. After digestion, trace element analysis was conducted by ICP-MS at the Savannah River Ecology Laboratory, SC. Calibration standards were prepared daily by serial dilution ranging from 1–500 μg/L from National Institute for Standards and Technology traceable primary standards. For quality control purposes blanks and certified reference material (Tort 2 and Mess 3; Canadian National Research Council, Ottawa, Canada) were included in the digestion and analysis procedures. Average percent recoveries for trace elements ranged from 91.19% to 117.76%.

2.3. Biomarker assays

All chemicals were purchased from Fisher Scientific (Pittsburg, PA), Sigma Chemical Company (St. Louis, MO), or Gibco (Grand Island, NY). The procedures for the COMET assay were modified from those described by Mitchelmore et al. (1998), Steinert et al. (1998), and Hook and Lee (2004). Shrimp were held unfed overnight to allow for voiding of gut contents to reduce the potential for contamination of cell suspensions with food. Shrimp were sacrificed, the hepatopancreas removed, and the tail frozen and stored in liquid nitrogen for total antioxidant potential analysis (see below). Cell suspensions were prepared by homogenization of the hepatopancreas in 750 μl of Hank's Buffered Salt Solution (HBSS; $Ca^{2+}$and Mg^{2+} free, pH 7.8, 4 °C) and passing the suspension through a 70 μm filter. All suspensions were tested for cell viability via the trypan blue exclusion method and were consistently >90% viable. A positive control experiment was conducted ($n=3$), in which cell suspensions ($n=6$ shrimp) were incubated for 30 min in the dark on ice in one of four concentrations of H_2O_2 (0 μM, 25 μM, 50 μM, 100 μM). Cell suspensions were centrifuged at 700 ×*g* for 2 min, and the resulting cell pellets resuspended in 20 μl of HBSS. This enabled us to choose a suitable positive control dose (50 μM) which was run with each set of *in vivo* treatment samples. For the COMET assay 10 μl of cell suspension was added to 100 μl of 0.65% low melting-point agarose (LMPA) in HBSS and placed onto a prepared agarose-coated slide and overlaid with LMPA. Two replicate slides were prepared for each sample. After solidification cells were lysed (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris-HCl, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10) for at least 2 h at 4 °C. Slides were gently rinsed twice with ice-cold distilled water and transferred into an electrophoresis chamber (Thermo EC Maxicell Primo EC340) filled with electrophoresis buffer (0.1 M NaOH, 1 mM EDTA, pH >12) to unwind the DNA for 15 min. Electrophoresis was conducted at 25 V, 300 mA for 15 min. Slides were then rinsed 3 times for 2 min with neutralization buffer (0.4 M Tris, pH 7.5) and stained with 50 μl of ethidium bromide (2 μg/ml). The extent of DNA damage was analyzed using an Olympus BX50 microscope (×200 magnification). A Q Imaging Retiga 1300 camera and a computerized imaging system (Komet 5.5, Kinetic Imaging) was used to analyze percent tail DNA (the amount of DNA in the tail), tail length, and tail moment (the amount of DNA in the tail × tail length) which are expressed as means ± SEM. Fifty randomly chosen cells per slide were used for analysis. The coefficients of variation (100 * SD/mean) were calculated for each treatment for each of the COMET parameters.

Total antioxidant potential was measured using the Bioxytech AOP-490 biotech kit from Oxis Research (Portland, OR). Grass shrimp tail tissue was homogenized in a 1:4 volume of HBSS (containing a 100 μM solution of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF)). Twenty microliters was reserved to analyze for protein content (see below). Samples were then centrifuged for 10 min at 10,000 ×*g* (4 °C) and the supernatant removed for use in the assay. Samples and standards were then diluted in 1:40 R1 buffer, mixed, and 200 μl placed into wells, on a 96 well plate. Fifty microliters of R2 solution was added to each well, mixed, and incubated for 3 min at room temperature. Fifty microliters of stop solution was then added to

each well and mixed. The plate was then read on a Spectramax Plus 384 plate reader at 490 nm. A buffer blank and set of standards was run on each plate and all samples, blanks and standards were run in triplicate. The uric acid equivalent concentration of each sample was determined using the standard curve. The reserved samples from the AOP analysis were diluted 1:20 and then analyzed using a BCA Protein Assay Kit (Pierce, Rockford, IL) using the microplate technique as per manufacturer's instructions. All samples and standards were run in triplicate. The plates were read on a Spectramax Plus 384 (Molecular Devices, Sunnyvale, CA) plate reader at 562 nm. A buffer blank and set of standards was run on each plate and a standard curve constructed. The protein concentration of each sample was determined using the standard curve generated using bovine serum albumin (BSA). The total antioxidant potential was determined by normalizing the uric acid equivalent to the protein content of each sample, and is expressed as means±SEM.

3. Statistical analysis

Due to non-normal data distributions (but equal variances), a Kruskal–Wallis test was used to test for treatment effects on tail DNA, tail moment, and tail length. Total antioxidant potential data were tested for normality and then analyzed using a one-way ANOVA ($\alpha=0.05$). A directional *t*-test was used to test for differences in trace element concentrations between reference and CCR treatments, because prior analysis of CCR had shown higher concentrations of trace elements. For the survival data the proportion survived was transformed by arcsine square root prior to analysis using a one-way ANOVA ($\alpha=0.05$). All statistical tests were conducted using Minitab software for Windows, version 13 (Minitab, State College, PA).

4. Results

4.1. Trace element analysis

P. pugio exposed to CCR accumulated trace elements (e.g. Se, Cd, Pb, Cu, As, Fe, V), although body burdens only differed significantly from controls for Se, Cd and Hg (Table 1). Table 2 shows the trace element concentrations of the food, sediment, and water to which the shrimp were exposed. *Artemia* spp. did not differ in trace element concentrations between treatments. Crayfish from the CCR contaminated site had higher concentrations of all trace elements than reference crayfish (except for Hg and Fe). Sediment samples from CCR treatments had higher concentrations of all trace elements of interest than the reference sediments (Table 2; small sample sizes of the initial sediment samples precluded statistical analysis of these samples). Fe and Cu were significantly elevated in the larval reference water, and V was significantly elevated in the larval CCR water, although none of these elements were significantly elevated in shrimp tissues. The final water samples did not differ significantly in the concentrations of trace elements of interest, consistent with previous studies of CCR-exposed systems (Rowe et al., 2002).

4.2. Survival and biomarker assays

Significant reductions in survival were observed in the CCR treatments, but this effect was dependent on lifestage. Survival in larvae (mean proportion ±1 SE)

Table 1
Whole body trace element concentrations (ppm dry mass) of *P. pugio* from coal combustion residue (CCR) and reference treatments

Shrimp tissue samples	*n*	Trace element				
		Se	Cd	Pb	Cu	As
Reference	4	2.56±0.28	0.22±0.13	0.51±0.04	135.60±9.31	6.23±0.62
CCR	3	9.44±0.15	2.15±0.22	6.12±5.80	151.78±0.40	7.05±0.94
p value		<0.001	0.01	0.44	0.18	0.52
Shrimp tissue samples	*n*	V	Ni	Hg	Sr	Fe
Reference	4	BDL	12.49±5.13	0.97±0.10	605.68±15.88	186.02±48.38
CCR	3	0.89±0.34	6.74±2.73	0.09 ±0.01	595.89±31.07	441.58±108.43
p value		0.08	0.38	0.003	0.8	0.16

Results are expressed as means±standard error. Minimum detection limits (ng/g) for food and tissue were: Se 90.42, Cd 0.03, Pb 0.71, Cu 1.66, As 0.10, V 0.05, Ni 0.88, Hg 0.02, Sr 0.58, Fe 1.19. Minimum detection limits (μg/L) for water were: Se 0.35, Cd 0.08, Pb 1.96, Cu 3.34, As 0.34, V 0.12, Ni 1.47, Hg 0.04, Fe 63.48.

Table 2
Trace element concentrations (ppm dry mass) in food, sediment, and water in CCR and reference treatments

Exposure type	*n*	Trace elements				
		Se	Cd	Pb	Cu	As
Food						
Artemia						
Reference		2.48– 3.59	BDL	152.09– 176.28	9.27– 12.72	19.82– 24.14
CCR		3.38– 4.30	0.07– 0.17	46.29– 47.39	10.74– 16.40	25.02– 36.33
Crayfish						
Reference		1.77– 1.92	0.44– 0.46	0.79– 0.84	62.86– 67.71	1.92– 1.95
CCR		16.29– 16.46	16.78– 19.98	6.73– 9.01	275.64– 277.31	7.03– 7.07
Sediment						
Initial						
Reference		0.01– 0.23	BDL– 0.01	0.87– 1.23	0.57– 0.75	0.14– 0.15
CCR		6.97– 9.19	3.80– 5.18	35.75– 39.29	46.90– 47.68	154.08– 192.52
Final						
Reference	4	0.22±0.20	0.02±0.001	0.41±0.03	1.06±0.08	0.17±0.05
CCR	3	4.75±0.28	4.44±0.11	34.12±0.63	48.31±1.72	136.06±6.78
p value		0.001	0.001	<0.001	0.001	0.002
Water						
Larval						
Reference	4	20.35±6.20	1.10±0.12	4.32 ±1.47	58.52±7.84	75.99±14.11
CCR	3	15.55±2.95	1.40±0.46	2.40±0.65	29.55 ±3.09	110.00±13.59
p value		0.52	0.59	0.30	0.04	0.16
Final						
Reference	4	2.42±0.31	0.22±0.05	0.28±0.55	16.03 ±2.02	15.24±2.05
CCR	3	2.90±0.50	0.22±0.03	1.31±0.15	17.56±3.90	14.92±4.01
p value		0.47	0.99	0.17	0.75	0.95

Exposure type	*n*	V	Ni	Hg	Sr	Fe
Food						
Artemia						
Reference		0.03– 0.04	2.83– 3.41	0.03– 0.04	25.66– 35.51	115.27– 145.94
CCR		4.23– 7.32	3.67– 6.64	0.06– 0.07	36.27– 47.08	742.51– 1697.89
Crayfish						
Reference		2.47– 2.72	3.16– 3.29	0.16– 0.17	73.25– 84.06	1510.46– 1573.22
CCR		4.37– 4.43	16.63– 17.25	0.10– 0.11	189.23– 212.76	1080.40– 1104.08
Sediment						
Initial						
Reference		0.068– 0.073	BDL	BDL– 0.01	1.36– 1.52	15.72– 40.78
CCR		64.03– 68.10	32.53– 39.00	0.20– 0.25	356.52– 439.39	15088.85– 16450.81
Final						
Reference	4	0.13±0.02	BDL	0.01±0.001	2.32±0.28	34.05±17.02
CCR	3	59.50±1.55	36.42±0.86	0.18±0.01	328.39±7.54	19329.58±680.22
p value		0.001	0.001	0.01	0.001	0.001
Water						
Larval						
Reference	4	BDL	117.92±37.99	0.10±0.04	11174.97±2190.43	15.87±4.09
CCR	3	27.16±1.18	112.67±23.51	0.25±0.02	4265.11±898.97	BDL
p value		0.002	0.91	0.04	0.06	0.31
Final						
Reference	4	BDL	11.17±10.40	0.01±0.01	1073.23±0.31	BDL
CCR	3	BDL	41.57±7.18	0.02±0.01	1035.05±249.01	BDL
p value		0.39	0.07	0.19	0.90	

Results are expressed as means±standard error. The food and initial sediment results are expressed as the range of the two measured values. Minimum detection limits (ng/g) for food and tissue were: Se 90.42, Cd 0.03, Pb 0.71, Cu 1.66, As 0.10, V 0.05, Ni 0.88, Hg 0.02, Sr 0.58, Fe 1.19. Minimum detection limits (μg/L) for water were: Se 0.35, Cd 0.08, Pb 1.96, Cu 3.34, As 0.34, V 0.12, Ni 1.47, Hg 0.04, Fe 63.48.

were 70±13 for the control (reference) treatment but significantly ($p=0.021$) reduced in the CCR treatment to 17±4%. However, during the juvenile and adult phase of the exposure no differences in survival were observed between reference and CCR exposures (53±13 and 56±12% respectively). Grass shrimp hepatopancreas showed a dose-dependant response in DNA damage to hydrogen peroxide. The average percent tail DNA was 6.7, 16.7, 20.6, and 23.3% for 0, 25, 50, and 100 μM respectively (Fig. 1a), and was significantly different ($p \leq 0.016$) in all doses from the control dose. The average DNA tail moment and average tail length followed a similar response as the percent tail DNA and are presented in Fig. 1b and c respectively.

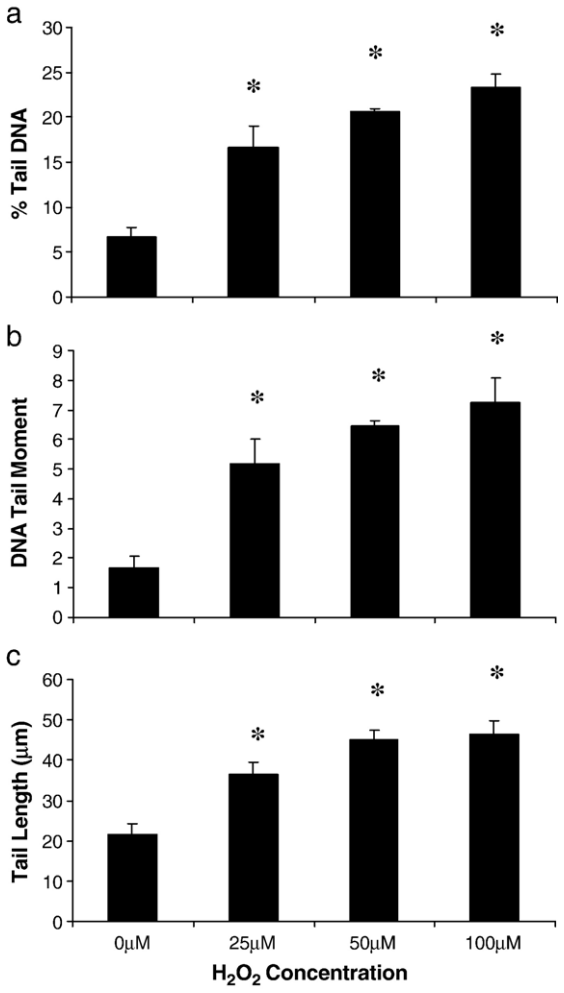

Fig. 1. Effect of 0 μM, 25 μM, 50 μM, and 100 μM *in vitro* (30 min) hydrogen peroxide exposures on percent tail DNA (a), DNA tail moment (b), and tail length (c) in isolated *P. pugio* hepatopancreas cells. Data was compiled from 100 cells total from two replicate slides per treatment ($n=3$) results were considered significant if $p \leq 0.05$.

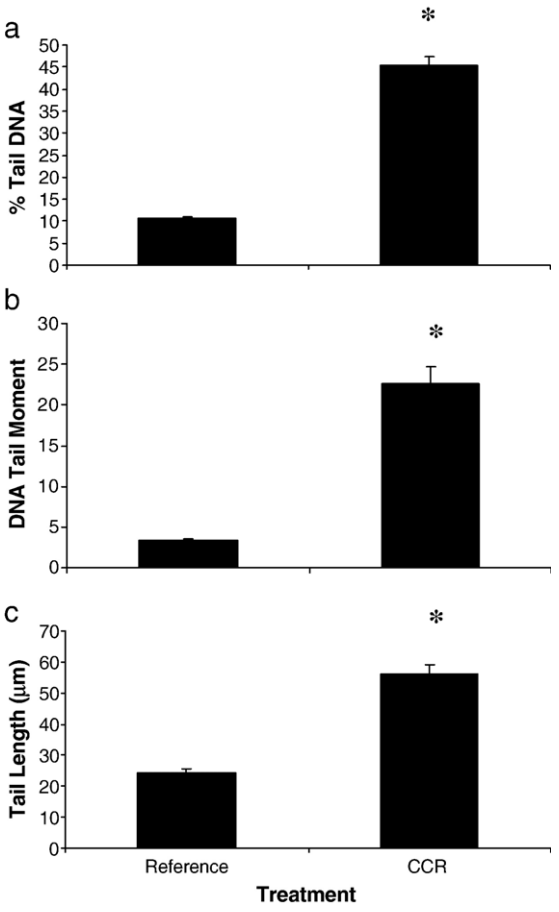

Fig. 2. Effect of 156 day coal combustion residue exposure and hydrogen peroxide, 50 μM, (positive control) on percent tail DNA (a), DNA tail moment (b), and tail length (c) of *P. pugio* hepatopancreas tissue. Data was compiled from 100 cells total from two replicate slides per treatment (reference $n=4$, CCR $n=3$, replicates $n=3$–4), results were considered significant if $p \leq 0.05$.

Grass shrimp exposed to CCR showed significantly ($p<0.05$) greater DNA damage in all parameters measured, than non-exposed shrimp (Fig. 2). The average percent of DNA in the COMET tail (Fig. 2a) in reference treatments was 10.66±0.83%. In CCR treatments the average percent tail DNA was significantly increased ($p=0.034$) to 45.35±3.79%. The coefficients of variation (CV) for percent tail DNA for non-exposed and CCR-exposed grass shrimp were 42.3 and 39.3% respectively. The average DNA tail moment and average tail length followed a similar response as the percent tail DNA and are presented in Fig. 2b and c respectively. A consistent increase in the percent tail DNA (58.1±23.9%, CV=41.1%) in the hydrogen peroxide positive control was observed for each COMET assay.

There was no significant difference ($p=0.53$) in total antioxidant potential between CCR-exposed and non-exposed grass shrimp. Average total antioxidant potential in reference treatments was 0.12±0.008 Uric Acid Equivalents (UAE)/mg protein. In CCR exposed treatments the average total antioxidant potential was 0.10±0.008 UAE/mg protein.

5. Discussion

Sediment and food from the D-Area settling basin were elevated in Se, Cd, Pb, Cu, As, V, Ni, Hg, Sr, and Fe compared to reference sediment (except Fe and Hg in crayfish). However, compared to reference shrimp, only Se and Cd were found to be significantly accumulated by the grass shrimp exposed to CCR. Both of these elements have been shown to be readily bioaccumulated by aquatic organisms in other studies (Chen and Chen, 1999; Besser et al., 1996; Goodyear and McNeill, 1999; Lemly, 2002; Lohner et al., 2001a,b,c; Sorensen et al., 1982; Staub et al., 2004; Thomas et al., 1999). The two main sources of exposure for juvenile and adult grass shrimp in this experiment were via sediment and/or the food as water-borne trace elements appeared to present a negligible exposure route given the low dissolved concentrations. Similarly, Thomas et al. (1999) found that the corixid *Trichocorixa reticulata* accumulated Se through food, but not through water-borne exposure. May et al. (1997) also suggested that Se is likely to be accumulated through the diet rather than sediment. In addition, the present study showed little variation in sediment trace element concentrations between initial and final samples, suggesting limited mobilization of trace elements from sediment to water. The use of a flow through system likely also contributed to low entrainment of dissolved or suspended sediment constituents.

Mercury levels were decreased in our CCR exposed shrimp compared with reference samples. Indeed many studies (see Southworth et al., 2000; Hopkins et al., 2006) have demonstrated a similar antagonistic bioaccumulation pattern of Hg with elevated Se levels.

If trace elements are bioaccumulated in resident organisms above threshold levels a variety of effects (and/or adaptive responses) may be elicited. For example, many heavy metals (e.g. Cd) can elicit conditions of oxidative stress and may decrease antioxidant levels (e.g. GSH) or alternatively they may induce a compensatory elevation of various antioxidant enzyme systems (e.g. glutathione peroxidase). Grass shrimp significantly accumulated Cd, which potentially may elicit toxicity via oxidative stress mechanisms (see Valko et al., 2006). Shaikh et al. (1999) found in a long-term twenty-two week study significant Cd induced increases in both liver and renal cortex lipid peroxidation in rats. Various studies in aquatic species, including in *P. pugio* have demonstrated compensatory increases in levels of GSH or antioxidant enzymes (e.g. SOD, GST and GPx; Downs et al., 2001; Griffitt et al., 2006; Sheader et al., 2006; Mouchet et al., 2006). The effect of Se on oxidative stress and antioxidant responses, however, is not as clear-cut. For example, Se has been shown to cause oxidative stress and altered GSH metabolism in semi-aquatic birds (Hoffman, 2002; Hoffman et al., 2002; Spallholz and Hoffman, 2002). However, as Se is a required trace metal in antioxidant enzymes, such as glutathione peroxidase, it is also possible that Se may reduce levels of oxidative stress if availability of Se is the limiting step for the increased production of Se-requiring antioxidant enzymes (e.g. see Keek and Finley, 2006; Wang et al., 2006). We did not observe any differences in the total antioxidant assay in our CCR exposed shrimp, although it is possible that any changes to specific scavengers/antioxidant enzymes may have been masked by the multitude of other potential antioxidants that would also have been measured using this pooled antioxidant response assay.

Prahalad et al. (2000) suggested that it is the bioavailability of metals, rather than their total concentrations, that is critical in eliciting DNA base damage. They also pointed out the importance of the availability of a catalytically active form, or of chelators, in formation of ROS and subsequent DNA damage. In a sample of CCR, all metals examined by Prahalad et al. (2000) (Ni, V, Fe) induced DNA damage (via hydroxylation of dG to 8-oxo-dG). Although no studies have investigated the extent of DNA SSB in organisms exposed specifically to CCR, some of the metals accumulated by the shrimp in this study are known to cause DNA damage in other organisms. For example, Forrester et al. (2000) found that both Cd and Se administered individually to rats caused DNA SSB. Yu et al. (2006) and Wycherly et al. (2004) also demonstrated that Se (at high doses) caused DNA damage (DNA SSB or DNA oxidation products). Cadmium is a known potent carcinogen as are many of the other trace elements found in CCR (see Valko et al., 2006). This may be via direct genotoxicity (DNA damage), although recent studies have suggested that the carcinogenic mechanism for Cd is via it's effect on inhibiting DNA repair enzymes and in preventing apoptosis (Pruski and Dixon, 2002). Therefore, in a mixed exposure (like CCR) Cd may potentiate the effects of other direct acting genotoxicants. However, Risso-de Faverney et al. (2001) demonstrated an increase in DNA SSB (and apoptosis) in Cd treated fish hepatocytes,

attributed (at least in part) to the production of genotoxic ROS.

The increase in SSB observed in our study, therefore, may be a result of damage to DNA potentially either due to direct damage (especially via metal-mediated ROS production) or disruption of one or more pathways that aid in maintaining DNA integrity (e.g. DNA repair enzymes). In this present study was that CCR exposure significantly ($p < 0.05$) decreased survival in larval shrimp (~70% loss compared with reference) although the exact mechanism of toxicity is unknown. Hook and Lee (2004) found that early embryonic stages of *P. pugio* are more likely to have developmental effects from genotoxicant exposure than later stage embryos. Our results demonstrating DNA damage in adults following chronic exposure agree with those of Steinert et al. (1998), who reported a reduction in DNA repair capacity in *Mytilus edulis* with increasing duration of exposure to genotoxic agents. Genotoxicity often persists in aquatic organisms as DNA repair is slow in comparison to mammalian cells (Mitchelmore and Chipman, 1998). Although there was very low mortality in juveniles and adults relative to larvae, CCR-exposed adults exhibit significantly more DNA SSB than non-exposed adults. It should be noted that we are only measuring biomarker responses in the surviving larvae, the prior loss of over 70% of the CCR exposed larvae alone may have significant population level consequences and is addressed in a following manuscript. The coupling of larval mortality with elevation of DNA SSB in the resultant surviving population may result in further substantial population level consequences with respect to disease states (carcinogenesis), teratogenicity and/or reproductive impairment in adults.

6. Conclusions

Organisms making use of open CCR settling basins and drainage systems for all or parts of their lives may be exposed to and accumulate contaminants to the extent that toxic responses are induced. By using molecular biomarkers such as the COMET assay and antioxidant potential we may be able to determine if these species are being adversely affected before there is permanent damage to the resident populations. Sediment and water chemistry can provide information about the concentration of contaminants, while the tissue chemistry gives information about the actual bioavailability of these contaminants. The COMET assay and total antioxidant status provide insights as to the actual effect of the contaminants. Repercussions of genotoxicity can be manifested at the population-level through impacts on DNA integrity and ultimate physiological processes (teratogenesis, mutagenicity, lethality) or through increased metabolic cost of repair. Here we have shown that chronic exposure to CCR can elicit genotoxic responses, and thus DNA damage is a sensitive biomarker for CCR exposure. Because CCR enters natural systems downstream of disposal facilities, employing a biomarker-based monitoring program will be useful in determining whether animals in those systems might also experience deleterious effects at the lower concentrations to which they are exposed.

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