ABSTRACT

Title of Document:	INDIVIDUAL AND POPULATION-LEVEL EFFECTS OF SOLID COAL COMBUSTION RESIDUE ON THE ESTUARINE GRASS SHRIMP (*PALAEMONETES PUGIO*)
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Burning coal for electricity produces solid coal combustion residue (CCR), which is rich in potentially toxic trace elements, and is frequently discharged into natural and man-made aquatic systems as a method of disposal. Lethal and sublethal effects of CCR on the estuarine grass shrimp, *Palaemonetes pugio*, were assessed. Grass shrimp were exposed in the laboratory to CCR-enriched sediments and food over a full life cycle. Grass shrimp exposed to CCR significantly accumulated selenium and cadmium. Chronic CCR-exposure caused significantly decreased larval survival, increased time to metamorphosis, and increased DNA strand breaks in shrimp compared to non-exposed conditions. Stage-classified matrix population models were constructed to assess the population-level effects of CCR on grass shrimp. The population models suggested that CCR-exposed grass shrimp would experience a decreased population growth rate, altered stable stage structure, stage-

specific reproductive value, and elasticity patterns relative to shrimp in reference conditions.

INDIVIDUAL AND POPULATION-LEVEL EFFECTS OF SOLID COAL
COMBUSTION RESIDUE ON THE ESTUARINE GRASS SHRIMP
(*PALAEMONETES PUGIO*)

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2006

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Acknowledgements

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Chapter 1: Introduction

In recent decades there has been an increase in global reliance on coal for electrical energy production. Trace element-enriched coal combustion residue (CCR) is formed when coal is burned for large-scale energy production. As of 1998 the United States produced 57 million tons of CCR annually (ACAA 1998). Production of such a large volume of waste presents a challenge for disposal. A primary disposal method, accounting for approximately one-third of the annual disposal in the U.S. (EPRI 1997), involves the pumping of a water-based CCR slurry into natural or man-made basins in an effort to allow settling of solids. The receiving waters from CCR-disposal systems often support unique ecosystems that may be negatively affected by trace elements contained in the CCR, and as they are often connected to a local waterway this allows for a potential release of CCR into nearby natural systems.

Environmental release of CCR is of concern primarily due to potentially toxic effects of trace elements, although in relatively static systems, smothering of sedentary benthic organisms may sometimes occur (Bamber 1984). A summary of the most common trace elements present in CCR and their concentrations at specific sites is provided in Table 1.1 (summarized from Rowe et al. 2002). CCRs can have long-lasting effects due to its persistence in sediments, chronically exposing and affecting organisms even decades after input into the system has ceased (Lemly 2002).

Coal combustion residue has been shown to cause a variety of sublethal effects in numerous species (Rowe et al. 2002). Chronic exposure to CCR has been linked to increased metabolic costs in aquatic invertebrates and vertebrates (Rowe et al. 1998a, 2001a). Chronic exposure to CCR has been shown to result in developmental,

Table 1.1. The average range of concentrations (ppm dry mass) of common trace elements in CCR measured in sediments in several contaminated sites.

	Trace Elements					
Site	Cr	Cu	As	Se	Cd	Pb
D Area Power Facility, SC Primary Settling Basin	NR	71.8	70.8	6.21	0.57	45.2
Belews Lake, NC	NR	NR	31.2 – 59.8	6.08 – 8.93	NR	NR
Hyco Reservoir, NC, Cooling Reservoir	24 – 197	15 – 104	1.8 – 13.3	0.68 – 5.50	NR	NR
Stingy Run, OH	45.4 – 132	40.6 – 57	27.6 – 58	5 - 20	1 – 1.9	19.8 – 30

(NR=not reported)

Data compiled from Rowe et al. (2002)

histopathological, teratogenic, and reproductive effects in fish and amphibians (Sorensen et al. 1984; Rowe et al. 1998b, 2001a; Lemly 2002). Specifically, exopthalmus, anemia, swollen and vacuolated gill lamellae, decreased hematocrit, inflammatory cell-filled pericardial spaces, and necrotic and ruptured egg follicles in fish (Sorensen et al. 1984; Lemly 2002), and malformed oral structures and spinal flexures in larval amphibians (Rowe et al. 1998b, 2001b) have been observed.

Because of the potential for CCR to exert effects both at the individual- and population-levels, my research examined the potential effects of CCR on grass shrimp (*Palaemonetes pugio* Holthius) from two distinct perspectives. First, I examined the sublethal effects of CCR on molecular and cellular functions of individuals to determine whether chronic exposure to CCR resulted in significant biological changes. Second, I examined the potential for chronic exposure to CCR to influence population dynamics as a result of the cumulative impact of individual responses.

CCR exposure is often not directly lethal to adults but can lead to reproductive failure or developmental defects in offspring, in some cases resulting in population declines (Lemly 2002). Reductions in species diversity resulting from population-level effects of CCR certainly suggest that CCR can have ecological effects (Lemly 2002). Yet, from a remediation and pro-active management perspective, it is desirable to identify potential effects prior to their emergence at the ecosystem level. The use of biomarkers as "early warning signals" for potential ecological effects of contaminants can provide evidence that a system may be in jeopardy prior to ecosystem changes being observed (van der Oost 2003) and allows the mechanisms of toxicity involved to be examined. Thus, by identifying molecular, biochemical, and cellular alterations in contaminant-

exposed organisms, it is possible to detect sublethal effects of CCR potentially before population-level effects are observed.

While measures of sublethal effects of contaminants are useful for identifying potential responses by individuals, environmental managers are generally concerned with population-level effects. Thus, the endpoints for ecological risk assessments of chemical contaminants should not necessarily be based upon effects on individuals, but rather on changes to populations (Lin et al. 2005). Population models are commonly employed to project the population-level effects of contaminants and other environmental variables. Using population models allows an integration of the multiple effects of chemicals on all life-stages (Munns et al. 1997). By using models to assess the potential population-level effects of contaminants, regulators may be able to develop risk assessments to determine whether specific contaminants require regulatory actions to prevent ecological damage. Matrix-based population models (Caswell 2001) have been of particular utility in quantifying the impacts of contaminant exposure on populations (Munns et al. 1997, Levin et al. 1996). An advantage of matrix models is their relative simplicity. The models capture processes occurring in discrete time units, and they can easily incorporate demographic data from laboratory toxicity bioassays. Additionally, modern computing languages make computations very simple (Usher 1972).

The use of stage-classified matrix models is valuable when the study organism has clearly discernable life-stages. Stage-classified models have ecological applications when the age of individuals in populations are poorly known or cannot be determined by inspection (Lefkovitch 1965). Stage-classified matrix models have been used to assess the population-level effects of dioxins and PCBs on the estuarine fish *Fundulus*

heteroclitus (Munns et al. 1997), to examine the effects of chemical contaminants (including hydrocarbons) associated with urban areas of Puget Sound on flatfish populations (Johnson et al. 1998), and to assess the effects of cadmium on gastropod population growth rate (Jensen et al. 2001, Salice and Miller 2003). Spencer and McGee (2001) also applied stage-based matrix models to assess natural fluctuations in populations of *Leptocheirus plumulosus*, a species commonly used in sediment toxicity tests.

Elasticity analyses and stage-based matrix models are useful for quantifying the population-level responses of organisms to toxicants, and as a tool for the basis of management decisions. It has been suggested that the demographic parameters with the largest elasticities are where management efforts should be focused because they would have the most influence on the population growth rate (Crouse et al. 1987, Doak et al. 1994, de Kroon et al. 2000, Vonesh and De la Cruz 2002). However, caution must be taken before undertaking any management strategy. Elasticity analysis may not necessarily lead to the best management choice if used alone (Caswell 2001, de Kroon et al. 2000, Ehrlén and van Groenendael 1998), and uncritically accepting the highest elasticity value to designate the focus of management strategies will be unlikely to fulfill management aims (Benton and Grant 1999). Elasticity does not take into consideration whether it is possible for a particular transition variable to be influenced by management (de Kroon et al. 2000), and not all transitions vary equally (Pfister 1998). Knowledge of biological constraints, actual transition values, and management options should all accompany evaluations of elasticity for conservation purposes (de Kroon et al. 2000).

The D-Area Power Facility site near Aiken, South Carolina is well characterized as a CCR-disposal location and thus was chosen as a model site for this study (Rowe et al. 2002). Slurried coal combustion residue is pumped into settling basins, which then flows into a drainage swamp and into the Savannah River. Invertebrates, reptiles, and amphibians living in this site have elevated body burdens of many trace elements, including selenium (Se), arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and copper (Cu) (Rowe et al. 2002, summarized in Table 1.2). Associated with accumulation of trace elements in the D-Area site, vertebrates and invertebrates have experienced sublethal effects on morphology, metabolism, and behavior (Rowe 1998, Rowe et al. 1998 a and b, Hopkins et al. 2000).

Coal combustion residue is disposed of into estuarine and marine systems as well as freshwater systems. However, effects of CCR in the former systems have received relatively little attention (Rowe et al. 2002). The grass shrimp, *Palaemonetes pugio* (Holthius), was chosen as a study organism because it is widely used as a model test organism for estuarine systems. *Palaemonetes pugio* ranges from Texas to Maine and is an ecologically important species in estuaries and tidal marshes, in salinities ranging from 0 to 35‰ (Knowlton and Kirby 1984). Grass shrimp are epibenthic omnivores and spend most of their lives associated with the sediments (Gregg and Fleeger 1998), and frequently ingest sediments during normal feeding activities. As a result, *P. pugio* may act as an important vector of CCR-derived contaminants to higher trophic levels.

Table 1.2. Concentrations of select trace elements measured in abiotic and biotic matrices in the primary CCR settling basin at the D-Area Power Facility, SC. Values for sediment and tissues are ppm (dry mass); values for water are ppb.

	Trace Elements					
Primary Settling Basin	**Cr**	**Cu**	**As**	**Se**	**Cd**	**Pb**
Sediment	NR	71.8	70.8	6.21	0.57	45.2
Water	0.44	2.53	17.17	7.0	0.11	0.08
Crayfish, whole body	2.46	158.52	8.71	14.92	2.78	NR
Mosquitofish, whole body	1.56	4.97	2.89	14.28	0.32	NR
Bullfrog, recent metamorph, whole body	1.58	13.79	15.55	26.85	0.8	NR

NR = not reported

Data compiled from Rowe et al. (2002)

To date most studies looking at the effects of coal combustion residue have been based on individual endpoints such as growth and mortality, and few studies have quantified the effects of CCR on populations or higher levels of organization. Therefore, I examined CCR-induced effects on both individual traits and population dynamics using a life-cycle experiment in which *P. pugio* were exposed to CCR through diet and sediment to address both individual- and population-level responses. I examined the sublethal effects of CCR on individuals through the use of subindividual-level biomarkers, which have shown to be responsive to some of the individual contaminants known to be present in CCR (see chapter 2). Specifically, I conducted the Comet (single-cell gel electrophoresis) assay to measure the extent of DNA strand breaks in shrimp hepatopancreas caused by genotoxicants present in CCR. I also measured total antioxidant potential as an indicator of oxidative stress. The extent of heavy metal bioaccumulation by the test organisms was also quantified for comparison with the biological endpoints. The exposure experiment also provided the parameter estimates for stage-classified matrix models, which were used to assess the impact of CCR on *P. pugio* over a full life cycle. I analyzed the models to assess the intrinsic rate of population increase (population growth rate), stable stage structure, and reproductive value, under both treatment conditions. I also conducted an elasticity analysis of the models to determine the life-history stage that is likely to have the largest effect on the population growth rate, as well as to examine any changes in elasticity among treatments.

Chapter 2: Sublethal effects of solid coal combustion residue on grass shrimp (*Palaemonetes pugio* Holthius)

Abstract

   Burning coal for electricity produces solid coal combustion residue (CCR), which is rich in trace elements including Se, Cd, Cu, As, Pb, and Cr, that have the potential to induce sublethal effects including DNA single strand breaks (SSB) and oxidative stress. Coal combustion residue is frequently disposed of into natural and man-made aquatic systems. As the effects of CCR have received relatively little attention in estuarine systems, the estuarine grass shrimp, *Palaemonetes pugio*, was chosen for this study. In the laboratory grass shrimp were exposed to CCR-enriched sediments and food over a full life cycle. The Comet assay, a general but sensitive assay for genotoxicity, was used to quantify DNA SSB. Total antioxidant potential was examined to assess the overall antioxidant scavenging capacity of CCR-exposed and non-exposed grass shrimp. Grass shrimp exposed to CCR significantly ($p<0.05$) accumulated Se and Cd compared to unexposed shrimp. Chronic CCR exposure caused DNA SSB in hepatopancreas tissue, as evidenced by the significantly ($p<0.05$) increased percent tail DNA, tail moment, and tail length as compared to reference shrimp. However, no significant difference ($p>0.05$) was observed in total antioxidant potential. It is important to quantify the sublethal effects of CCR as they have the potential to give insights into the mechanisms of toxicity as well as aid in an overall biomonitoring regime by providing 'early warning signals' of potentially higher-order impacts.

Introduction

Burning coal for electricity produces solid coal combustion residue (CCR), which is enriched in a variety of elements. These residues have been found to contain potentially toxic concentrations of trace elements, such as, selenium, cadmium, and copper, (see Table 2.1, compiled from Rowe et al. 2002). In addition, CCR may also contain trace concentrations of organic compounds depending upon facility-specific waste comanagement practices (EPRI 1997) and combustion conditions. Of forty-five organic compounds measured in water from CCR disposal sites at 21 facilities in the U. S., only two compounds (bis(2-ethylhexyl)phthalate Di-n-octylphthalate) were found in concentrations above detection limits (EPRI 1987).

A common disposal method for CCR is to pump it as a slurry into settling basins, which are designed to capture solids prior to discharge of the water into local systems. However, often these basins do not retain all of the particulate and dissolved materials from CCR. Therefore, there may be serious environmental implications regarding the release of CCR derived contaminants into local waterways. The trace elements in which CCR is typically enriched can potentially have detrimental effects on the organisms that live in these systems (for a review see Rowe et al. 2002). For example, negative effects of CCR, including increased standard metabolic rate, were observed in a freshwater shrimp, *Palaemonetes paludosus* (a congener of the species studied here), when exposed chronically through diet and sediment (Rowe 1998). Lemly (2002) demonstrated that chronic exposure to CCR-enriched sediment caused developmental, histopathological, and teratogenic effects in numerous fish species, and associated population declines in 19 out of 20 species studied. Increased metabolic costs have been observed in crayfish (*Procambarus acutus*) (Rowe et al. 2001a) following exposure to CCR-enriched

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Table 2.1. The average range of concentrations (ppm dry mass) of common trace elements in CCR measured in sediments in several contaminated sites.

Site	Trace Elements					
	Cr	Cu	As	Se	Cd	Pb
D Area Power Facility, Primary Settling Basin	NR	71.8	70.8	6.21	0.57	45.2
Belews Lake	NR	NR	31.2 – 59.8	6.08 – 8.93	NR	NR
Hyco Reservoir, NC, Cooling Reservoir	24 – 197	15 – 104	1.8 – 13.3	0.68 – 5.50	NR	NR
Stingy Run, OH	45.4 – 132	40.6 – 57	27.6 – 58	5 - 20	1 – 1.9	19.8 – 30

(NR=not reported)

Data compiled from (Rowe et al. 2002)

sediment. Although population declines and sometimes extirpation of organisms exposed to CCR clearly demonstrates its detrimental effects, from a pro-active management perspective, quantifying biomarker (sublethal) responses in resident organisms is preferred (van der Oost 2003). Through the use of molecular, biochemical, and cellular biomarkers in addition to investigating mechanisms of toxicity, it is potentially possible to detect sublethal effects of CCR in exposed organisms, before serious population-level effects are observed. Currently, there is a need to develop and apply suitable biomarkers as 'early warning signals' of exposure to and/or effect of CCR in resident organisms.

Biomarkers of exposure and/or effect have been developed for many of the trace elemental components of CCR and not only have potential for use as 'early-warning' signals but have given insights into the mechanisms resulting in CCR toxicity (for a review of biomarkers see van der Oost 2003). For example, copper has multiple pathways of toxicity including the generation of reactive oxygen species (ROS; Correia et al. 2002), and results in conditions of oxidative stress (Doyette et al. 1997). The production of reactive oxygen species is a natural consequence of aerobic life (Arun et al. 1999), and transition metals, including Cu, Cr, Fe, Ni, V, and Co, are able to redox cycle electrons and thus initiate the production of ROS (Prahalad et al. 2000). The contaminants associated with CCR can, therefore, be an anthropogenically-related source of ROS and pro-oxidant, free-radical production (Livingstone 2001). Reactive oxygen species can impair nucleotides, proteins, lipids, and carbohydrates, which are all important cellular constituents (Yu 1994). To overcome endogenous and anthropogenic elevations of ROS, organisms have developed effective antioxidant systems. Exposure to

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oxidative stressors, may decrease cellular pools of antioxidant scavengers; however, this may simply mean that the oxidative defense systems have functioned normally, and oxidative damage has not necessarily occurred (Doyette et al. 1997, Diaz et al. 2004). For example, Viarengo et al. (1990) found that exposure of mussels (*Mytilus galloprovincialis*) to copper resulted in lipid peroxidation and a concurrent significant decrease in glutathione concentrations, an important antioxidant defense mechanism against oxidative damage to cellular components, after one day of exposure. Exposure to cadmium and zinc did not increase lipid peroxidation (oxidative injury) nor show significant variations in glutathione concentrations (Viarengo et al. 1990), however, Shaikh et al. (1999) found glutathione to be important in protecting rat cells against cadmium toxicity, and Downs et al. (2001) found increased oxidative stress in *P. pugio* exposed to cadmium as exhibited by increased concentrations of glutathione compared to non-exposed shrimp.

Many biomarkers have been employed routinely to assess the status of an organism's oxidative stress or antioxidant capacity (see Van der Oost, 2003, Livingstone 2001). The measurement of total antioxidant potential (total oxidant radical scavenging capacity) is a recent approach (Winston et al., 1998, Regoli and Winston, 1998, 1999). The total antioxidant potential (AOP) test covers a wide array of antioxidants collectively as opposed to individually, and is advantageous because it is an integrative approach and gives a good overall picture of the organism's free radical scavenging capacity. However, this can be disadvantageous if the toxicant has a very specific response, such as the induction or depletion of glutathione, which can be masked amongst the overall effect.

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Besides evidence that the elemental constituents of CCR may result in conditions of oxidative stress, Smith-Sonneborn et al. (1981), Kubitscheck and Ventra (1979), Li et al. (1983) and others have shown CCR to be mutagenic to a variety of organisms (including *Paramecium tetraurelia*, *Salmonella typhimurium*, and Chinese hamster ovary 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 2000). However, there is limited information regarding oxidative stress and genotoxicity endpoints in organisms exposed *in vivo* to CCR.

Many trace elements characteristic of CCR may be genotoxic and result in DNA damage. Genotoxic compounds can alter the chemical structure of DNA, causing various perturbations (e.g. strand breaks or DNA adducts) that may affect efficient DNA repair and ultimately lead to various disease states including teratogenesis and cancer (Mitchelmore and Chipman, 1998). Toxic compounds may be direct-acting genotoxicants (e.g. UV light, hydrogen peroxide) or indirect-acting in that they require metabolism to exert their effects. For example, indirect-acting genotoxicants can result in damage to DNA by their metabolism / oxidation reactions leading to the formation of ROS that are highly damaging to DNA (Livingstone 2001). Heavy metals can alter DNA directly or initiate the production of ROS.

DNA single strand breaks (SSB) can be used as a rapid and relatively 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. The detection of SSB has been classified as a non-specific and general biomarker for exposure to genotoxicants and is our reasoning for using it in this

experiment (Mitchelmore and Chipman, 1998). Many assays for detecting SSB have been employed, although I have focused on a simple and reproducible assay, the Comet assay (Mitchelmore et al. 1998). Previously, the Comet assay has been used to assess the presence of DNA SSB and 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). An advantage of the Comet assay is that it can be used on small samples of cells (Steinert et al. 1998), and may also be used for non-destructive and/or repeated sampling of the same individual (Nacci et al. 1992), which could be used to monitor effects over time in a biomonitoring program.

Palaemonetes pugio, is a species ubiquitous to estuarine and coastal systems from Maine to Texas (Knowlton and Kirby 1984) where it serves as important prey for estuarine fish (Wood 1967, Poole 1988). Individuals mature at approximately 3 months of age (Wood 1967), produce four to six broods per year of up to 400 eggs per brood (dependent on body size; Alon and Stancyk 1982), achieve a maximum weight of 3 grams, and live approximately 2 years (Poole 1988). Grass shrimp are epibenthic omnivores, and have the potential to accumulate and uptake trace elements from the sediment (Poole 1988), transferring them to higher trophic levels. This species is a model organism (APHA 1985) in many toxicological studies and preliminary data suggest that it (and its congener *P. paludosus*) may be chronically affected by CCR-contaminated sediment (Rowe 1998, Rowe and Hopkins, unpublished). The hepatopancreas was chosen for assessment of DNA damage because it is homologous to the mammalian liver and pancreas and it is responsible for the major metabolic events in crustaceans,

including uptake and storage of inorganic nutrients, synthesis and secretion of digestive enzymes, lipid and carbohydrate metabolism, and molting (Gibson and Barker, 1979).

A full life-cycle experiment in which *P. pugio* was exposed to CCR through diet and sediment was conducted to examine the chronic and sublethal effects of CCR on individuals. Specifically, the Comet assay (single-cell gel electrophoresis) was used to measure the extent of DNA SSB in shrimp hepatopancreas, and the total antioxidant potential of shrimp tail tissue was used as an indicator of oxidative stress. These results were analyzed in combination and interpreted with respect to concentrations of accumulated trace elements following exposure.

Materials and Methods
CCR Exposure

This study involved a full life cycle laboratory exposure of *P. pugio* in order to examine potential sublethal (biomarker) responses under CCR-exposed and non-exposed conditions. Stage-specific lethal effects were also quantified for use in population models (Chapter 3). There were two treatments (four replicates per treatment); one with CCR sediment and CCR-contaminated food, and a reference treatment in which black sand served as the sediment and food was uncontaminated. The black sand was used to mimic the color of CCR. Contaminated sediment was derived from CCR dredge piles surrounding a drainage basin at the D-Area Power Facility on the U. S. Department of Energy operated Savannah River Site, SC. Contaminated food for juvenile and adult shrimp (crayfish tissue; see below) was collected from the drainage basins and swamp at

the D-Area site. Reference food 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 gravid adults collected from the Patuxent River, MD (salinity ~ 9 – 15 ‰). Gravid *P. pugio* were suspended in mesh baskets in individual glass jars containing 400 ml of aerated, filtered water from the Patuxent River (adjusted to 20‰ salinity with Instant Ocean) and placed in a 24ºC incubator until hatching and release of larvae from the egg clutch. The chelae of the females were clipped to prevent the females from cannibalizing the egg mass (Little 1968), as sometimes occurs in laboratory conditions. Daily, newly-hatched larvae were removed and placed in a shallow glass dish and the larvae were allowed to mix fully.

Three days prior to the predicted peak of hatching, eight 1500 ml beakers were set up with 200 cm^3 of sediment and filtered, Patuxent River water to be used in larval exposures. Two hundred larvae were placed in each beaker on the day that they hatched (Day 1). In an effort to provide genetic diversity, all replicates had a mixture of larvae from three to fourteen females. The initial salinity in each beaker was 20‰ (optimal for larval survival; Knowlton and Kirby, 1984) and the salinity was gradually decreased to the ambient salinity of Patuxent river water (approximately 10‰) throughout the duration of the larval stage. Salinity, temperature (22.52 ± 0.91°C), and dissolved oxygen (7.02 ± 0.72 mg/L) were measured every three days prior to a fifty percent water change. Larvae were fed newly hatched *Artemia spp.* (see above). The duration of the larval stage was

estimated based upon the number of larvae metamorphosing in each replicate on each day.

Thirty-nine newly metamorphosed shrimp from each replicate were used to start the juvenile stage of the experiment, with the same replicates carried over from the larval stage. I chose to use 39 juveniles because this represented the minimum number per replicate that survived the larval exposures. During the transition to the juvenile stage one of the CCR replicates was lost due to unusually low survival. Juvenile exposures were conducted in 57-L, flow-through tanks (with mesh netting over the outflow to prevent loss of shrimp) containing ambient salinity filtered (20μm) Patuxent River water, and 1000 cm^3 of sediment (approximately 3 mm depth). Salinity, temperature, and dissolved oxygen were monitored weekly. Juveniles were fed ground crayfish *ad libitum.* Reference crayfish were obtained commercially; crayfish for the contaminated treatment were collected in the D-Area site. Crayfish were prepared by separating the tissue from the exoskeleton, drying, and grinding the soft tissue (I rarely observed sediment in the gut contents) and was stored at 4°C. Juvenile exposures lasted for 60 days based on the stage duration reported by Wood (1967), at the end of which (Day 96 overall) shrimp were surveyed for survival and maturation (defined as having attained a total length of 16 mm, Alon and Stancyk 1982). Total length was defined as the distance from the base of the rostrum to the posterior tip of the telson.

The adult phase of the experiment took place in the same flow-through system as the juvenile phase, and lasted for 77 days. The feeding regime for the adult exposure was the same as in the juvenile exposure. As adult females became gravid they were removed and suspended in mesh baskets in individual glass jars containing 800 ml of aerated,

filtered water from their replicate tank, and their chelae were clipped to prevent them from eating the egg mass, however, sediment was not placed in the jars. The number of larvae that hatched from each female was counted. Survival, growth, and reproduction were measured, and are reported elsewhere (see Chapter 3).

Trace Element Analysis

Tissue samples (ten pooled shrimp) were collected from each replicate at the termination of the experiment (Day 156). 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. Tissue and food samples (*Artemia spp.* and crayfish) were freeze-dried and ground with a mortar and pestle. Sediment samples were oven-dried at 60º C for approximately 24 hr.

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 0.45μm filter and acidified with ten percent ultrex nitric acid (Sigma Chemicals).

Trace elements of primary interest in this study were Se, Cd, Cu, Pb, As, and Cr as these elements are the most often elevated in environmental matrices in most sites characterized to date (Rowe et al. 2002). Samples were acid digested following EPA Method 200.3. After digestion, trace element analysis was conducted by ICP-MS at the Savannah River Ecology Lab, 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 Counsil, Ottawa, Canada) were included in the digestion and analysis procedures. Average percent recoveries for trace elements ranged from 97.27% to 117% (with the exceptions of Cr and Se, 159% and 142% respectively). Average variability of percent recovery of certified reference materials in digestion sets ranged from 0.0003 to 0.0059.

Comet Assay Protocol

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). Glass microscope slides were coated with 1% normal melting-point (NMA) agarose in Dulbecco's phosphate buffered saline solution (Gibco) (1x, pH 7.5) and dried and stored in an oven.   Shrimp were held unfed overnight to allow for the emptying of gut contents to reduce the potential for contamination with food. Shrimp were sacrificed by severing the tail from the base of the carapace with a razor blade. The hepatopancreas was dissected out using forceps, and the tail was frozen and stored in liquid nitrogen for total antioxidant potential analysis (see below). For each sample, the hepatopancreas was ground in a glass homogenizer with 750ml of Hank's Buffered Salt Solution (HBSS; Ca^{2+} and Mg^{2+} free, pH 7.8) at 4ºC, passed through a 70μm filter and placed in a microcentrifuge tube on ice. A positive control was run with each set of samples, in which the filtered cell homogenate was centrifuged at 700xg for 2 minutes, and the pellet resuspended in 100μL of 50μM

hydrogen peroxide in HBSS and incubated for thirty minutes in the dark on ice (this dose was determined as follows). A positive control experiment was also conducted (n=3), in which the hepatopancreas' from six shrimp were pooled and prepared in the same manner as the hydrogen peroxide sample above, except the pellet was resuspended in 800μL total of HBSS. One hundred microliters of this cell suspension was then incubated for 30 minutes in the dark on ice in one of four concentrations of H_2O_2 (0μM, 25μM, 50μM, 100μM), with duplicate incubations for each concentration. All samples were centrifuged at 700xg for 2 minutes, and the resulting cell pellets resuspended in 20μl of HBSS, the aliquot was tested for cell viability via the trypan blue method (>90%). Ten microliters of this cell suspension was added to 100μl of 0.65% low melting-point agarose (LMPA) in HBSS and placed onto a prepared NMA agarose-coated slide. A cover slip was added and the gel allowed to solidify (10 minutes at 4ºC). Two replicate slides were prepared for each sample. The cover slip was then removed and 100μl of LMPA was added and covered with a cover slip. After solidification the cover slip was removed and the slides placed in a coplin jar with fresh lysing solution (2.5M NaCl, 0.1M EDTA, 0.01 M Tris-HCl, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10) for at least 2 hours at 4 ºC. Slides were placed on a wire rack and gently rinsed twice with ice-cold distilled water. Slides were transferred into an electrophoresis chamber (Thermo EC Maxicell Primo EC340) filled with electrophoresis buffer (0.1M NaOH, 1mM EDTA, pH >12) to unwind for fifteen minutes. Electrophoresis was conducted at 25V, 300mA for fifteen minutes. Slides were then placed on a wire rack and rinsed 3 times for two minutes with neutralization buffer (0.4M Tris, pH 7.5). Slides were placed on a paper towel and stained with 50μl of ethidium bromide (2 μg/ml).

The extent of DNA damage was analyzed using an Olympus BX50 microscope (x200 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 moment (the amount of DNA in the tail x tail length), and tail length which are expressed as means ± SEM. Figure 2.1 represents an example of an undamaged and damaged cell, and illustrates the head and tail region parameters. Fifty randomly chosen cells per slide were used for analysis. The coefficients of variation (100*stdev/mean) was calculated for each treatment for each of the Comet parameters.

Undamaged Cell   Damaged Cell

Figure 2.1. An example of an undamaged and damaged cell, and illustrates the head and tail region parameters.

Assessment of Total Antioxidant Potential

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 100μM solution of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF)). Twenty microliters was reserved to analyze

for protein content (see below) and was tested for cell viability via the trypan blue method (>90%). Samples were then centrifuged for ten minutes at 10,000xg (4ºC) 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. All samples and standards were run in triplicate. An HBSS buffer blank was also run. Fifty microliters of R2 solution was added to each well, mixed, and incubated for three minutes 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 490nm. A buffer blank and set of standards was run on each plate and a standard curve was made. 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 manufacture'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.

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. Total

antioxidant potential data were tested for normality and then analyzed using a one-way ANOVA (α=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. All statistical tests were conducted using Minitab software for windows, version 13 (Minitab, State College, PA).

Results

Trace element analysis

Trace element concentrations in grass shrimp tissues are shown in Table 2.2. *Palaemonetes pugio* exposed to CCR significantly accumulated selenium and cadmium. Table 2.3 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.

Sediment samples from CCR treatments had higher concentrations of all trace elements of interest than the reference sediments (Table 2.3; small sample sizes of the initial sediment samples precluded statistical analysis of these samples). Neither the initial nor the final water samples differed significantly in the concentration of trace elements of interest, concurrent with previous studies of CCR-exposed systems (Rowe et al. 2002).

Table 2.2. Whole body trace element concentrations (ppm dry mass) of *P. pugio* from coal combustion residue (CCR) and reference treatments.

Shrimp Tissue Samples	n=	Trace Elements					
		Cr	Cu	As	Se	Cd	Pb
Reference	4	28.50 ± 11.80	135.60 ± 9.31	7.33 ± 0.73	2.97 ± 0.29	0.24 ± 0.14	0.51 ± 0.04
CCR	3	13.93 ± 5.96	151.78 ± 0.40	8.64 ± 1.15	11.91 ± 0.11	2.08 ± 0.26	6.12 ± 5.80
p value		p=0.815	p=0.101	p=0.178	P<0.001	p=0.001	p=0.949
T value		T_5=0.98	T_5=1.47	T_5=1.02	T_5=24.76	T_5=6.80	T_4=2.12

Results are expressed as means ± standard error

Table 2.3. Trace element concentrations (ppm dry mass) in food, sediment, and water in CCR and reference treatments.

Exposure Type		n=	Cr	Cu	As	Se	Cd	Pb
					Trace Elements			
Food								
	Artemia							
	Reference	2	6.42 – 7.42	9.27 – 12.72	23.31 – 28.39	3.59 – 4.28	BDL	152.10 – 176.28
	CCR	2	5.48 – 8.46	10.74 – 16.40	29.43 – 42.72	4.46 – 5.20	0.02 – 0.04	46.29 – 47.39
	Crayfish							
	Reference	2	2.39 – 2.86	62.86 – 67.71	2.21 – 2.39	2.17 – 2.18	0.38 – 0.41	0.79 – 0.84
	CCR	2	2.60 – 2.68	275.64 – 2.77.31	8.62 – 8.66	20.34 – 20.57	17.14 – 20.46	6.73 – 9.01
Sediment								
	Initial							
	Reference	2	0.36 – 0.37	0.57 – 0.75	0.15 – 0.17	BDL	0.00 – 0.01	0.87 – 1.23
	CCR	2	26.33 – 30.94	46.90 – 47.68	181.20 – 218.61	8.98 – 9.15	0.64 – 0.75	35.75 – 39.29
	Final							
	Reference	4	0.42 ± 0.03	1.06 ± 0.08	0.21 ± 0.05	BDL	0.01 ± 0.00	0.41 ± 0.03
	CCR	3	29.60 ± 0.62	48.31 ± 1.72	154.50 ± 7.70	4.38 ± 0.32	0.58 ± 0.03	34.12 ± 0.63
	p value		p=0.00	p=0.00	p=0.00	p=0.00	p=0.00	p=0.00
	T value		T_5=56.15	T_5=32.69	T_5=23.95	T_5=9.08	T_5=22.16	T_5=63.37
Water								
	Larval							
	Reference	4	3.27 ± 0.21	58.52 ± 7.84	75.99 ± 14.11	434.41 ± 79.15	0.76 ± 0.17	4.32 ± 1.47
	CCR	4	2.58 ± 0.69	42.48 ± 13.11	185.79 ± 76.40	253.21 ± 100.57	1.28 ± 0.32	4.90 ± 2.54
	p value		p=0.82	p=0.83	p=0.10	p=0.90	p=0.10	p=0.83
	T value		T_6=0.97	T_6=1.05	T_6=1.41	T_6=1.42	T_6=1.45	T_5=1.05
	Final							
	Reference	4	1.06 ± 0.13	16.03 ± 2.02	15.24 ± 2.05	48.98 ± 7.36	0.10 ± 0.03	0.28 ± 0.55
	CCR	3	1.08 ± 0.10	17.56 ± 3.38	14.92 ± 3.47	44.74 ± 10.59	0.09 ± 0.02	1.31 ± 0.13
	p value		p=0.45	p=0.36	p=0.53	p=0.62	p=0.59	p=0.09
	T value		T_5=0.13	T_5=0.38	T_5=0.08	T_5=0.32	T_5=0.24	T_5=1.57

Results are expressed as means ± standard error. The food and initial sediment results are expressed as the range of the two measured values.

Single cell gel electrophoresis (Comet assay)

The results from the hydrogen peroxide positive control experiment are presented in Figure 2.2. 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 100µM respectively (Figure 2.2a), 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 Figures 2.2b and 2.2c respectively. For a positive control 50µM hydrogen peroxide was chosen for use in the subsequent CCR-exposure study.

Grass shrimp exposed to CCR showed significantly ($p<0.05$) greater DNA damage, using all parameters measured, than non-exposed shrimp (Figure 2.3). The average percent of DNA in the Comet tail (Figure 2.3a) 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 Figures 2.3b and 2.3c 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 undertaken.
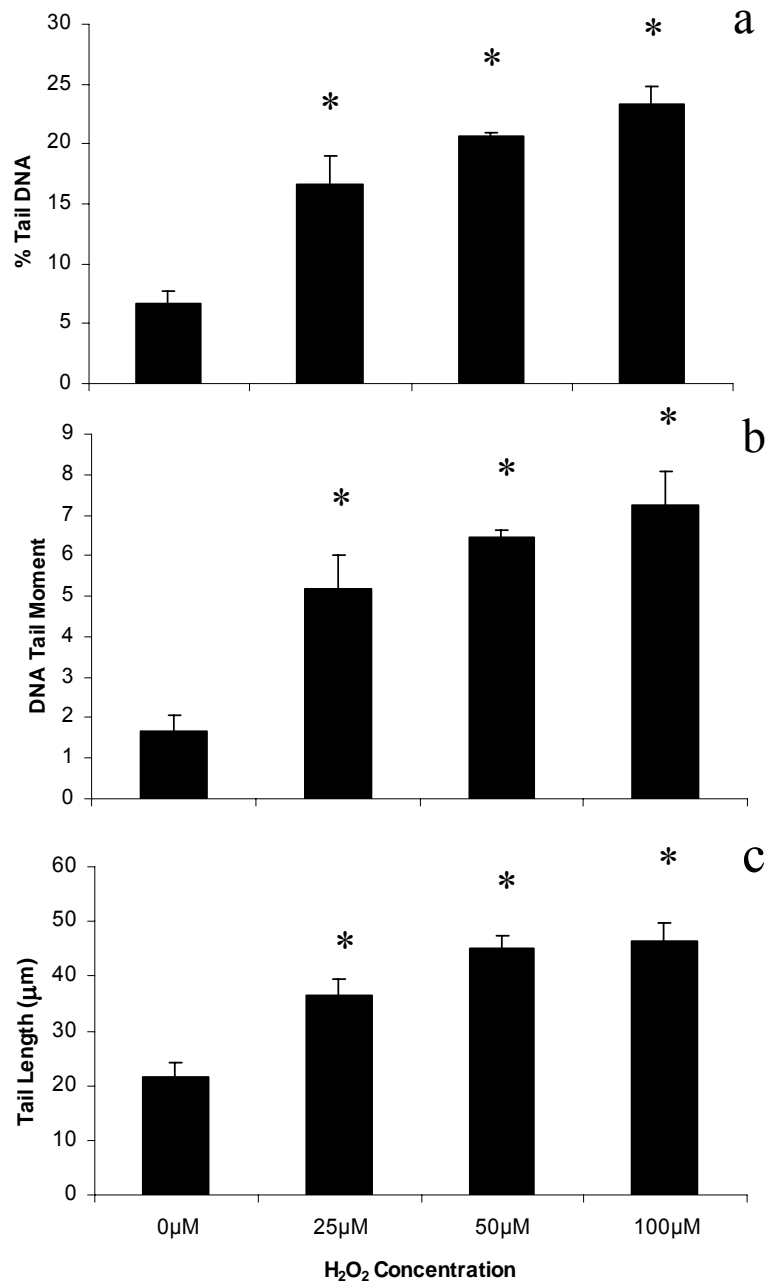
Figure 2.2. Effect of 0μM, 25μM, 50μM, and 100μM *in vitro* (30 minutes) hydrogen peroxide exposures on percent tail DNA (a), DNA tail moment (b), and tail length (c) in isolated *P. pugio* hepatopancreas cells. Data was compiled from 100 cells total from two replicate slides per treatment (n=3) results were considered significant if $p \leq 0.05$.
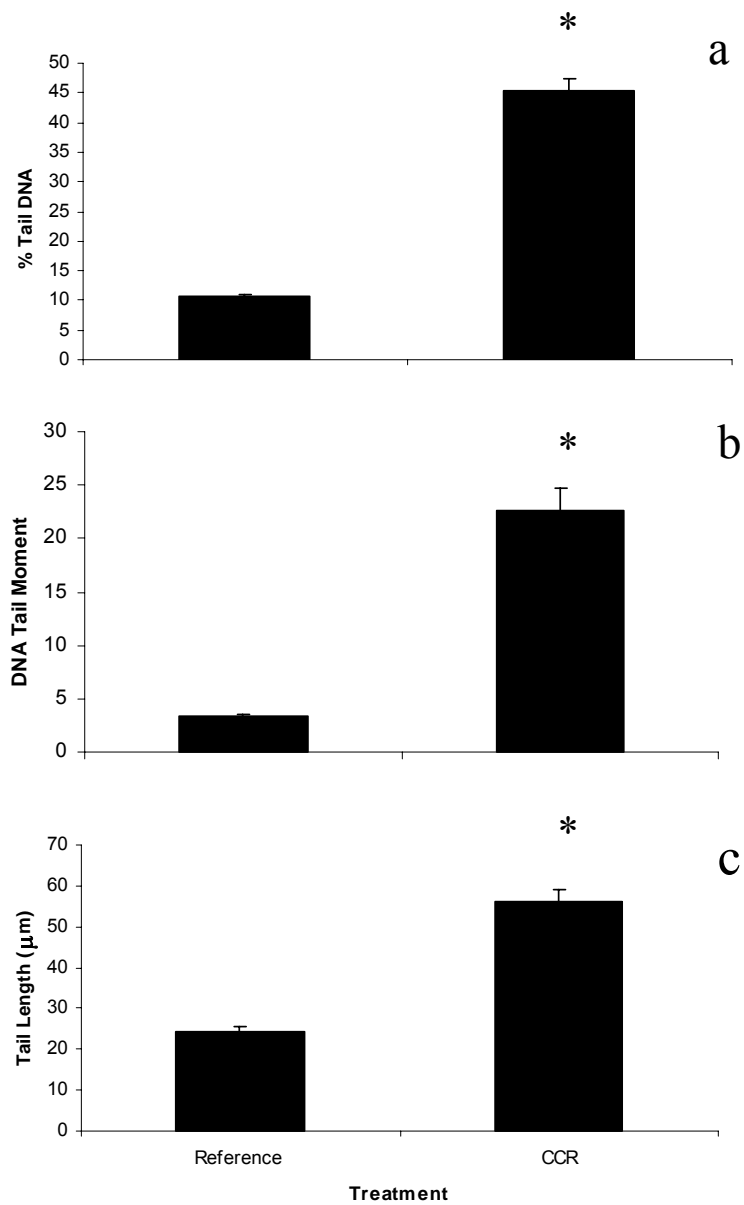
Figure 2.3. Effect of 156 day coal combustion residue exposure and hydrogen peroxide, 50μM, (positive control) on percent tail DNA (a), DNA tail moment (b), and tail length (c) of *P. pugio* hepatopancreas tissue. Data was compiled from 100 cells total from two replicate slides per treatment (reference n=4, CCR n=3, replicates n=3-4), results were considered significant if p ≤ 0.05.

Total antioxidant potential

There was no significant difference (p=0.53) in total antioxidant potential between CCR-exposed and non-exposed grass shrimp (Figure 2.4). 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±0.008UAE/mg protein.
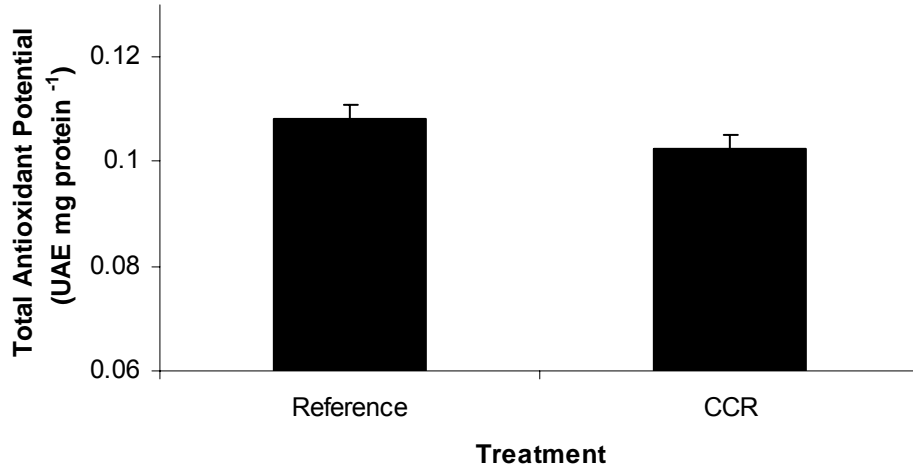
Figure 2.4. Effect of 156 day coal combustion residue exposure on the total antioxidant potential of *P. pugio* tail tissue. All samples were run in triplicate (reference n=4, CCR n=3, replicates n=3-4), results were not significantly different (p>0.05) amongst treatments.

Discussion

Samples from the D-Area settling basin were elevated in Cr, Cu, Se, Pb, Cd, and As compared to reference sediment (refer to Table 2.3). The two main exposure routes for juvenile and adult grass shrimp in this experiment were via sediment and/or the food. Water-borne trace elements appeared to present negligible exposure as dissolved concentrations were very low relative to concentrations in sediment. There was little variation in sediment trace element concentrations between initial and final samples, suggesting little mobilization of trace elements from sediment to water. I also used a flow-through system to mimic the dynamics of aquatic CCR disposal systems, which typically have low water-borne trace element concentrations (Rowe et al. 2002).

Cadmium and selenium (respectively) had the lowest concentrations of the trace elements of interest in the sediment (refer to Table 2.3). However, both of these element's concentrations were greatly elevated in the crayfish food source. As there was significant accumulation of Se and Cd by shrimp, and their concentrations were relatively low in the sediment, shrimp most likely accumulated these elements primarily from their food. Both selenium and cadmium have been shown to be accumulated into aquatic organisms (Chen and Chen 1999, Besser et al. 1996, Goodyear and McNeill 1999, Lemly, 2002, Sorensen et al. 1982, Thomas et al. 1999). Thomas et al. (1999) found that the corixid *Trichocorixa reticulata* accumulated selenium through food, but not through water-borne exposure, and May et al. (2001) suggest that in the Republican River Basin, in the mid-western U.S., Se is likely to be accumulated through the food chain but not the sediment. Note that, although highly concentrated in sediment, As was not significantly accumulated shrimp.

The persistence of CCR impact is likely due to the uptake of contaminants from the sediment, trophic transfer, and the bioaccumulative properties of selenium. The toxicity of selenium is caused by the enzyme system's inability to distinguish the molecular structure of selenium from sulfur (Garrett and Inman 1984), which can cause the malformation of proteins during synthesis. Effects of selenium on aquatic organisms include teratogenesis (which suggests DNA damage), exopthalmus, anemia, and reproductive failure in fish (Lemly 2002). Sorensen et al. (1984) reported swollen and vacuolated gill lamellae, decreased hematocrit, swollen and inflammatory cell-filled pericardial spaces, necrotic and ruptured egg follicles in ovaries in green sunfish (*Lepomis cyanellus*) from two study sites in Belews Lake, NC. Female bluegills (*Lepomis macrochirus*) with high body burdens of selenium produced larvae with edema, which did not survive to the swim-up stage (Gillespie and Baumann 1986). Selenium has been shown to cause oxidative stress and altered glutathione metabolism in aquatic birds (Hoffman 2002, Hoffman et al. 2002, Spallholz and Hoffman, 2002).

Grass shrimp significantly accumulated cadmium. However, Kamiyama et al. (1995) saw minimal effects in liver function tests and infiltration of inflammatory cells in parenchyma and degeneration of proximal tubules of kidneys of rats chronically exposed (one year) to cadmium. Bagchi et al. (1997) found an increase in lipid peroxidation in the liver of rat chronically exposed to cadmium and chromium. Shaikh et al. (1999) found in a twenty-two week study significant cadmium induced increases in both liver and renal cortex lipid peroxidation in rats. These studies show evidence of cadmium causing ROS generation, however I did not see any difference in the overall antioxidant potential.

Perhaps if I had measured glutathione alone I may have seen a difference amongst treatments.

The highest concentration of any element in the crayfish food source was copper. However, as there was no significant accumulation of copper by shrimp, it may be that the copper was not in a bioavailable form. This is possible because the total copper concentration was measured and it does not reflect the speciation of the copper. It is also possible that the shrimp are metabolizing and excreting the copper (Variego et al. 1990), thereby still causing DNA damage, yet not being accumulated in the tissues. Borgmann et al. (1995) found that the freshwater *Hyalella azteca* accumulated copper in short-term experiments, but were able to regulate it in long-term experiments. The digestive gland of mussels can excrete metals bound to lipid peroxidation products and therefore may provide a mechanism for the exocytosis of the residual copper bodies, accounting for its short half-life (6-8 days) in mussels compared to cadmium (6 months) (Viarengo et al. 1990).

Oxidative stress is a very dynamic process and must be alleviated quickly. Doyette et al. (1997) found decreases in glutathione reductase, selenium-dependent glutathione peroxidase, and reduced glutathione concentrations in bivalves (*Unio tumidus*) within seven days of exposure to cokery effluents contaminated with PCBs and PAHs. Correia et al. (2002) found that amphipods (*Gammarus locusta*) had increasing malondialdehyde (MDA) concentrations (indicative of increased lipid peroxidation) with exposure to increasing doses of copper, with concentrations peaking at exposure day four and returning to control concentrations by day ten. Because mine is a long-term experiment and indicative of chronic exposure, any oxidative stress would most likely be

compensated for early in the exposure period, which may be why I did not see any difference in total antioxidant potential.

Prahalad et al. (2000) suggested that the availability and not the concentration of metals in CCR discharges are critical in controlling DNA base damage. They also suggest the importance of the availability of a catalytically active form, or of chelators, in the making of ROS and subsequent DNA damage. All metal species examined (Ni, V, Fe) in a sample of CCR induced DNA damage (hydroxylation of dG to 8-oxo-dG) (Prahalad et al. 2000). The Comet assay was used in this study to assess the DNA damage in grass shrimp caused by chronic *in vivo* exposure to CCR. I found significantly increased percent tail DNA, tail moment, and tail length in CCR exposed compared to reference animals. Although no studies have specifically investigated the extent of DNA SB in organisms exposed to CCR, previous studies have demonstrated an increase in DNA SB following exposure to metals that were shown to be accumulated by the shrimp in this study. For example, Forrester et al. (2000) found that both cadmium and selenium administered individually to rats caused DNA single strand breaks. Steinert et al. (1998) found increased DNA SB in *Mytilus edulis* exposed to sediments contaminated with PAHs and trace metals including Cu, Hg, Zn, and Pb.

I demonstrated that grass shrimp hepatopancreas had a dose-dependant response in DNA damage to hydrogen peroxide, a known inducer of DNA SSB. The coefficients of variation in the hydrogen peroxide positive control (41.1) was higher than previously reported for other invertebrate species (see Mitchelmore and Chipman, 1998) and quite likely reflects inter-animal variability. Ideally a repeated sampling from a single organism is preferred for a positive control.

In a concurrent study (see Chapter 3) there was significantly decreased survival in CCR-exposed larval shrimp. Hook and Lee (2004) found that early embryonic stages of *P. pugio* are more likely to have developmental effects from genotoxicant exposure, and when exposed to the same concentration of genotoxicant they had lower hatching rates than later stage embryos. My 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. Although I found that there was very low mortality in juveniles and adults, CCR-exposed adults exhibit significantly higher DNA SSB than non-exposed adults. Genotoxicity often persists in aquatic organisms as DNA repair is slow in comparison to mammalian cells (Mitchelmore and Chipman 1998).

Release of CCR into aquatic systems can have detrimental effects on resident organisms. By using molecular biomarkers such as the Comet assay and antioxidant potential as biomarkers of effect, I may be able to determine if these systems are being adversely affected before there is permanent damage to the system. Repercussions of genotoxicity can be manifested at the population-level through impacts on DNA integrity and ultimate physiological processes (teratogenesis, mutagenicity, lethality) or through the increased metabolic cost of repair. Thus employing a biomarker-based monitoring program in combination with exposure and accumulation indices can provide the basis for pro-active management of systems receiving CCR.

Chapter 3: Effects of solid coal combustion residue on population dynamics of grass shrimp (*Palaemonetes pugio* Holthius)

Abstract

   Burning coal for electricity produces solid coal combustion residue (CCR), which is rich in potentially toxic trace elements, and is frequently discharged into natural and man-made aquatic systems as a method of disposal.   CCR is known to induce lethal and sublethal effects in aquatic and semi-aquatic organisms. However, the potential for CCR to elicit population-level effects has received relatively little attention in freshwater systems and has not been examined in contaminated estuarine or marine systems. I thus chose to study population-level responses of the estuarine grass shrimp, *Palaemonetes pugio*, which may result from exposure to CCR in sediment and food over the full life cycle of the shrimp. I exposed grass shrimp in the laboratory to CCR-enriched sediments and food derived from a coal-fired power plant in South Carolina. Exposure to CCR decreased larval survival and increased time to metamorphosis, but embryonic and adult survival and fecundity appeared unaffected. I constructed stage-classified matrix population models that reflected life stage-specific responses measured during the exposures. The population models suggested that CCR-exposed grass shrimp would experience a decreased population growth rate and altered stable stage structure and stage-specific reproductive value relative to shrimp in reference conditions. Elasticity analysis indicated that the survival of juvenile and gravid adult females would have the largest effects on population growth rate. Changes in population structure, as indicated by the models, could have ecosystem-wide consequences due to the trophic importance of grass shrimp in many estuarine systems. As many CCR disposal basins are not

isolated bodies of water, but integral components of larger aquatic systems, my results suggest that actions to regulate aquatic disposal of CCR could serve to protect the integrity of aquatic ecosystems local to CCR disposal sites.

Introduction

Traditionally toxicity tests have focused on acute mortality as a primary endpoint. However, because chronic low-level exposure from contaminated sediments can have subtle and sublethal effects on reproduction and growth in exposed organisms, an understanding of these effects at the population level is required to assess potential ecological risks (Spencer and McGee 2001). For example, Hummon and Hummon (1975) pointed out that analyses based upon life table information are more applicable to ecological systems than are traditional, acute toxicity tests, and Lin et al. (2005) argued that endpoints for ecological risk assessments should not be focused at the individual-level, but rather at the population-level (Lin et al. 2005).

A widespread source of contaminants known to cause sublethal stresses on aquatic organisms, and having potential to elicit population-level effects, is trace-element rich coal combustion residue (CCR). Large quantities of CCR are produced when coal is burned to generate electrical power. A common disposal method for CCR is to pump slurried ash into settling basins that are meant to retain solids prior to discharge of the water into local systems (Rowe et al. 2002). However, the basins rarely retain all of the suspended solids, and thus the release of CCR into downstream receiving waters is not uncommon. Because contaminants present in CCR are persistent and bioaccumulative,

there is growing concern about CCR disposal into aquatic basins and downstream systems where aquatic communities may be chronically exposed (Rowe et al. 2002).

The D-Area Power Facility site near Aiken, SC was chosen as a source of CCR for this study because it has been well characterized (Hopkins et al. 2000, Rowe 1998, Rowe et al. 1998b, 2002). At the D-Area site, coal combustion residue is pumped into settling basins, which then flows into a drainage swamp and creek. Invertebrates, reptiles, and amphibians living in this site display elevated body burdens of many trace elements, including Se, As, Cd, Cr, Pb, and Cu (Rowe et al. 2002, summarized in Table 3.1). In the D-Area site, sublethal effects of CCR on physiological, morphological, and behavioral processes have been reported in numerous vertebrates and invertebrates. For example, dietary and sediment-borne exposure of CCR to freshwater grass shrimp (*Palaemonetes paludosus*) and crayfish (*Procambarus acutus*) resulted in elevated standard metabolic rate and reduced growth rates compared to unexposed individuals (Rowe 1998, Rowe et al. 2001a). Elevated metabolic rates were observed in larval bullfrogs (*Rana catesbeiana*) (Rowe et al. 1998a), which also displayed oral deformities (Rowe et al. 1998b), spinal flexures (Hopkins et al. 2000), and behavioral anomalies that affected predator avoidance (Raimondo et al., 1998). Studies from other locations provide similar evidence for negative impacts of CCR. Lemly (2002) found that chronic exposure to CCR caused developmental, histopathological, and teratogenic effects, including exopthalmus and anemia, in numerous fish species inhabiting a CCR-contaminated lake (Belews Lake, NC). Sorensen et al. (1984) reported that green sunfish (*Lepomis cyanellus*) from two study sites in Belews Lake, NC contaminated with CCR

Table 3.1. Concentrations of select trace elements measured in abiotic and biotic matrices in the primary CCR settling basin at the D-Area Power Facility, SC. Values for sediment and tissues are ppm (dry mass); values for water are ppb.

Primary Settling Basin	Trace Elements					
	Cr	Cu	As	Se	Cd	Pb
Sediment	NR	71.8	70.8	6.21	0.57	45.2
Water	0.44	2.53	17.17	7.0	0.11	0.08
Crayfish, whole body	2.46	158.52	8.71	14.92	2.78	NR
Mosquitofish, whole body	1.56	4.97	2.89	14.28	0.32	NR
Bullfrog, recent metamorph, whole body	1.58	13.79	15.55	26.85	0.8	NR

NR = not recorded

Data compiled from Rowe et al. 2002.

exhibited swollen and vacuolated gill lamellae, decreased hematocrit, swollen, inflammatory cell-filled, pericardial spaces, and necrotic and ruptured egg follicles.

Palaemonetes pugio, hereafter "grass shrimp", is a species ubiquitous to estuarine and coastal systems from Maine to Texas (Knowlton and Kirby 1984) where it serves as important prey for estuarine fish (Wood 1967, Poole 1988). Individuals mature at approximately 3 months of age (Wood 1967), produce four to six broods per year of up to 400 eggs per brood (dependent on body size; Alon and Stancyk 1982), achieve a maximum weight of 3 grams, and live approximately 2 years (Poole 1988). Grass shrimp are benthic omnivores, and have the potential to accumulate and uptake trace elements from the sediment (Poole 1988). This species is a model organism (APHA 1985) in many toxicological studies and preliminary data suggest it may be chronically affected by CCR-contaminated sediment (Rowe and Hopkins, unpublished). Observations of sublethal effects of CCR on a congeneric freshwater shrimp (*P. paludosus;* Rowe 1998) further suggest that *P. pugio* may be sensitive to CCR and thus provide a model for studying effects of CCR in estuarine systems.

To assess potential population-level effects of contaminants, population models can be employed to examine the integrative effects on specific life stages (Munns et al. 1997; Salice and Miller 2003). Often sensitivity to contaminants is life-stage specific, and thus demographic population models allow for an evaluation of the overall effects of contaminants on the ecology of individual organisms and population growth rates (Caswell 1996b).

There are many types of population models, all of which can be used to determine the consequences of sublethal effects of contamination on the population growth rate.

Individual-based models (Kooijman and Metz 1984, Power et al. 1994), life table response experiments (Bridges et al. 1994, Caswell 1996a, 1996b, Levin et al. 1996), and age- and stage- classified matrix models (Caswell 2001, Munns et al. 1997, Salice and Miller 2003) have all been employed to examine population responses to contaminants. A feature common to all of these models is the use of demographic-specific bioassays to attain a set of vital rates, which are used in the model to forecast the integrated population-level responses of stage-specific toxicant effects on population growth rate (Caswell 1996b).

Individual-based models have been used to explore sublethal effects in several systems. For example, Power et al. (1994) used an individual-based model to examine the effects of copper, derived from laboratory data, on Atlantic salmon, and Kooijman and Metz (1984) used an individual-based population model to study the effects of chemical contaminants on the population growth rate of *Daphnia*. An individual-based model was used to examine the effects of a nonpolar narcotic on populations of *Daphnia magna* (Koh et al. 1997). Jaworska et al. (1997) used an individual-based population model to simulate the effects of polychlorinated biphenyls (PCBs) on young-of-the-year largemouth bass (*Micropterus salmoides*).

Life table response experiments (LTREs) integrate population modeling with life-history data to allow the quantification of effects of environmental factors on populations (Caswell 1996a, 1996b). LTREs allow the effects of treatments on vital rates (survival, growth, and reproduction) to be measured directly, and integrated into a model to assess the projected population-level effects of treatments, particularly the population rate of increase, λ. Life-history tables have been used to model population recovery of herring

gulls and common terns following oil spills (Samuels and Ladino 1984), to examine the effect of dichlorodiphenyltrichloroethane (DDT) on a freshwater gastrotrich (Hummon and Hummon 1975), to model the impacts of exposure of polychaetes to oil pollution (Levin et al. 1996, Bridges et al. 1994), and to assess the toxicity of Kepone on copepods (Allan and Daniels 1982).

Matrix-based approaches to population modeling (Caswell 2001) have been of particular utility in quantifying the impacts of contaminant exposure on populations (Munns et al. 1997, Landahl et al. 1997). Advantages of matrix models are that they directly incorporate specific life stages, and that their processes are assumed to take place in discrete time units, which is reminiscent of biological processes (Usher 1972). These features allow demographic characteristics and test endpoints from laboratory bioassays to be incorporated directly into matrix population models (Kuhn et al. 2001). Both age- and stage-based models have been developed to assess the importance of sublethal effects. Schaaf et al. (1987) used an age-classified matrix model to estimate potential pollution effects on estuarine fish populations, and Rose et al. (2003) used a matrix model to examine the effects of PCBs on Atlantic croaker (*Micropogonias undulatus*) population dynamics.

Stage-classified matrix models have been used to assess the population-level effects of dioxin and PCBs to the estuarine fish *Fundulus heteroclitus* (Munns et al. 1997), to examine the effects of chemical contaminants (including hydrocarbons) associated with urban areas of Puget Sound on flatfish populations (Johnson et al. 1998), and to assess the effects on cadmium on gastropod population growth rate (Jensen et al. 2001, Salice and Miller 2003). Stage-based matrix models were also used to assess

natural populations of *Leptocheirus plumulosus*, which are used in sediment toxicity tests (Spencer and McGee 2001).

Application of age- and stage-based projection models provides several insights into the impact of sublethal levels of contamination. Lefkovitch (1965) suggested the use of two matrices to examine the differences between reference populations and populations affected by a contaminant directly. More sophisticated analysis of the eigen structure of the projection matrix provides information on the population growth rate as well as the stable age/stage distribution and relative reproductive values of the life-history stages. Furthermore, elasticity analysis allows for the sensitivity of population growth rate on a proportional scale, to changes in any of the vital rates, such as survival and fecundity (de Kroon et al. 1986). By combining demographic elasticity analysis with more traditional toxicological studies on survival, growth, and reproduction it is possible to quantify the relative contributions of different life-stages and the organism's physiology in determining its susceptibility to toxicant exposure (Forbes and Callow 2002).

I conducted a series of exposure studies to determine effects of CCR exposure on *P. pugio* over a complete life cycle to provide parameter estimates for a stage-based matrix model. Experiments were conducted using CCR-contaminated sediments from the D-Area Power facility. Vital rates were estimated from these experimental results. I developed and analyzed models to compare the intrinsic rate of population increase (population growth rate), stable stage structure, and reproductive value in CCR-exposed and unexposed cohorts. I conducted an elasticity analysis of the models to determine the

life stages that have the largest effects on the population growth rate, as well as to examine any changes in elasticity between treatments.

Materials and Methods

Experimental Design

The study involved laboratory experimentation to provide estimates of vital rates (survival and stage duration for larval, juvenile, and adult stages) of *P. pugio* populations under reference conditions and when exposed to CCR. There were two treatments (four replicates per treatment); one with CCR sediment and CCR-contaminated food, and a reference treatment in which black sand served as the sediment and food was uncontaminated. The black sand was used to mimic the color of CCR. Contaminated sediment was derived from CCR dredge piles surrounding a drainage basin at the D-Area Power Facility on the U. S. Department of Energy operated Savannah River Site, SC. Contaminated food for juvenile and adult shrimp (crayfish tissue; below) was collected from the drainage basins and swamp at the D-Area site. Reference food for juveniles and adults was purchased commercially. 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 gravid adults collected from the Patuxent River, MD (salinity ~ 9 – 15 ‰). Gravid *P. pugio* were suspended in mesh baskets in individual glass jars containing 400 ml of aerated, filtered water from the Patuxent River (adjusted to 20‰ salinity with Instant Ocean) and placed in a 24ºC incubator until hatching and release of larvae from the egg clutch. The chelae

of the females were clipped to prevent the females from cannibalizing the egg mass (Little 1968), as sometimes occurs in laboratory conditions. Newly hatched larvae were removed daily and placed in a shallow glass dish and the larvae were allowed to mix fully.

Three days prior to the predicted day of peak hatching, eight 1500 ml beakers were set up with 200 cm^3 of sediment and filtered, Patuxent River water to be used in larval exposures.   Two hundred larvae were placed in each beaker on the day that they hatched (Day 1). In an effort to provide genetic diversity, all replicates had a mixture of larvae from three to fourteen females. The initial salinity in each beaker was 20‰ (optimal for larval survival; Knowlton and Kirby, 1984) and the salinity was gradually decreased to the ambient salinity of Patuxent River water (approximately 10‰) throughout the duration of the larval stage. Salinity, temperature (22.52 ± 0.91°C), and dissolved oxygen (7.02 ± 0.72 mg/L) were measured every three days prior to a 50% water change. Larvae were fed newly hatched *Artemia spp.* (above).

Thirty-nine newly metamorphosed shrimp from each replicate were used to start the juvenile stage of the experiment, with the same replicates carried over from the larval stage. I chose to use 39 juveniles because this represented the minimum number per replicate that survived the larval exposures. During the transition to the juvenile stage one of the CCR replicates was lost due to unusually low survival. Juvenile exposures were conducted in 57-L, flow-through tanks (with mesh netting over the outflow to prevent loss of shrimp) containing ambient salinity, filtered (20μm), Patuxent River water at ambient salinity (~10‰), and 1000 cm^3 of sediment (approximately 3 mm depth). Salinity, temperature, and dissolved oxygen were monitored weekly. Juveniles were fed

ground crayfish *ad libitum.* Reference crayfish were obtained commercially, and crayfish for the contaminated treatment were collected in the D-Area site. Crayfish were prepared by separating the tissue from the exoskeleton, drying, and grinding the soft tissue, the guts generally contained very little sediment as the crayfish had been maintained in traps for 24-96 hours prior to freezing. Thus crayfish likely fed only on other organisms that had become trapped. Juvenile exposures lasted for 60 days based on the stage duration reported by Wood (1967), at the end of which (Day 96 overall) shrimp were surveyed for survival and maturation defined as having attained a total length of 16 mm (Alon and Stancyk 1982). Total length was defined as the distance from the base of the rostrum to the posterior tip of the telson.

The adult phase of the experiment took place in the same flow-through system as the juvenile phase, and lasted for 77 days. The feeding regime for the adult exposure was the same as in the juvenile exposure. As adult females became gravid they were removed and suspended in mesh baskets in individual glass jars containing 800 ml of aerated, filtered water from their replicate tank, and their chelae were clipped to prevent them from eating the egg mass, however, sediment was not placed in the jars. The number of larvae that hatched from each female was counted. The average number of larvae hatched for each treatment was adjusted for a 50:50 sex ratio and used to estimate fertility in the model.

Survival and hatching success data were tested for normality and equivalence of variance and analyzed using one-way ANOVA. Time to metamorphosis data were log transformed prior to analysis by one-way ANOVA. All statistical tests were conducted using Minitab software for Windows, version 13 (Minitab, State College, PA).

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Trace Element concentrations in shrimp tissues, food, sediment, and water were analyzed using ICP-MS. Detailed methods and results for trace element analyses are provided in Chapter 2.

Model Development

I used a stage-based matrix model to examine population responses to contaminant exposure. The model was based on a pre-reproductive census, which characterized the life cycle as being composed of larvae, juveniles, gravid females, and non-gravid females. Gravid and non-gravid adults were considered to represent separate stages, as changes in the survival of gravid shrimp will have a different effect on the population growth rate than changes in the survival of non-gravid shrimp, and individual females may spend an unequal amount of time in each of these stages.

The life cycle graph of *P. pugio*, that formed the basis of the model, is shown in Figure 3.1 and was used to develop the projection matrix:

$$A = \begin{bmatrix} P_1 & 0 & 0 & F_4 \\ G_1 & P_2 & 0 & 0 \\ 0 & G_2 & P_3 & G_4 \\ 0 & 0 & G_3 & P_4 \end{bmatrix}$$

The model had a time step of one day. A pre-breeding census was used to estimate fecundity, represented by F_i, and adjusted for a 50:50 sex ratio. In the projection matrix P_i represents the probability of surviving and remaining in the same stage over one time step, G_i represents the probability of surviving and growing to the next stage over one time step. P_i and G_i represent the overall probability of surviving for each stage i, and is by definition ≤ 1. The transition probabilities P_i and G_i are calculated from estimates of

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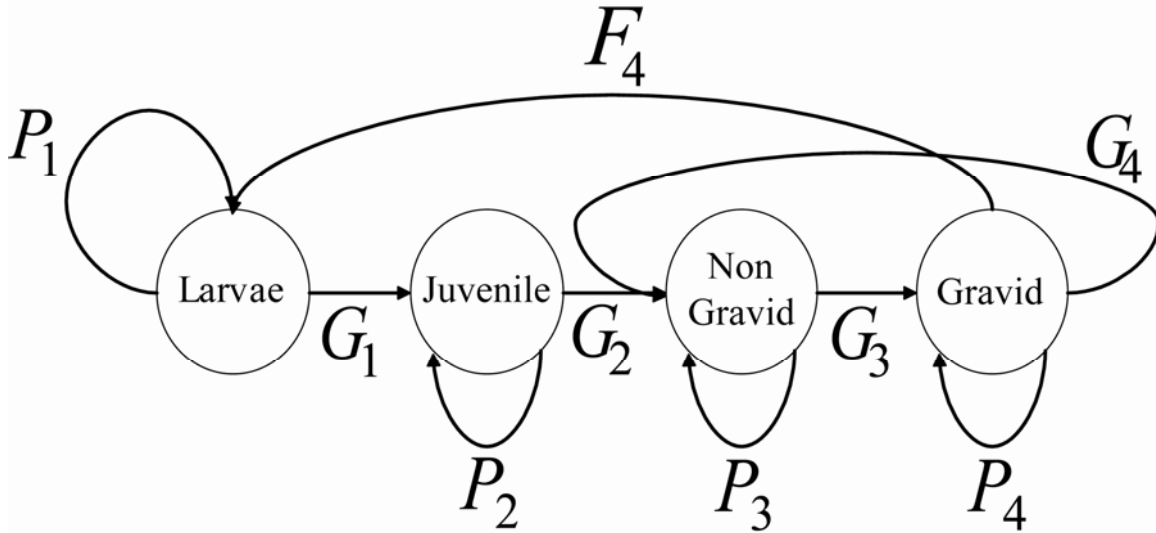
Figure 3.1. Life history diagram of *P. pugio*. P_i represents the probability of surviving and remaining in the same stage. G_i represents the probability of surviving and growing to the next stage and F_i represents fertility.

the probability of an individual of stage i surviving during a single time step σ_i, and the probability that an individual of stage i grows to the next stage, γ_i. The probability of surviving a stage and growing to the next stage was calculated with Equation 1.

Equation 1: $\qquad G_i = \sigma_i\, \gamma_i$

The probability of surviving a particular stage and not growing to the next stage was calculated using Equation 2.

Equation 2: $\qquad P_i = \sigma_i(1 - \gamma_i)$

The probability of surviving a stage, σ_i, was calculated with Equation 3,

Equation 3: $\qquad \sigma_i = e^{\frac{\ln s_i}{T_i - 1}}$

where s_i is the stage survival probability derived from the experiments (see below), and T_i is the number of daily time steps in stage i. The estimates of γ_i were obtained using Caswell's (2001) method (Equation 4) of assuming fixed stage duration, and assuming that all individuals in a stage have the same probability of surviving,

Equation 4: $$\gamma_i = \frac{\left(\sigma_i / \lambda_{init}\right)^{T_i} - \left(\sigma_i / \lambda_{init}\right)^{T_i - 1}}{\left(\sigma_i / \lambda_{init}\right)^{T_i - 1}}$$

where λ is the dominant eigenvector of the transition matrix A and represents the finite rate of population increase. I started with an initial value of λ_{init}=1, and iterated λ until the value of λ given by eigenanalysis of the transition matrix A equaled the value of λ_{init} used in equation 4.

Separate models were developed for *P. pugio* populations that were either exposed or unexposed to CCR contamination. The models were run in Excel, and analyzed using the PopTools add-in (http://sunsite.univie.ac.at/Spreadsite/poptools/). PopTools was used to calculate the discrete rate of population growth λ, given by the dominant eigenvalue of the projection matrix. Values of $\lambda > 1$ indicate positive population growth, λ=1 indicates no population growth, and values of $\lambda < 1$ indicate population declines. The calculated λ was transformed to the more common intrinsic rate of population increase, r, as $r = e^{\lambda}$. The left and right eigenvectors associated with the dominant eigenvector were examined to quantify the stable stage structure and reproductive values of each life history stage. I also calculated the elasticity matrix for each population. Elasticities can be defined for all possible life history transitions and represent the proportional change in λ to equal proportional changes in the probability of the transition. The elasticities were calculated using Equation 5.

Equation 5: $\quad e_{ij} = \dfrac{a_{ij}}{\lambda} \dfrac{\partial \lambda}{\partial a_{ij}}$

Parameter estimation

Stage durations (Ti) were estimated from laboratory observations. The larval stage duration was 21 days and was estimated from the laboratory estimates of peak metamorphosis (Figure 3.2). The juvenile stage duration was defined as 77 days; the end of the juvenile exposure (Day 96) minus the larval stage duration (21days). The adult gravid stage duration was defined as 18 days, and the adult non-gravid stage duration was 7 days, which were estimated from laboratory observations.

The proportion of individuals that survived each life stage was used as the survival probability parameter, s_i. The proportion of individuals in each stage that had grown to the next life-stage by the final day of that exposure was used to calculate the initial value of the growth probability parameter, γ_i, which was then, iterated using the method described above to estimate the probability of growing to the next stage for a single time step. Fecundity, F_i, was estimated by counting the total number of larvae hatched per female and averaging over each of the two treatments, and adjusting for gravid female stage duration and a 50:50 sex ratio.
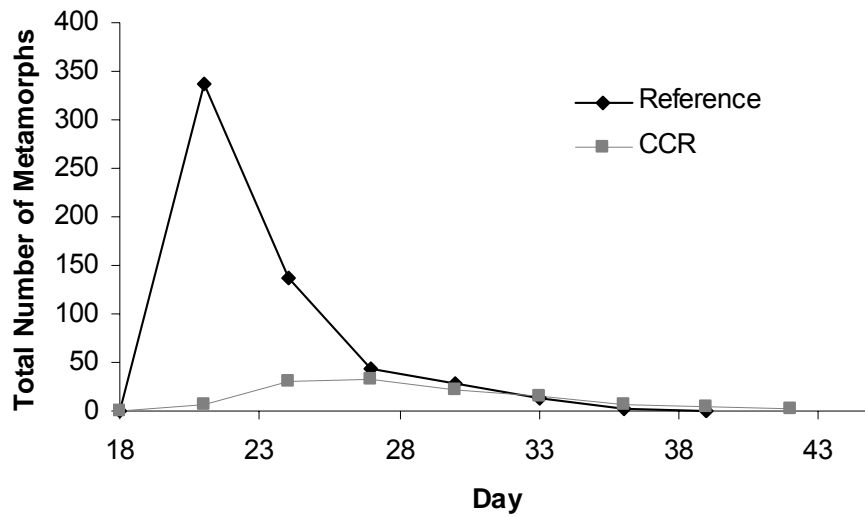
Figure 3.2. Time to metamorphosis of *P. pugio* larvae in reference and CCR conditions

Results

Survival of larvae differed significantly (p=0.007) between the reference and CCR treatments (Table 3.2). Survival ranged from 31% to 90% and 7.5% to 22% in the reference and CCR treatments respectively. Successful metamorphosis occurred in both treatments, however time to metamorphosis was significantly delayed (p=0.018) in the CCR treatment (Figure 3.2). In the reference treatment most larvae metamorphosed on day 21, whereas in the CCR treatment most larvae metamorphosed on day 27. In contrast to the larval stage, there was no significant difference in juvenile or adult survival between treatments (Table 3.2). Nor were there significant differences between treatments in the number of larvae hatched (Table 3.2).

Table 3.2. Survival probability of specific life stages of *P. pugio* and average number of larvae hatched in reference and CCR treatments.

| Treatment | | Survival Probability | | | |
		Larval	Juvenile	Adult	Total Hatch
Reference	Mean	0.70	0.58	0.99	86.43
	St. Dev	0.26	0.24	0.01	37.96
CCR	Mean	0.15	0.68	0.99	72.54
	St. Dev	0.07	0.17	0.02	40.34
p		0.01	0.57	0.73	0.46

Survival and hatch data were used to estimate the transition probabilities and fecundities shown in Table 3.3. The main difference in P_i was in the larval stage with probabilities of 0.95 and 0.90 in the reference and CCR treatments respectively. Larvae in the reference treatment had a slightly higher probability of surviving and growing to the next stage than did larvae in the CCR treatment (G_i = 0.03 and 0.01 respectively). Reference females had higher fecundity (2.32) than CCR-exposed females (1.91), though this was not statistically significant. Analysis of the resultant projection matrices indicated that populations in both treatments had positive population growth rates; however the CCR-exposed shrimp population growth rate was reduced relative to the reference population (1.02 and 1.03 respectively) (Table 3.4). After ten simulated generations the CCR grass shrimp populations were projected to be six orders of magnitude reduced relative to reference populations (Figure 3.3). The model indicates an increase in generation time in CCR exposed grass shrimp populations, as well as a decrease in the average age at maturity and the expected number of replacements (Table 3.4).

Table 3.3. Transition probabilities and fecundities of *P. pugio*

	Reference			CCR		
Stage	P_i	G_i	F_i	P_i	G_i	F_i
Larvae	0.95	0.03	0.00	0.90	0.01	0.00
Juvenile	0.99	0.00	0.00	0.99	0.00	0.00
Non Gravid	0.85	0.15	0.00	0.84	0.16	0.00
Gravid	0.95	0.04	2.32	0.95	0.05	1.91

P_i = probability of surviving and remaining in the same stage over one time step, G_i = probability of surviving and growing to the next stage over one time step, and F_i = fertility, estimated with a pre-breeding census

Table 3.4. Life history parameters from the analysis of the transition matrix.

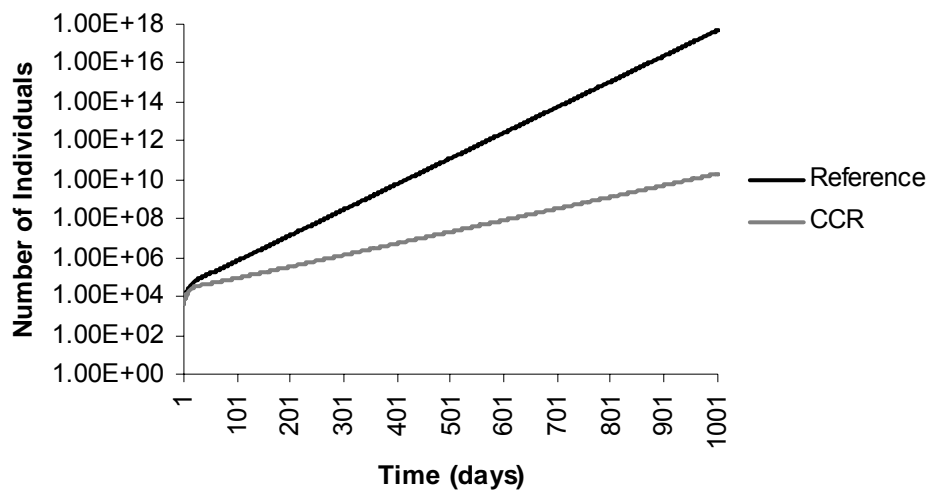| Parameter	Reference	CCR
Intrinsic rate of increase	0.03	0.02
Population growth rate (λ)	1.03	1.02
Generation time (days)	196.72	226.17
Average age at maturity (days)	1842.22	1098.84
Expected number of replacements	460.25	78.83

Figure 3.3. Projected population size after ten generations

The model was used to examine the reproductive value of shrimp life stages and the stable stage structure for reference and CCR populations (Table 3.5). In CCR populations there was a decrease in reproductive value for gravid adults and a subsequent increase in reproductive value for juveniles compared to reference populations. There was a shift in the stable stage structure of CCR exposed shrimp populations compared to reference populations, with a decreased proportion of the shrimp population in the juvenile stage, and a subsequent increase in the proportion of the population in the larval stage.

Table 3.5. Reproductive value and stable stage structure for reference and CCR-exposed grass shrimp populations

Stage	Reproductive Value		Stable stage structure	
	Reference	CCR	Reference	CCR
Larvae	0.9%	0.7%	56.7%	66.2%
Juvenile	2.5%	6.8%	40.4%	27.6%
Non Gravid	43.6%	43.3%	1.0%	1.9%
Gravid	53.0%	49.2%	1.9%	4.3%

Elasticity values of model parameters are presented in Table 3.6. In reference populations the parameters with the highest elasticities were survival of juveniles (0.322) and survival of gravid adults (0.320). In the CCR population model the parameters with the highest elasticities were survival of gravid adults (0.373) and survival of juveniles (0.348). The largest changes in elasticity between reference and CCR populations was an increase in the elasticity values of the survival of larvae and the survival of gravid adults, which increased to a greater degree.

Table 3.6. Elasticities of model parameters (P_i = probability of surviving and remaining in the same stage over one time step, G_i = probability of surviving and growing to the next stage over one time step, and F_i = fertility, estimated with a pre-breeding census)

	Reference			CCR		
Stage	P_i	G_i	F_i	P_i	G_i	F_i
Larvae	0.16	0.01	0.00	0.07	0.01	0.00
Juvenile	0.32	0.01	0.00	0.35	0.01	0.00
Non Gravid	0.12	0.03	0.00	0.13	0.03	0.00
Gravid	0.32	0.01	0.01	0.37	0.02	0.01

Discussion

Population growth rate is considered a better measure of fitness than individual-level effects (Forbes and Callow 2002), and thus I used a matrix population model to examine the effects of CCR induced changes of individual life-history traits on the population growth rate. The matrix population models for CCR-exposed and reference grass shrimp populations suggested decreased population growth rates in the CCR-exposed populations. The effect of CCR on projected population growth rates was primarily due to a decrease in larval survival in these populations. Allan and Daniels (1982) found a similar decrease in population growth rate in copepods exposed to Kepone. Copepods in this study exhibited lowered survivorship, reduced fecundity, and delayed the onset of reproduction. Population growth rate (λ) was also shown to be reduced by hydrocarbon exposure in a polychaete, *Streblospio benedicti*, due to reduced fertility combined with excessively delayed maturation (Levin et al. 1996).

A principal advantage of the approach taken here is the decomposition of population growth into its constituent parts so that the contribution of sublethal effects on different life stages can be understood. Coal combustion residue has a detrimental effect on the survival of larval grass shrimp, and caused a delay in the time to metamorphosis. However, no significant differences were found in either juvenile or adult survival, or in hatch success. In a concurrent study (Chapter 2), I found that CCR-exposed grass shrimp significantly accumulated selenium and cadmium, as well as had significantly increased DNA strand breaks. Exposure to CCR, and specifically the accumulation of selenium, has been shown to reduce early life stage survival in numerous organisms including amphibians (Rowe et al. 2001b) and by causing developmental defects in fish (Lemly 2002, Gillespie and Baumann 1986). Increased DNA damage from CCR-exposure may have also contributed to the decrease in larval survival (Chapter 2). Lee et al. (2000) and Hook and Lee (2004) found that DNA damage in early stage larval *P. pugio* affected development, and subsequently survival, after exposure to genotoxicants. Delays in metamorphosis are also a common response of organisms to contaminants. Exposure to toxicants, including herbicides, atrazine, and perfluorooctanesulfonate have been shown to increase the time to metamorphosis in amphibians (Howe et al. 2004, Ankley et al. 2004, Sullivan and Spence 2003).

Changes in fecundity are also a likely significant component of the change in population growth rates. Salice and Miller (2003) found altered fecundity in two strains of gastropods exposed to cadmium. Spurgeon et al. (2003) observed a decrease in the number of offspring produced in earthworms exposed to cadmium and copper. As grass

shrimp in the CCR experiments accumulated cadmium as well as selenium (See Chapter 2) it may be that this contributed to the decrease in fecundity seen in this study.

Coal combustion residue-exposed populations were projected to produce 5.8 times fewer replacements than reference populations, which would contribute to the decrease in population size and population growth rate indicated by the model. Model projections suggest that, under the conditions tested, after ten generations CCR-exposed populations would be three million times smaller than reference populations. The model also indicated an increase in generation time in CCR-exposed populations, which suggests that a population that has been exposed to CCR will require longer to replace itself. The model indicates a decrease in the average age at maturity. This decrease is likely an artifact of the model due to the small decrease in reference juvenile shrimp survival compared to CCR-exposed shrimp, and which was not significantly different. Increased generation times and smaller population sizes may mean that CCR-exposed grass shrimp populations are more vulnerable than reference populations to natural environmental fluctuations, competition, or predation.

Many studies have used population growth rate as an index of toxic effects on populations; however Barnthouse et al. (1987) argue that reproductive potential is the simplest index to integrate the effects on contaminants on all life stages for toxic risk assessment. In CCR-exposed grass shrimp there was a shift in reproductive value, with a decrease in the reproductive value of gravid adults leading to a subsequent increase in the reproductive value of juveniles. This shift is likely due to the decreased probability of a CCR-exposed individual reaching adulthood. This indicates that environmental factors that may change the growth or survival of juvenile shrimp, such as predation, could

further effect the population as the individuals nearing sexual maturity are likely to make the greatest contributions to the population in terms of overall reproductive potential. In contrast to our study Gentile et al. (1982) found that the exposure of mysid shrimp to nickel and mercury shifted the reproductive value to older age classes due to delays in sexual maturity.

In CCR-exposed grass shrimp populations there was a shift in the stable stage structure to a smaller proportion of juveniles and a subsequent increase in the number of larvae and gravid adults, as compared with reference populations. Most likely this was caused by the decrease in larval survival and fewer individuals reaching the juvenile stage. Levin et al. (1996) found that in polychaete (*Stebelspio sp.*) populations with higher population growth rates (λ) displayed a stable age distribution biased toward younger stages. The same appears to be the case for both populations of grass shrimp in our study, since both had the highest proportions of their populations in the early life stages. However, the CCR-exposed population had a higher proportion of individuals in the earliest life stage compared to the reference population.

Exposure to toxicants can cause changes in the population growth rate and elasticity patterns (Hansen et al. 1999). Elasticity analysis has become a popular tool in ecological risk assessment and conservation biology (de Kroon et al. 2000). Thus, an elasticity analysis was conducted on our models to examine how individual parameters can affect population growth rate. In general, parameters with the largest elasticities have the highest impact on population growth rate; and thus anything that further affects these parameters will most likely have a larger impact on population growth rate. It must be

kept in mind that elasticities of matrix elements are not independent of each other, as they sum to one, and negative correlations may arise between them (Shea et al. 1994).

In the reference grass shrimp population, survival of juveniles and survival of gravid adults had the highest elasticities, with each having near equal weight. Larval survival had the next highest elasticity, emphasizing the importance of this parameter. There was a shift in the elasticity pattern of CCR-exposed grass shrimp in which there was a general increase in the elasticity values of older life stages. In CCR-exposed populations gravid adults had the highest elasticity value, with the next highest being juvenile survival; however, both of these values are larger than their counterparts in the reference model. This indicates that further changes in these life-history parameters in CCR-exposed populations will have a larger effect on the population growth rate than changes in these same parameters in reference populations. Factors that change demographic parameters, such as toxicants or fishing pressure, have been shown to change the elasticity patterns of many organisms (for example Salice and Miller 2003, Frisk et al 2002, Heppell et al. 1996).

Elasticities are relative contributions and thus when there is a change in one parameter, the others must shift to compensate. The largest change in elasticity was the decrease in the elasticity of larval survival in the CCR-exposed shrimp. Hansen et al. (1999) found that when the probability of juvenile survival is low, then the elasticity of λ to further toxicant induced changes in juvenile survival is reduced. Similarly, Heppell et al. (1996) found that in loggerhead sea turtles adult survival elasticity decreased as the survival rate of adults decreased. However, they suggested that this was not an indication of reduced adult value, but rather that sub-adults become relatively more valuable as the

population reached a steady state. This may be the case in the current study; reduced larval survival may have brought about a concomitant decrease in elasticity in this parameter, which may indicate that further changes in larval survival will not have as great of an effect on the population growth rate. However, this may also indicate that because of decreased larval survival, juvenile and gravid adult survival has become relatively more influential on population growth rate.

The results of this study are similar to other studies of CCR-impacted systems. Decreases in early life-stage survival are common in CCR contaminated systems (Lemly 2002, Sorensen et al. 1984). In this study CCR affected grass shrimp vital rates including larval survival and fecundity, and the model indicated decreases in population growth rate and shifts in the reproductive value and elasticity. However, contaminants do not always have the same effect on every species. Levin et al. (1996) found that polychaete population growth rate was decreased because of excessively delayed maturation and reduced fertility due to hydrocarbon exposure. Salice and Roesijadi (2002) found a decrease in time to death as well as percent hatch due to cadmium exposure in a parasite-resistant strand of gastropod in comparison to a parasite-susceptible strand, which indicated that sensitivity to cadmium was a cost of being resistant to parasitic infection. Salice and Miller (2003) found that cadmium exposure to gastropods also caused a significant affect on the population growth rate which was caused by changes in percent hatch, juvenile and adult survival in both strains, and by changes in fecundity and time to maturity in the parasite-susceptible strain. Vonesh and De la Cruz (2002) found that amphibian population growth rate was more sensitive to changes in juvenile survival than egg mortality. The population growth rate can be affected by changes in any of the vital

rates and thus it is important for toxicity tests to look at all life-history parameters in order to determine which vitals rates are causing contaminant-induced changes in the population growth rate or other model parameters.

The effects of contaminants may be influenced by the trophic status of a community or the stability of the ecosystem (Clements and Kiffney 1994). Grass shrimp are an important prey item in estuarine systems (Poole 1988). Elasticity analysis indicates that CCR-exposed grass shrimp populations may be more sensitive than non-exposed populations to predation or other factors that may decrease adult survival. Thus predation or other processes influencing adult survival may exacerbate contaminant effects, ultimately affecting the availability of these prey items to the predators. The potential interaction of predation and contaminant effects as suggested in this study supports the argument by Clements and Kiffney (1994) that assessing effects of contaminants on populations can be complicated by competitive and predatory interactions.

Although toxicants exhibit initial effects on individuals at the molecular level, severe enough effects can modify higher-order, ecological processes (Caswell 1996b). Interactions with potential competitors and predators, the physical environment, and the life-history characteristics of a species may all contribute to changes in the population growth rate and consequently have important effects on population stability, and ultimately on the ecosystem (Gentile et al. 1982). Because populations function as individual entities within ecosystems, if their size or structure is changed by chemical toxicity, there may be functional and structural ecosystem-wide consequences (Lin et al.

2005). Thus, decreased population sizes, such as those indicated by our model, can have effects on the larger community.

Disposal of coal combustion residue is largely unregulated and many of the aquatic-disposal settling basins are connected to local waterways, and thus the effects of CCR exposure can also occur down-stream. Nearby terrestrial systems can also be affected by CCR through contamination of soil and groundwater, and by reduced fitness of terrestrial or semi-terrestrial organisms that use the contaminated aquatic sites for feeding or reproducing (such as amphibians and some reptiles). Therefore, effective management must incorporate both toxicity and bioavailability data into assessments of the localized sources and less directly affected habitats (Kuhn et al. 2002).

For effective management, laboratory toxicity studies must be made predictive and representative of contaminant impact at the population-level (Gentile et al. 1982). By identifying important stages in an organism's life history, population models can provide environmental managers with a place to focus their efforts and set protection limits based on these relatively sensitive life stages (Kuhn et al. 2002). However, the life-history stage that shows the most sensitivity to a toxicant may not be the most important in terms of population growth rate, and thus may not be the best place to focus management efforts as has been previously done. Also, it may not be feasible to manage the life-history stage that is most sensitive to toxicants. This must all be taken into account for management strategies to be effective.

The findings of this study not only have implications for grass shrimp, but also for some other species that inhabit environments local to CCR disposal facilities. More studies are needed to assess the potential population-level effects that other species might

experience in CCR-contaminated systems. For toxicity test endpoints to be ecologically relevant, they must be applicable to population, community, and ecosystem traits (Kuhn et al. 2001). Population models are powerful tools that managers can utilize to link traditional toxicity tests to higher order consequences. Population-level risk assessments should be a vital part of any management strategy for CCR affected systems. As these CCR disposal basins are not isolated bodies of water, but integral components of larger aquatic systems, management efforts based upon population-level assessments are required to minimize the impacts of CCR on these ecosystems.

Chapter 4: Synopsis

Overview: properties of coal combustion residues and toxicological effects:

Coal combustion residue (CCR) is a solid waste product derived from large-scale coal burning facilities such as coal-fired power plants. Coal combustion residue contains a complex mixture of trace elements concentrated from the parent coal as a result of combustion. The high volume of CCR produced in the USA creates challenges for its disposal, as of 1998 57 million tons annually (ACAA 1998). A primary disposal method is to pump CCR as a slurry into aquatic basins in an effort to allow the settling of solids. The aquatic settling basins often support unique ecosystems that may be negatively affected by the trace elements contained in the CCR. These settling basins are often connected to a local waterway, providing the potential for release of CCR into nearby systems.

There can be serious environmental effects of CCR disposal into aquatic basins and downstream habitats. The trace elements characteristic of CCR can have negative effects on organisms that inhabit the contaminated aquatic systems and nearby terrestrial habitats (Rowe et al. 2002). Lemly (2002) found that chronic exposure to CCR caused developmental, histopathological, and teratogenic effects in numerous fish species. Increased metabolic costs have been observed in crayfish (Rowe et al. 2001a), grass shrimp (Rowe 1998), and larval bullfrogs (Rowe et al. 1998a) exposed to CCR. The latter also displayed oral deformities (Rowe et al. 1998b), spinal flexures (Hopkins et al. 2000), and behavioral anomalies that affected predator avoidance (Raimondo et al. 1998). Although such freshwater organisms have received considerable attention with respect to

CCR exposure, very little research has addressed organisms inhabiting estuarine systems receiving CCR effluent (Rowe 2003).

Selenium (Se) is perhaps the contaminant of greatest concern with respect to the toxicity of CCR (Lemly 1997, 2002). Selenium is persistent in CCR-contaminated sediments, is highly bioaccumulative, and has been linked to toxicological and ecological effects in numerous CCR-impacted systems (Besser et al. 1996, Lemly 2002, Sorensen et al. 1984). The toxicity of Se is likely related to its chemical similarities with sulfur (S) and its substitution for S in cysteine-rich proteins/enzymes (Garrett and Inman 1984). Substitution of S with Se can lead to abnormal tertiary structures during protein synthesis, and has been linked to effects including teratogenesis, exopthalmus, anemia, and reproductive failure in fish (Lemly 2002). Also, selenium has been shown to cause oxidative stress and altered glutathione metabolism in aquatic birds (Hoffman 2002, Hoffman et al. 2002, Spallholz and Hoffman 2002).

CCR exposure in the current studies

Because of the dearth of information regarding CCR effects on estuarine systems (Rowe 2003), I conducted full life cycle exposures to CCR on the estuarine grass shrimp (*Palaemonetes pugio* Holthius) to quantify lethal and/or sublethal effects (see Chapters 2 and 3). Grass shrimp were exposed to either CCR or reference sediment and food for 156 days, with larval-stage exposure taking place in 1500mL beakers, and juvenile- and adult-stage exposure taking place in 57-L flow through tanks. Sediments consisted of solid CCR derived from the D-Area settling basin on the Savannah River Site, SC or black sand as a reference (to mimic the color of CCR). Food for larval grass shrimp consisted

of brine shrimp (*Artemia spp.*) hatched over CCR or reference sediment; juvenile and adult grass shrimp were fed ground, dry tissues of crayfish collected from the site in which CCR was derived, or from uncontaminated sites.

At the end of the exposure the sublethal effects of CCR were examined through the use of molecular biomarkers; specifically, I conducted the Comet assay (single-cell gel electrophoresis) to measure the extent of DNA damage in shrimp hepatopancreas, and the total antioxidant potential of shrimp tail tissue as an indicator of oxidative stress. The exposure experiment also provided the parameter estimates for stage-classified matrix models. The models were analyzed to assess the population growth rate, stable stage structure, and reproductive value, and an elasticity analysis was conducted under both treatment conditions.

Contaminant concentrations in sediments and food used in exposures

Sediment and crayfish were enriched in Cr, Cu, Se, Pb, Cd, and As compared to the reference sediment and crayfish. The primary exposure routes for grass shrimp in the experiments were through the sediment and the food provided to juvenile and adult life stages (consisting of tissues from crayfish captured in the study sites). Dissolved concentrations of trace elements did not differ significantly between CCR and reference treatments, suggesting that water-borne exposure was negligible. Cadmium and Se (respectively) were the least concentrated of the potentially toxic trace elements detected in the sediment. However, both of these elements concentrations were significantly elevated in the food source. The shrimp significantly accumulated Cd and Se, suggesting that the primary exposure route was most likely their food.

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Sublethal effects of CCR on grass shrimp – measurement of DNA damage and oxidative stress

Adult grass shrimp were assessed for DNA single strand breaks (SSB) at the end of the exposure experiment. Damage to DNA, if not repaired prior to transcription, can alter protein translation and, in some cases, result in mutagenesis. I chose to use DNA damage as a biomarker because contaminants associated with CCR have been shown to be mutagenic in a variety of organisms (Smith-Sonneborn et al. 1981, Kubitscheck and Ventra 1979, Li et al. 1983). I specifically examined DNA SSB because they have been shown to be a rapid and relatively sensitive indicator of genotoxicant exposure and combines many DNA damage endpoints (Mitchelmore and Chipman 1998, Steinert et al. 1998). Therefore, the Comet assay was used to assess the extent of DNA SSB in grass shrimp.

At the end of the exposure experiment adult grass shrimp were also examined for changes in total antioxidant potential. The measurement of total antioxidant potential (total oxidant radical scavenging capacity) has recently been applied to aquatic organisms (Winston et al., 1998, Regoli and Winston, 1998, 1999), and is advantageous because it is a combined approach and provides an overall indication of the organism's free radical scavenging capacity. Reactive oxygen species can impair nucleotides, proteins, lipids, and carbohydrates, (Yu 1994), and may ultimately lead to tissue damage (Diaz et al. 2004). I included antioxidant potential in my biomarker assessments of sublethal effects because transition elements, including Cu, Cr, Fe, Ni, V, and Co, can initiate the production of reactive oxygen species (ROS) (Prahalad et al. 2000), and selenium

specifically has been shown to cause oxidative stress and altered glutathione metabolism in aquatic birds (Hoffman 2002, Hoffman et al. 2002, Spallholz and Hoffman 2002).

Significant increases in percent tail DNA, tail moment, and tail length in CCR-exposed shrimp compared to reference shrimp suggested that chronic exposure to CCR caused DNA damage to adult grass shrimp which was not repaired. Prahalad et al. (2000) found that Ni, V, and Fe induced DNA damage (hydroxylation of dG to 8-oxo-dG), and that the availability rather than total exposure of metals was critical in inducing DNA base damage. Additionally, both cadmium and selenium administered individually to rats (Forrester et al. 2000) and water-borne exposure of mussels to a combination of PAHs and metals (Steinert et al. 1998) displayed DNA SSB. Thus, the occurrence of DNA SSB that I observed in CCR-exposed grass shrimp is consistent with observations of DNA damage in other species exposed to several constituents of CCR.

There was no significant difference in total antioxidant potential between reference and CCR-exposed grass shrimp. Because of the chronic exposure period in my experiment, it is possible that oxidative stress was compensated for early in the exposure period. However, Shaikh et al. (1999) found that chronic cadmium toxicity caused increased hepatic and renal cortex glutathione concentrations in rats, indicative of ROS generation. It is possible that, had I measured glutathione alone, I may have seen a difference; because the total antioxidant potential test covers a wide array of antioxidants as a collective as opposed to individually, which can be disadvantageous if the toxicant has a very specific response, as it may be masked amongst the overall effect.

Chronic, population level effects

In addition to sublethal effects, CCR caused significant changes in larval survival and time to metamorphosis, which have the potential to exert population-level effects. CCR exposure is often not directly lethal, but can lead to reproductive failure or developmental defects in offspring, in some cases resulting in population declines (Lemly 2002). As the population growth rate is considered a better measure of fitness than individual-level effects (Forbes and Callow 2002) I modeled population growth rates to examine the potential for CCR-exposure to affect on grass shrimp population dynamics.

I employed matrix population models to examine the effects on population growth rates of CCR-exposure to specific life-stages. I used stage-classified matrix models because grass shrimp have clearly discernable life-history stages. Separate models were constructed for reference and CCR-exposed grass shrimp. Responses to CCR among all life stages (survival, growth, and fecundity) were measured in the laboratory exposure and used to calculate the parameters for the models. Population growth rate has often been used as an index of toxic effects on populations, although Barnthouse et al. (1987) argued that reproductive potential is the simplest index to integrate effects of contaminants on all life stages. Thus, I measured both population growth rate and reproductive potential. I also conducted an elasticity analysis on the models to examine how individual parameters could affect the population growth rate, as exposure to toxicants can cause changes in elasticity patterns (Hansen et al. 1999), and as elasticity analyses have become an important tool in ecological risk assessment and conservation biology (de Kroon et al. 2000).

The models indicate a decreased population growth rate in CCR-exposed grass shrimp populations compared to reference populations, (1.02 and 1.03 respectively). This is due to the changes in the transition probabilities, mainly in decreases in the larval stages (probability of surviving and remaining in stage i, P_i = 0.95 and 0.90; and the probability of surviving and growing to stage $i+1$, G_i = 0.03 and 0.01, in the reference and CCR treatments respectively). Also reference females had a higher fecundity (2.32) than CCR exposed females (1.91), though this was not statistically significant. Similar projected declines in population growth rate were seen in copepods exposed to Kepone (Allan and Daniels 1982), and in polychaetes exposed to hydrocarbons (Levin et al. 1996).

The model results primarily reflect a reduction in larval survival and delayed time to metamorphosis in CCR-exposed shrimp relative to reference shrimp. I found no effects of CCR on juvenile and adult survival and as a result, these vital rates were relatively unimportant in explaining differences in projected population growth rates. Effects of CCR on projected population sizes resulting from altered larval traits were striking. Model projections suggest that, under the conditions tested, after ten generations CCR-exposed populations would be three million times smaller than reference populations, and that they would produce 5.8 times fewer replacements. The model also indicates an increase in generation time in CCR exposed populations, which indicates that a population that has been exposed to CCR will require longer to replace itself. The model also indicated a decrease in the average age at maturity, which was an artifact of the decrease in reference shrimp juvenile survival (which was not significantly different from CCR-exposed juvenile shrimp survival).

In CCR-exposed grass shrimp there was a shift in reproductive value, with a decrease in the value of gravid adults and a subsequent increase in the reproductive value of juveniles, which was likely due to the decreased probability of a CCR-exposed individual reaching adulthood. In CCR-exposed grass shrimp populations there is a shift in the stable stage structure to a smaller proportion of juveniles and a subsequent increase in the number of larvae and gravid adults, as compared with reference populations. This is most likely caused by the decrease in larval survival and fewer individuals entering the juvenile stage.

In general, parameters with the largest elasticities have the greatest influences on population growth rates. Thus anything that further affects these parameters, such as toxicants or fishing pressure, will most likely have a larger impact on population growth rate. However, it must be kept in mind that elasticities are not independent of each other, and negative correlations may arise between them (Shea et al. 1994). In reference grass shrimp populations survival of juveniles and survival of gravid adults had the highest elasticities, each having near equal weight, and the survival of larvae had the next highest elasticity, emphasizing the importance of this parameter. The elasticity analysis indicated a shift in the elasticity pattern of CCR-exposed grass shrimp, with gravid adults having the highest elasticity value, and juvenile survival having the next highest, and both of these values were larger than their reference model counterparts. This suggests that further changes in these life-history parameters in CCR-exposed populations will have a larger effect on the population growth rate than changes in these same parameters in reference populations. Altered elasticity patterns in response to contaminant exposure have been reported in other studies, including cadmium-induced changes in two strains of

gastropod (Salice and Miller 2003), and changes in skate populations experiencing heavy fishing exploitation (Frisk et al. 2002).

The largest change in elasticity was the decrease in the elasticity of larval survival in the CCR-exposed shrimp. Similarly, Hansen et al. (1999) found that when the probability of juvenile survival was low, the elasticity of juvenile survival was reduced. Heppell et al. (1996) found that elasticity in adult survival for loggerhead sea turtles decreased as the survival rate of adults decreased. Such decreases in elasticity do not mean that these life-history stages are unimportant to population dynamics, but rather that another life-history stage has greater influence. Because elasticities are not independent of each other and must sum to one, a change in one parameter requires a compensatory change in other parameters. This may be the case in this study, where decreased larval survival, and a subsequent decrease in elasticity of this term, resulted in the survival of other life-stages becoming relatively more important to the population growth rate.

Elasticity analysis indicated that the CCR-exposed grass shrimp populations may be more sensitive than non-exposed populations to factors other than contaminants that may decrease adult survival. Factors such as predation and competition, which may regulate survival of adults in some systems, could intensify projected contaminant effects on population growth. Because adult grass shrimp are important to the trophic dynamics in many estuarine systems, combined influences of contaminants and natural factors on adult survival (and thus population growth) could have overall implications for community or ecosystem health.

Summary

This study approached the effects of coal combustion residue on grass shrimp from two distinct perspectives. CCR-exposed grass shrimp displayed increased DNA SSB, which may have contributed to the observed decrease in larval survival, and the subsequent projected population-level effects. Lee et al. (2000) and Hook and Lee (2004) found that DNA damage in early stage larval *P. pugio* affected development, and subsequently survival, after exposure to genotoxicants. Other studies have found that CCR exposure caused decreased survival in the early life stages of several species. Gillespie and Baumann (1986) found that female bluegills (*Lepomis macrochirus*) with high body burdens of selenium, from a CCR contaminated reservoir, produced larvae with edema that did not survive to the swim-up stage. At Belews Lake, North Carolina CCR exposure resulted in extensive reproductive failure in 19 out of 20 fish species, reflecting embryonic and larval mortality, and deformities in vertebrae, head, mouth, and fins of larvae and juveniles (Lemly 2002).

Although toxicants exhibit initial effects on individuals at the cellular level, severe enough effects can modify higher-order, ecological systems (Caswell 1996b). My study suggests that a combination of measured and unmeasured effects of CCR on specific life stages have the potential to exhibit population-level effects, as indicated by the models. Because populations function as individual entities within ecosystems, alteration of their size or structure by chemical toxicity may have functional and structural ecosystem-wide consequences (Lin et al. 2005). Thus, decreased population sizes such as those projected in my models could have effects on the larger community.

The disposal of coal combustion residue is largely unregulated. Many aquatic-disposal basins are connected to local waterways, and thus the effects of CCR exposure can also emerge down-stream. Nearby terrestrial systems can also be affected by CCR through contamination of soil and groundwater, and by reduced fitness of terrestrial or semi-terrestrial organisms that use the contaminated aquatic sites for feeding or reproducing (such as amphibians and some reptiles). Therefore, effective management of these disposal sites must include assessments of the localized sources and less directly affected habitats (e.g., Kuhn et al. 2002). Further studies of sublethal endpoints, as well as studies to assess the potential population-level effects of CCR exposure to other species are necessary for regulation of CCR disposal strategies.

Future directions

The results of this work pose interesting questions for future studies. Induction of oxidative stress may be examined more closely by measuring oxidative parameters, such as glutathione, individually as opposed to the total antioxidant potential test conducted here. As many of the contaminants in CCR are metals, it may be useful to examine if chronic CCR exposure would lead to any changes in metallothionein. The Comet assay may also be used to dissect out the pathways of DNA damage, i.e. how much of the damage was oxidative.

It would be interesting to determine if there is a difference in biomarker expression based upon duration of exposure to CCR. For example, determining whether DNA damage occurs during the larval stage may provide insights into the mechanisms behind the decrease in larval survival and metamorphosis. A factorial laboratory

exposure design would also be useful in further determining which exposure routes are likely causing the trace element accumulation seen in grass shrimp. As well, multi-generational studies would provide insights into maternal transfer of contaminants (Roe et al. 2004) and potentially heritable DNA alterations, either of which may affect reproductive fitness.

A next step would be to examine the chronic effects of CCR through a dose-dependent exposure. Changing the proportion of CCR in sediments would allow the dose-dependent effects on grass shrimp vital rates to be examined. These changes could be further examined by running additional matrix population models. This may allow for recommendations to be made for the management of CCR.

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