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The Effect of Dietary Exposure to Coal Ash Contaminants within Food Ration on Growth and Reproduction in *Daphnia magna*

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Abstract: Coal ash contains numerous contaminants and is the focus of regulatory actions and risk assessments due to environmental spills. We exposed *Daphnia magna* to a gradient of coal ash contamination under high and low food rations to assess the sublethal effects of dietary exposures. Whereas exposure to contaminants resulted in significant reductions in growth and reproduction in daphnids, low, environmentally relevant food rations had a much greater effect on these endpoints. *Environ Toxicol Chem* 2020;00:1–10. © 2020 SETAC

Keywords: *Daphnia magna*; Coal ash; Dietary exposure; Multiple stressors; Food ration

INTRODUCTION

Coal ash, the residual from coal combustion, has been the focus of regulatory actions and ecological risk assessments due to a number of recent environmental spills (Lemly and Skorupa 2012; Mathews et al. 2014; Lemly 2015). Although some coal ash can be recycled for use in different products and materials (concrete, structural fill, gypsum wallboard, etc.), it contains elevated concentrations of a number of trace metals that, if released into the environment, can adversely affect aquatic ecosystems. Although toxicity standards and bioaccumulation models most often consider individual contaminants, coal ash spills, like most other environmental spills, consist of a mixture of contaminants. The constituents of these mixtures can interact in complex ways, potentially affecting the toxicity and bioavailability of each of the contaminants in the mixture.

Bioaccumulation of many metals in aquatic organisms is largely a function of dietary exposure, with dissolved concentrations being poor predictors of their bioaccumulation and toxic effects (Stewart et al. 2004). Many coal ash–associated metals are essential micronutrients at low concentrations (e.g., zinc, selenium [Se], copper), but become toxic at elevated concentrations. Others (e.g., mercury [Hg], cadmium, arsenic) are only toxic, having no known biological function. Among coal ash–associated contaminants, Se and Hg are of particular interest because they are recognized to biomagnify in aquatic food webs, becoming increasingly concentrated from the base of the aquatic food chain to fish (Mathews and Fisher 2008a, 2008b) such that regulatory guidelines for these 2 metals include fish tissue concentrations for the protection of human and ecological health (US Environmental Protection Agency 2001, 2016).

Ecological risk assessments often rely on short-term toxicity tests that involve exposure to aqueous contaminants (Stewart and Konetsky 1998; US Environmental Protection Agency 2002; Sherrard et al. 2015). These tests may not be sensitive enough to detect sublethal impacts of contaminants deriving from exposure to Se or Hg that may occur through chronic dietary exposure. Indeed, dietary exposure to metals can elicit sublethal effects at much lower concentrations than aqueous exposure (Hook and Fisher 2001a). Sublethal effects (e.g., declines in growth, reproduction) can have impacts on populations over multiple generations that can lead to significant underestimations of risk if ignored.

Even in the absence of other stressors, resource availability (e.g., food quality and quantity) can directly affect an

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organism's growth and reproduction. Resources are often limited in natural systems and may even be reduced in contaminated habitats, but food rations recommended in standard toxicity tests are often hundreds of times higher than environmentally relevant concentrations, outside of algal blooms, which can significantly affect how organisms cope with exposure to a given stressor (Stevenson et al. 2017). Several laboratory toxicity tests suggest that nutritional or energy deficits resulting from resource limitations can increase an organism's sensitivity to pollutants (Chandini 1988a, 1988b; Hopkins et al. 2002; Conley et al. 2011). Evidence from both laboratory and field studies suggests that food quality and quantity can also affect contaminant bioaccumulation, especially for contaminants that are efficiently assimilated from the diet (Pickhardt et al. 2002; Karimi et al. 2007, 2010; Chen et al. 2008).

We examined the effect of food ration and exposure to coal ash contaminants in the freshwater crustacean *Daphnia magna*. We exposed individual daphnids, through their diet, to a gradient of coal ash concentrations under high (0.1 mg C/daphnid/d) or low (0.01 mg C/daphnid/d) food rations. We followed the survival, growth, and reproduction of individuals under the different treatments and examined the implications for populations. Our results are relevant to the broader evaluation of the environmental impacts of coal combustion wastes in aquatic ecosystems.

MATERIALS AND METHODS

Algae/ash exposure

Coal ash was air-dried and homogenized, and 10 samples were analyzed for trace metals (Method SW846-6010C; US Environmental Protection Agency 2007c), mercury (Method SW846-7471; US Environmental Protection Agency 2007b), and uranium and rubidium (Method SW846-6020A; US Environmental Protection Agency 2007a) at 3 different contract laboratories, Frontier Geosciences (Vancouver, BC, Canada), Galbraith (Knoxville, TN, USA), and RJ Lee (Monroeville, PA, USA). Average dry weight concentrations are presented in Table 1. Six samples of homogenized, air-dried ash were analyzed for total carbon content using a LECO TruMac CN Analyzer (Nelson and Sommers 1996). Axenic clonal cultures of the chlorophyte *Chlamydomonas reinhardtii* (UTEX 2243) were grown on a 14:10-h light:dark cycle at 25 ± 0.5 °C in sterile-filtered (0.2 μm) WC medium (Guillard 1975) prior to experiments. Throughout the experiments, algal cultures were handled aseptically, and all glassware used for experiments was put through a rigorous acid washing protocol and rinsed 3 times with deionized water before drying.

TABLE 1: Concentrations of coal ash constituents (dry weight; mean ± 1 SD; $n = 30$ for all elements except carbon for which $n = 6$)

Element	Mean		SD	Unit
Aluminum	4.44	±	1.09	μg/g
Antimony	11.85	±	3.83	μg/g
Arsenic	31.59	±	1.54	μg/g
Barium	0.14	±	0.02	μg/g
Beryllium	10.53	±	1.07	μg/g
Boron	160.41	±	33.69	μg/g
Cadmium	10.53	±	1.07	μg/g
Calcium	3.01	±	0.30	μg/g
Carbon	5.94	±	0.08	%
Chromium	65.31	±	26.07	μg/g
Cobalt	32.32	±	11.65	μg/g
Copper	76.90	±	21.80	μg/g
Iron	2.20	±	0.51	μg/g
Lead	22.37	±	7.36	μg/g
Lithium	22.44	±	1.43	μg/g
Magnesium	0.66	±	0.09	μg/g
Manganese	87.37	±	19.65	μg/g
Mercury	117.53	±	6.73	ng/g
Molybdenum	10.53	±	1.07	μg/g
Nickel	47.73	±	13.13	μg/g
Potassium	0.56	±	0.21	μg/g
Rubidium	21.49	±	6.63	μg/g
Selenium	11.14	±	2.17	μg/g
Silver	8.61	±	3.14	μg/g
Sodium	69.72	±	202.28	μg/g
Strontium	686.07	±	112.82	μg/g
Thallium	11.14	±	2.17	μg/g
Thorium	33.26	±	13.57	μg/g
Titanium	0.26	±	0.07	μg/g
Uranium	5.76	±	0.68	μg/g
Vanadium	143.33	±	33.09	μg/g
Zinc	64.43	±	16.03	μg/g

SD = standard deviation.

A minimum of 1 wk prior to coal ash additions, phytoplankton cells were cultured in WC medium without ethylenediaminetetraacetic acid. To 4 separate 125-mL algal culture flasks, 0, 42.9, 214.3, and 428.6 mg of coal ash were added to create the 4 different treatments: No ash (control), low ash, medium ash, and high ash. These treatments were selected to correspond to nominal concentrations of 0, 0.2, 1.0, and 2.0 nM Hg, respectively. Phytoplankton cells were exposed to coal ash for 4 h before feeding to daphnid grazers. Previous studies have shown that this amount of time is sufficient for significant uptake of many of the metals found in coal ash in phytoplankton, by both active and passive accumulation (e.g., Liu et al. 2002; Obata et al. 2004; Xu and Wang 2004; Pickhardt and Fisher 2007). We allowed cells (and coal ash particles) to sink during the exposure period, and only cells in suspension were recounted via hemocytometer to determine volumes to add for feeding to *Daphnia*.

Daphnia exposure

Daphnia magna were obtained from Aquatic Biosystems and were maintained in 30% dilute mineral water medium (US Environmental Protection Agency 2002) at 20 °C under a 14:10-h light:dark cycle at Lakeland University (Plymouth, WI, USA). To ensure that all experimental *D. magna* were clonal, a single neonate (F1 generation) was extracted and placed into media until gravid. Second-generation neonates from the original F1 *D. magna* were placed into 1 of 8 treatments (Table 2; $n = 8$) of 45 mL dilute mineral water in 50-mL Falcon tubes at a food ration of either 10 000 cells/mL (low food) or 50 000 cells/mL

TABLE 2: Standardized mean differences (Cohen's *d* values) and associated confidence intervals (CI) comparing the effect of dietary coal ash exposure on average survival within 2 food rations[a]

Food ration	Compared with no ash at that food ration	Average survival (d)	Cohen's *d*	CI
High food	No ash	32		
	Low ash	32	NA	[NA NA]
	Medium ash	32	NA	[NA NA]
	High ash	32	NA	[NA NA]
Low food	No ash	24.5	–76[b]	[–1.78 0.25]
	Low ash	17	–0.5	[–1.5 0.5]
	Medium ash	28.25	0.3	[–0.68 1.29]
	High ash	32	0.76	[–0.25 1.78]

[a] Confidence intervals (CIs) that include 0 indicate nonstatistical significance ($p > 0.05$), and CIs that do not include 0 indicate statistical significance ($p < 0.05$). All individuals at the high food ration lived until the end of the experiment, so a difference between the coal ash treatments at this food ration could not be calculated.

[b] Comparing high food no ash with low food no ash treatments.

NA = not available.

(high food) of *C. reinhardtii* (freshwater green algae) exposed to coal ash as noted in the *Algae/ash exposure* section. Daphnids were moved to new feeding tubes using glass pipettes. Neonates were quantified daily. Media was changed every 48 h, and daphnids were fed a new ration of appropriately ash exposed food at either 10 000 or 50 000 cells/mL. Because the food rations used in our study were lower than standard regulatory protocols for toxicity testing, standard test acceptability criteria would be inappropriate to evaluate the results of this investigatory study. However, all measures were taken to ensure that phytoplankton cultures were axenic, and that the *Daphnia* neonates were clonal from the same brood mother, as previously described. There was 100% survival in the high food no ash treatment, which served as control, no ephippia were produced, and neonates were all <24 h old at start of the test, meeting most standard toxicity test requirements.

Four flasks containing increasing concentrations of coal ash and a control without ash exposure (0, 42.9, 214.3, and 428.6 mg) were used to treat the algal stocks. From each flask, 2 food rations: low and high (5 times the food concentration of low) were used to feed the *Daphnia*. Therefore, the 3 ash treatments (low, medium and high) were not consistent across food treatments. For example, because the high food ration was 5 times the food concentration of the low food ration, the low ash treatment at high food was 5 times the ash concentration of the low ash treatment at low food. For our analyses we focused on comparing ash treatments within food rations rather than between them.

Life history analysis

Daphnia magna were monitored over 30 d every day, and their survivorship, clutch initiation, clutch sizes, number of molts before first clutch, total reproductive output, final length, birth date, and date of expiration were recorded.

We estimated the intrinsic rate of increase (*r*) of a daphnid population at each food ration and coal ash contaminant level using the Euler equation:

$$1 = \sum_{x=0}^{k} e^{-rx} l(x) b(x)$$

Calculation of survivorship of individuals from birth to age *x*, *l*(*x*), and the fecundity schedule (average number of offspring born/d to a female of age *x*), *b*(*x*) from the start ($x = 0$) until the end of the experiment, and $x = k$ were as outlined in Stevenson et al. (2017) and are briefly described here. We calculated *r* from our entire data set by numerically solving the Euler equation using the uniroot function in R Statistical Software (Ver 3.6.1). To estimate the uncertainty around these values, we resampled the data with replacement 1000 times using a bootstrapping technique and recalculated values for *r* based on resampled data sets.

Cohen's d *calculations*

Standardized mean differences (Cohen's *d* values) and their associated confidence intervals (Nakagawa and Cuthill 2007) were calculated using R Statistical Software (Ver 3.6.3) using the compute.es package. We used standardized mean difference calculations for our statistical analyses because effect sizes and similar metrics emphasize the magnitude of the effect of interest rather than solely whether the effect is statistically significantly different based on *p* values.

RESULTS AND DISCUSSION

Food limitation

Less food led to lower survival rates (Figure 1 and Table 2), slower growth/smaller maximum sizes (Figure 2 and Table 3), and a delay in reproduction/less reproduction (Figure 3 and Tables 4 and 5) in daphnids. These results are consistent with previous studies showing that the amount of food a daphnid eats directly affects survival, growth, and reproduction (Bradley et al. 1991; Preuss et al. 2009; Kooijman 2010). Guidelines for standard toxicity tests recommend food rations (i.e., 0.1–0.2 mg C/daphnid/d) that are often hundreds of times higher than daphnids are likely to see in the environment, outside of an algal bloom (McCauley and Murdoch 1987; Murdoch et al. 1998). Under these ideal, constant conditions daphnids can produce hundreds of eggs, but under limiting food conditions they can slow or halt reproduction. Previous studies have shown that effects on daphnid survival, growth, and reproduction are observed at much lower toxicant concentrations when daphnids are provided with food rations that are more environmentally relevant than those used in standard toxicity tests (Coors et al. 2004; Stevenson et al. 2017), a finding that has broad implications for extrapolating results from toxicity tests to environmental risk assessments. The food rations used in the present study, 10 000 cells/mL/daphnid/d and 50 000 cells/mL/daphnid/d correspond to approximately

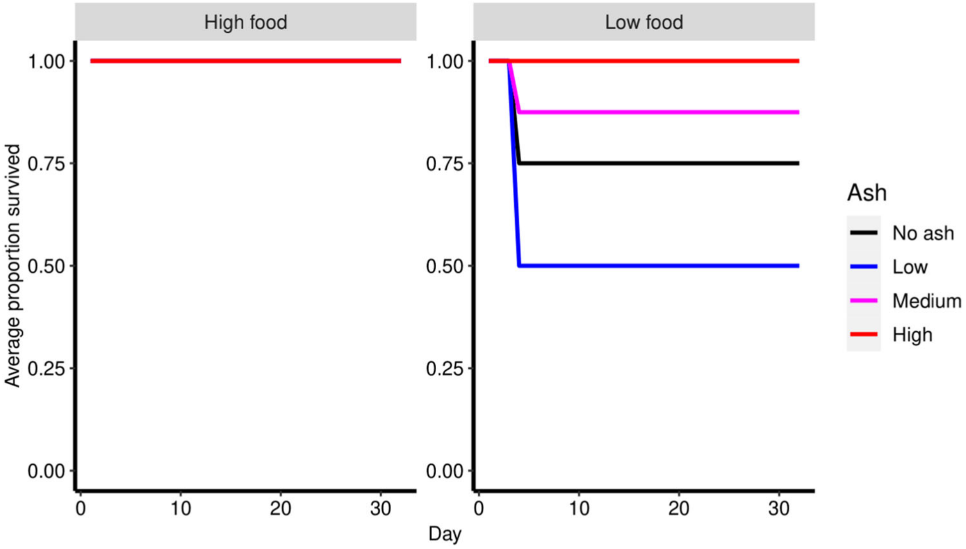

FIGURE 1: Through time survival of individual *Daphnia* ($n = 8$/treatment at start of experiment) fed high (0.1 mg C/daphnid/d) and low (0.01 mg C/daphnid/d) food rations. Prior to feeding daphnid food, *Chlamydomonas reinhardtii* cells were exposed for 4 h to ash at 4 levels: no ash, low, medium, and high ash (gray, blue, pink, and red lines, respectively; see *Materials and Methods* section for more details on ash concentrations).

0.01 and 0.1 mg C/daphnid/d, respectively, and represent an environmentally relevant range of food concentrations likely to be encountered by daphnids in temperate lakes (McCauley and Murdoch 1987; Murdoch et al. 1998; Stevenson et al. 2017).

Exposure to coal ash contaminants did not affect survival in daphnids exposed to high food rations but did affect survival in those exposed to low food rations (Figure 1 and Table 2). In the high food rations, 100% survival was observed at all coal ash concentrations for the entire 30 d of the exposure period. In the low food rations, mortality was seen within the first 5 d in the 3 lowest ash contaminant treatments (i.e., no ash, low, medium) leading to lower average survival over the 30 d of the experiment (Figure 1). These effects were most marked in the low ash contaminant treatment (Table 2).

Previous studies have shown that lower algal densities can lead to higher bioconcentration of metals on a per cell basis (Pickhardt et al. 2002; Karimi et al. 2007), leading to more efficient trophic transfer (Karimi et al. 2010) and greater toxicity (Conley et al. 2011) of metals under low food conditions. Although we were not able to obtain bioaccumulation data on individual daphnids in the present study, our results suggest that daphnids given low food rations may have obtained limiting nutrients from the coal ash. This could include either unburnt carbon, which can make up 1 to 10% of coal ash by weight (Yao et al. 2020), or trace metals, many of which (most

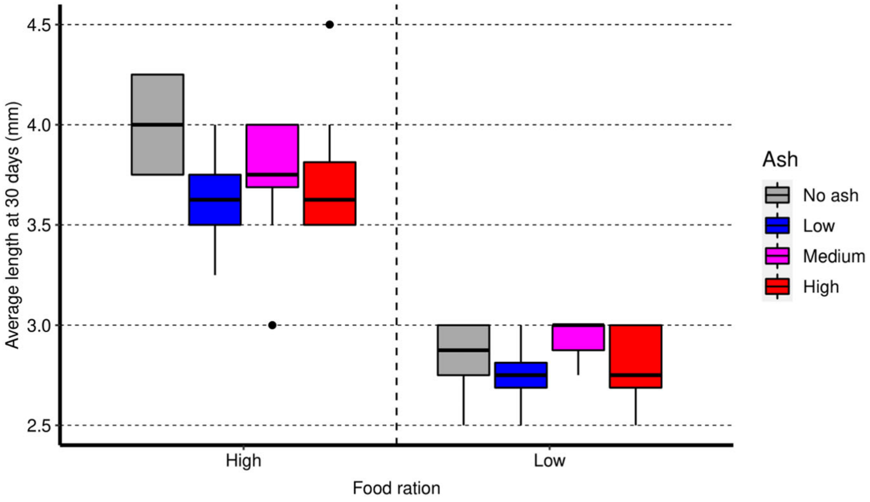

FIGURE 2: Average (±standard error) length of individual *Daphnia* ($n = 8$/treatment at start of experiment) fed high (0.1 mg C/daphnid/d) and low (0.01 mg C/daphnid/d) food rations. Prior to feeding daphnid food, *Chlamydomonas reinhardtii* cells were exposed for 3 h to ash at 4 levels: no ash, low, medium, and high ash (gray, blue, pink, and red boxes, respectively; see *Materials and Methods* section for more details on ash concentrations).

TABLE 3: Standardized mean differences (Cohen's *d* values) and associated confidence intervals (CI) comparing the effect of dietary coal ash exposure on average final length within 2 food rations[a]

Food ration	Compared with no ash treatment at that food ration	Average final length (mm)	Cohen's *d*	CI
High food	No ash	4		
	Low ash	3.62	−1.62	[−2.75 −0.49]*
	Medium ash	3.72	−0.97	[−2 0.07]
	High ash	3.75	−0.84	[−1.86 0.19]
Low food	No ash	2.83	−5.29[b]	[−7.52 −3.06]*
	Low ash	2.75	−0.41	[−1.69 0.87]
	Medium ash	2.93	0.58	[−0.53 1.69]
	High ash	2.78	−0.25	[−1.31 0.81]

[a] Confidence intervals (CIs) that include 0 indicate nonstatistical significance ($p > 0.05$), and CIs that do not include 0 indicate statistical significance ($p < 0.05$, denoted by an asterisk).

[b] Comparing high food no ash with low food no ash treatments.

*Statistically significant ($p < 0.05$).

notably Se) are micronutrients at low concentrations but become toxic at elevated concentrations. The addition of higher ash concentrations supplied the daphnids with higher concentrations of these essential elements, which may have led to the higher survival rates (Figure 1 and Table 2) and higher long-term growth rates (Figure 4 and Table 6) seen in the low food treatments.

From an energetic standpoint, the food an organism ingests can be used for somatic maintenance, growth, and/or reproduction. When food is limiting, somatic maintenance accounts for a larger proportion of an organism's total energy budget, because unlike growth and reproduction, maintenance costs often cannot be reduced (Kooijman 2010). Furthermore, exposure to contaminants can increase maintenance costs thorough various detoxification methods (Fan et al. 2009; Kwok et al. 2009). Exposure to ash contaminants was a stressor to daphnids in the high food treatment, because even the lowest

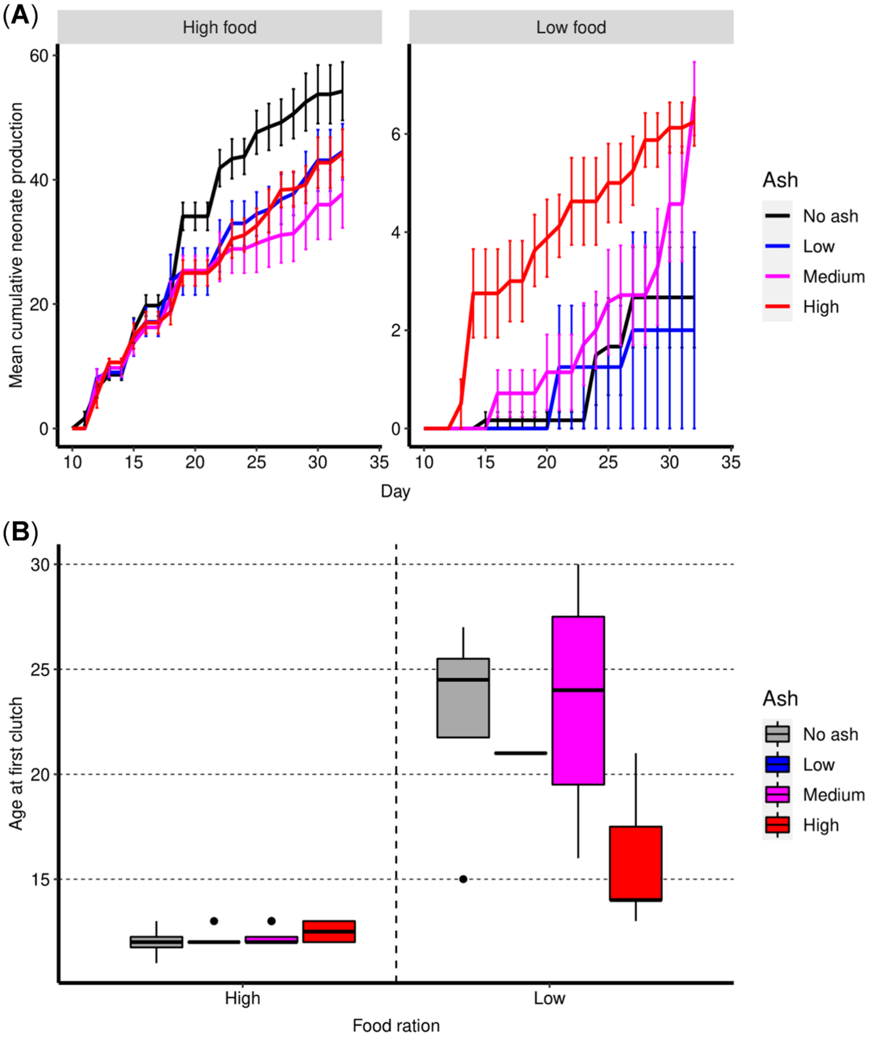

FIGURE 3: Impacts of dietary coal ash contaminant exposure on reproduction. Mean (±standard error) cumulative number of neonates produced on a given day (**A**) and the average age of the daphnia when the first clutch appeared (**B**). In (**B**), the middle line represents the median, the lower and upper hinges display the first and third quartiles, and single data points outside this range represent outliers of the data. Individual *Daphnia* ($n = 8$/treatment at start of experiment) were fed high (0.1 mg C/daphnid/d) and low (0.01 mg C/daphnid/d) food rations. Prior to feeding *Daphnia*, *Chlamydomonas reinhardtii* cells were exposed for 3 h to ash at 4 levels: no ash, low, medium, and high ash (gray, blue, pink, and red bars, respectively; see *Materials and Methods* section for ash concentrations).

TABLE 4: Standardized mean differences (Cohen's *d* values) and associated confidence intervals (CI) comparing the effect of dietary coal ash exposure on average cumulative neonate production within 2 food rations[a]

Food ration	Compared with no ash at that food ration	Cumulative neonate production	Cohen's *d*	CI
High food	No ash	54.25		
	Low ash	44.5	–0.75	[–1.76 0.26]
	Medium ash	37.75	–1.15	[–2.2 –0.09]*
	High ash	44.25	–0.82	[–1.84 0.2]
Low food	No ash	2.67	–5.04[b]	[–7.19 –2.89]*
	Low ash	2	–0.21	[–1.48 1.06]
	Medium ash	6.71	1.81	[0.52 3.11]*
	High ash	6.25	1.85	[0.59 3.12]*

[a] Confidence Intervals (CIs) that include 0 indicate nonstatistical significance ($p > 0.05$), and CIs that do not include 0 indicate statistical significance ($p < 0.05$, denoted by an asterisk).
[b] Comparing high food no ash with low food no ash treatments.
*Statistically significant ($p < 0.05$).

ash concentration resulted in smaller daphnids than the no ash treatment (Figure 2). In the low food treatment, however, daphnids had slower growth rates and lower maximal sizes than in the high food treatment regardless of ash contaminant exposure, suggesting that food limitation was a greater stressor than ash exposure in these treatments (Figure 2).

Similar to patterns seen for growth, food limitation had a greater effect on reproduction in daphnids than exposure to ash, with the mean number of cumulative neonates produced per daphnid ranging from 38 to 54 in the high food treatment and from 2 to 7 in the low food treatment (Figure 3A and Table 4); on average, individuals produced 10 times more neonates in the high food treatments across all exposures compared with low food (high food individuals produced 45.2 offspring on average compared with 4.4 offspring from low food *Daphnia*). We did not measure the size of the neonates—it is possible that the *Daphnia* fed lower food rations produced fewer but larger neonates in response to decreased food availability, as has been found in other studies (Coors et al. 2004). Also, similar to results seen for survival, exposure to ash may have provided micronutrients that were limiting in the low food treatment. *Daphnia* fed low food rations that did not contain ash were more likely to not reproduce at all than those fed low food rations with ash exposure. In the low food treatment, 2 of the surviving 6 no ash, 3 of the 4 surviving low ash-exposed, and 1 of the 7 surviving medium ash-exposed *Daphnia* never reproduced; all other individuals across the remaining treatments reproduced at least once. Interestingly, these *Daphnia* that did not reproduce were above the size threshold for maturity. Size at maturity is food-dependent for *D. magna*, but a commonly used threshold for *Daphnia* fed low food rations is approximately 1.7 to 1.8 mm (Ananthasubramaniam et al. 2015), and the *Daphnia* that did not reproduce in our study were all larger than 2.5 mm at the end of the experiment. Exposure to ash contaminants resulted in increases in reproduction in daphnids in the low food treatment but led to declines in reproduction in daphnids fed high food rations (Figure 3A). In addition to affecting overall neonate production, low food rations led to a delay in the age at first reproduction, and have been found to delay maturity in *D. magna* (Ananthasubramaniam 2015), and reduce mean clutch sizes. The daphnids in the high food treatment reached maturity on the same day (Figure 3B and Table 5), regardless of ash concentration, but had smaller clutch sizes with ash contaminant exposure, which led to the overall decrease in average neonate production compared with no ash exposure (Figure 3A and Table 4).

TABLE 5: Standardized mean differences (Cohen's *d* values) and associated confidence intervals (CIs) comparing the effect of dietary coal ash exposure on average age at maturity within 2 food rations[a]

Food ration	Compared with no ash at that food ration	Average age at maturity (d)	Cohen's *d*	CI
High food	No ash	12		
	Low ash	12.12	0.21	[–0.77 1.19]
	Medium ash	12.25	0.4	[–0.59 1.39]
	High ash	12.5	0.76	[–0.25 1.78]
Low food	No ash	22.75	3.61[b]	[1.73 5.49]*
	Low ash	21	NA[c]	[NA NA][c]
	Medium ash	23.43	0.12	[–1.11 1.35]
	High ash	15.75	–1.84	[–3.25 –0.43]*

[a] Confidence intervals (CIs) that include 0 indicate nonstatistical significance ($p > 0.05$), and CIs that do not include 0 indicate statistical significance ($p < 0.05$, denoted by an asterisk).
[b] Comparing high food no ash with low food no ash treatments.
[c] Unable to calculate Cohen's *d* or CIs because only one individual reproduced at this ash concentration and food ration, making it impossible to calculate the standard deviation (necessary for Cohen's *d*).
*Statistically significant ($p < 0.05$).
NA = not available.

Exposure to coal ash contaminants

The toxicity of coal ash–associated contaminants has been the subject of numerous studies because of recent spills and discharges. There has been a range of reported effects from coal ash exposure, in both laboratory and field studies. Perhaps the most well-known case of poisoning due to exposure to coal ash waste is Belews Lake in North Carolina (USA), where teratogenic, reproductive, and developmental effects in fish, birds, and other wildlife were attributed to Se exposure (Adams et al. 1998; Lemly 2002). However, at the site of the world's largest coal ash spill at the Tennessee Valley Authority's Kingston Fossil Plant in Tennessee (USA), no such effects have been observed up to 10 yr after the spill (Pracheil et al. 2016). The differences in observed effects are likely due to differences in exposure conditions. Contaminant concentrations in coal ash can vary based on the provenance of the coal, the temperature at which it was combusted, whether it is fly ash or bottom ash, and how it was stored (Ruhl et al. 2009). Furthermore, water chemistry, ecology, and hydrology can affect ecosystem responses to contaminants. For example, studies have shown that aqueous Se speciation, food web differences, and residence time affect Se bioaccumulation and toxicity (Conley et al. 2013), with lentic systems being much more susceptible to impacts from Se than lotic systems (Rowe et al. 2002).

Furthermore, interactions between contaminants can also affect the severity of effects of exposure to coal ash

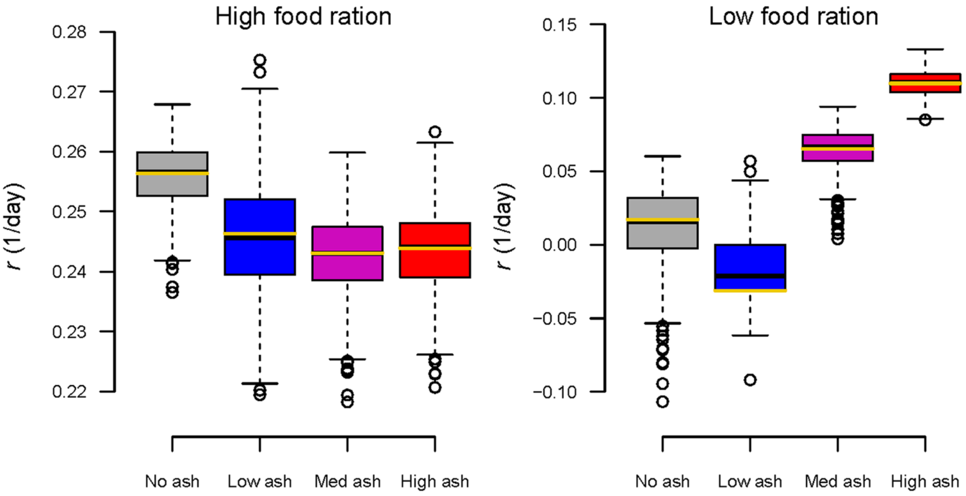

FIGURE 4: Comparison of specific population growth rate (*r*, yellow lines) and the bootstrapped values to estimate variability for all treatments between the 2 food rations. Yellow lines indicate the value of *r* for the entire data set, and the boxplots display the range of bootstrapped values (resampled 1000 times/treatment with replacement). The box of the boxplot is approximately the first to the third quartile of the bootstrapped data, the dark black line represents the median of the bootstrapped values, the whiskers extend to the most extreme data points that are no more than 1.5 times the interquartile range, and the circles represent outliers beyond this range.

contaminants in aquatic systems. For example, Hg and Se are associated with coal combustion and coal ash respectively (Sackett et al. 2010; Mathews et al. 2014) and have become textbook examples of metal interactions in biological and environmental sciences, because they can complex with one another, mediating the toxicity of the other metal in mammals and aquatic organisms (Cuvin-Aralar and Furness 1991; Deonarine et al. 2013). Although it is not possible to test each combination of contaminant and stressor on every organism, it is important for risk assessments to design tests that are appropriate for the context (e.g., site specific) and to develop new frameworks that are not stressor specific. Developing methods to incorporate suborganismal processes (i.e., omics data) into risk assessments will be a critical next step toward addressing the challenge of multiple stressors in aquatic ecosystems (Ormerod et al. 2010; Murphy et al. 2018), because molecular responses to stressors are often common to multiple stressors and conserved among taxa.

Although the relevance of dietary exposure to toxicants is increasingly recognized (Hook and Fisher 2001b; Wang 2011), most standard toxicological studies expose organisms to aqueous toxicants, which can significantly underestimate risk. In the present study dietary exposure to relatively low ash concentrations resulted in sublethal effects in *D. magna* (Figures 2 and 3), but previous laboratory studies have reported no toxicity in *Ceriodaphnia dubia* or in fathead minnows (*Pimephales promelas*) exposed to much higher coal ash concentrations through aqueous or sediment exposure (Greeley et al. 2014; Sherrard et al. 2015). Because most toxicants need to be taken up into the body (e.g., through respiration, absorption, ingestion, etc.) before they can elicit effects, direct exposure (i.e., aqueous, sediment) studies with contaminants that are primarily accumulated through dietary exposure may underestimate toxicity.

Although exposure to coal ash contaminants did not cause significant mortality in the present study (Table 2), we did observe significant effects of coal ash exposure on growth (Table 3) and reproduction (Tables 4 and 5) between some coal ash concentrations and their no ash controls. Reductions in growth and fecundity can have implications for the fitness of populations and although ecological risk assessments initially followed the framework of human health risk assessments that focus on the protection of individuals, it is increasingly recognized that protecting populations, communities, and ecosystems is often a more relevant goal for ecological risk assessments (Forbes and Calow 2001).

To assess what the implications of our results mean for population dynamics, we calculated the specific population growth rate (*r*) of *Daphnia* at high and low food rations at all ash concentrations based on survival and reproduction data (Figure 4). If *r* is >0, the population will grow exponentially. If *r* is <0, the population will decline to extinction. Coal ash exposure

TABLE 6: Standardized mean differences (Cohen's *d* values) and associated confidence intervals (CIs) comparing the effect of dietary coal ash exposure on average long-term growth rate (*r* value) within food rations[a]

Food ration	Compared with no ash treatment at that food ration	*r* value (1/d)	Cohen's *d*	CI
High food	No ash	0.26		
	Low ash	0.25	−1.4	[−1.49 −1.3]*
	Medium ash	0.24	−2.22	[−2.33 −2.11]*
	High ash	0.24	−2.08	[−2.19 −1.97]*
Low food	No ash	0.01	−12.29[a]	[−12.68 −11.90]*
	Low ash	−0.02	−1.02	[−1.13 −0.92]*
	Medium ash	0.06	2.44	[2.32 2.56]*
	High ash	0.11	4.82	[4.65 4.99]*

[a] Confidence intervals (CIs) that include 0 indicate nonstatistical significance ($p > 0.05$), and CIs that do not include 0 indicate statistical significance ($p < 0.05$, denoted by an asterisk).
*Statistically significant ($p < 0.05$).

significantly altered r values at every exposure concentration and food ration (Table 6). Interestingly, coal ash did not always decrease population growth rate compared with the no ash treatments (Table 6). The r values calculated for all ash concentrations were >0 at the high food ration, suggesting that although coal ash did have sublethal effects at the concentrations examined, these were not enough to have significant effects on population dynamics (although they are significantly lower than the no ash growth rate; Table 6). Interestingly, ash exposures at the high food rations did appear to increase the variability in predicted population responses, as indicated by the larger error bars from the resamples r values for coal ash exposures compared with no ash controls at the high food ration (see the *Life history analysis* section in *Materials and Methods* for details behind these analyses). Also, whereas the daphnids at the no and low ash treatments were on the brink of extinction (resampled data sets produced some negative r values for no and low ash treatments), exposure to ash increased population growth rates in *Daphnia* in the low food treatment with much less variability in resampled r values (Figure 4). It is important to note that these calculations do not take into account differences in offspring size; previous studies have found that *Daphnia* can adapt to lower food conditions by producing fewer but larger offspring, temporarily avoiding local extinctions (Cleuvers et al. 1997; Coors et al. 2004; Gergs et al. 2014).

In addition to trace metals, coal ash contains unburnt carbon, which was approximately 6% by mass in the ash used in the present study (Table 1). Because it was not possible to completely separate algal cells from coal ash particles, we estimate that the medium and high ash exposures may have added up to 0.02 and 0.04 mg C, respectively, at each transfer, which, if bioavailable, may represent a significant increase in a daphnid's carbon ration for individuals at the low food ration (0.01 mg C/daphnid/d). We estimate that the medium and high ash exposures added 0.08 and 0.16 ppb Se at each transfer. Previous research has shown that *Daphnia* populations cannot be maintained in media that contains <0.1 ppb Se (Keating and Dagbusan 1984; Lam and Wang 2008). In the high food ration treatments, exposure to coal ash contaminants had negative effects on growth and reproduction at all ash concentrations examined (Figures 2 and 3). At a food ration just 5 times less, the addition of coal ash improved survival, growth, and reproduction in daphnids (Figures 1, 2, and 3). Although these observed effects can be caused by many factors, most of the elements associated with coal ash are not essential. Of the essential elements associated with coal ash, Se has the smallest window between nutrient and toxicant status (Stewart et al. 2010). If the increase in survival and reproduction in the medium and high ash treatments was due to Se in daphnids fed low food rations, this may have implications for developing risk assessments for coal ash.

CONCLUSIONS AND IMPLICATIONS

The present study focused on the effect of dietary exposure to coal ash contaminants on *Daphnia* given a range of environmentally relevant food rations. Although we found significant effects of coal ash exposure on daphnid growth and reproduction, these effects were not severe enough to threaten the population at the concentrations of ash considered in our study as estimated by specific population growth rates (r). However, the concentrations of ash used in the present study were significantly lower than those used in previous studies (Greeley et al. 2014; Sherrard et al. 2015) and could be significantly lower than those that organisms would be exposed to in a coal ash–contaminated environment. Food limitation had a much greater impact on the viability of *Daphnia* populations, and under food-limiting conditions, exposure to coal ash contaminants had a positive effect on population viability because survival and reproduction increased with increasing ash concentration in the low food treatment. Although exposure to coal ash contaminants at low food rations appears to decrease risks to *Daphnia* populations, other research suggests that low food rations could lead to higher Se bioaccumulation rates in invertebrates (Conley et al. 2011), which could lead to greater risks to higher trophic levels such as fish. Future work should address the cascading effects of exposure to contaminants under environmentally realistic food concentrations.

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