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The effects of arsenic speciation on accumulation and toxicity of dietborne arsenic exposures to rainbow trout.

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Abstract

The effects on juvenile rainbow trout survival, growth, food consumption, and food conversion efficiency from dietborne exposures to inorganic arsenic (arsenite, arsenate) and to the organoarsenicals monomethylarsonate (MMA), dimethylarsinate (DMA), and arsenobetaine (AsB) were investigated in two experiments: (1) a 28-d exposure using a live diet of oligochaete worms separately exposed via water to these five arsenic compounds and (2) a 56-d exposure using a pellet diet prepared from commercial fish food to which arsenite, MMA, or DMA were added. In the live diet experiment, the degree to which worms could be contaminated with the organoarsenicals was limited by toxicity to the worms and other experimental constraints, so that their toxicity relative to inorganic arsenic could not be fully established, but AsB was concluded to have low toxicity, consistent with published results for mammals. For the pellet diet experiment, MMA and DMA were found to be at least an order of magnitude less toxic than inorganic As on the basis of concentration in the diet, as well as much less toxic on the basis of accumulation in the fish. The need to consider speciation in aquatic risk assessments for arsenic was further demonstrated by tissue analyses of three macroinvertebrate species from a mining-impacted stream, which showed large variations in both total arsenic and the relative amounts of inorganic and organic arsenic. Additionally, although effects of arsenic on trout appear to be well correlated with inorganic arsenic, worms were found to be more sensitive to waterborne DMA than to inorganic arsenic, showing that low toxicity of organoarsenicals cannot be assumed for all aquatic organisms. Various difficulties in evaluating and applying studies on dietborne exposures and fish growth are also discussed.

Keywords

Dietborne Exposure; Rainbow Trout; Growth; Arsenic Speciation; Accumulation

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Data availability – Data are available through the USEPA Environmental Dataset Gateway (<https://edq.epa.gov/metadata/catalog/main/home.page>) or by request from the corresponding author (erickson.russell@epa.gov)

1. INTRODUCTION

Dietborne arsenic (As) has been shown to reduce the growth of salmonid fish fed either pellet diets amended with inorganic As salts (Oladimeji et al., 1979; Cockell and Hilton, 1988; Cockell et al., 1991; Pedlar et al., 2002) or live diets of oligochaete worms contaminated by separate water exposure to inorganic As salts (Erickson et al., 2010, 2011). These effects occurred at As concentrations similar to those in benthic organisms which were exposed to mining-contaminated sediments and which also caused reduced growth when fed to salmonid fish (Woodward et al., 1994, 1995; Hansen et al., 2004). Additionally, it has been demonstrated that concentrations of inorganic As in water insufficient to cause direct toxicity to rainbow trout (*Oncorhynchus mykiss*) can sufficiently contaminate oligochaete worms to reduce the growth of trout fed a diet of these worms (Erickson et al., 2011), reinforcing the need to consider dietborne exposure in aquatic risk assessments for As.

The above findings concern exposure to inorganic As, but As occurs in a variety of biologically-derived organic species that differ in their relative toxicities and that vary in their relative accumulations across different organisms and exposure conditions. The occurrence (primarily in marine organisms) and the toxicity (primarily in mammals and mammalian cell lines) of organoarsenicals have been addressed in numerous studies, which have been the subject of various reviews. Reviews by Cullen and Reimer (1989), Francesconi and Kuehnelt (2003), Sharma and Sohn (2009), Caumette et al. (2012), Rahman et al. (2012), Kumari et al. (2017) and Popowich et al. (2016) form the basis for most of the following summary.

In surface waters, dissolved arsenic usually is primarily arsenate (iAsV), but appreciable concentrations of arsenite (iAsIII), monomethylarsonate (MMA), and dimethylarsinate (DMA) can occur as well. Upon uptake into organisms, the reducing environment in tissues will result in conversion of iAsV to iAsIII, which can be methylated to MMA, DMA, and trimethylarsenoxide (TMAO), and converted to more complex organics such as arsenobetaine (AsB), arsenocholine (AsC), and various arsenosugars (typically having two or three methyl groups and a ribose-containing moiety). These organoarsenicals, especially AsB, are usually substantially less toxic than iAsIII. Trivalent analogs of MMA and DMA have been reported to occur in organisms and to have greater cytotoxicity than iAsIII, as has thio-DMA, a metabolite of some arsenosugars (Moe et al. 2016). The trivalent analog of MMA has also been demonstrated to be highly toxic in water exposures to *Daphnia pulex* (Shaw et al. 2007); however, significant exposure to such reduced species in aquatic ecosystems is uncertain. Arsenolipids (e.g., arsenate-containing analogs of phospholipids) have also been reported. Therefore, dietborne arsenic exposures can involve a complex mixture of different As species with different toxicities.

In marine systems, arsenosugars usually constitute most of the total As in primary producers and some herbivores. AsB is usually the dominant species in fish and macroinvertebrates, commonly constituting more than 90% of the total As, although inorganic arsenic can be important when its environmental concentrations are high (e.g., Fattorini et al. 2004, Boyle et al. 2008). Accumulations in marine fish and shellfish can be very high (>50 mg As/kg

dwt), but risks from human consumption are limited due to very low toxicity and rapid excretion of AsB in mammals. Although this low mammalian toxicity and the high accumulations of AsB in marine fish argue for low toxicity to fish as well, this has not been directly tested.

Although less studied, reported arsenic speciation in freshwater organisms differs substantially from, and is more variable than, in marine organisms. Inorganic arsenic has often been reported to dominate arsenic extracted from freshwater phytoplankton and zooplankton, although DMA and arsenosugars are major species in some cases (see review by Caumette and Reimer, 2012). In freshwater macroinvertebrates, inorganic arsenic and arsenosugars are often major species, with AsB usually being relatively minor, especially in contaminated systems (Devesa et al., 2002; Soeroes et al., 2005a; Schaeffer et al., 2006; Miyashita et al., 2009; Williams et al., 2009; Lai et al., 2012). In wild freshwater fish, AsB is often the dominant species, especially in salmonids, but also is often relatively minor, with inorganic forms, simple methylated species, and/or arsenosugars being more important (Lawrence et al., 1986; Shiomi et al., 1995; Šlejkovec et al., 2004; Schaeffer et al., 2006; de Rosemund et al., 2008; Miyashita et al., 2009; Ciardullo et al., 2010). Some reports of the importance of AsB in cultured fish can be attributed to the AsB in commercial feed (Shiomi et al., 1995; Soeroes et al. 2005b). Also, in many of these studies, low extraction efficiencies for certain organisms and systems make the total arsenic speciation uncertain, although the unmeasured arsenic species are not likely to include AsB, which typically has a high extraction efficiency.

Aquatic risk assessments for As therefore require knowledge not only of the total concentration of As in water and diet, but also the concentrations and the relative toxicities of the various As species. The As speciation for exposures in natural systems is uncertain, especially in freshwater, and there is virtually no information on the relative toxicities of different organoarsenicals to any aquatic animal. Using pellet diets amended with As compounds, Cockell et al. (1988) reported juvenile rainbow trout growth to be reduced 31% by iAsIII at 180 µg As/g diet and 77% by iAsV at 137 µg As/g diet, whereas diets amended with 1500 µg As/g diet of DMA or arsanilic acid had no effect on fish growth, indicating these organoarsenicals were more than 10-fold less toxic than the inorganic As compounds for this fish species and endpoint.

The present study was conducted to further define the relative toxicities of different As species on rainbow trout growth when presented in live and pellet diets. To provide more information on arsenic species likely to occur in natural trout diets, As speciation also was measured in aquatic insects from a mining-contaminated stream (Panther Creek, ID, USA). Some difficulties and uncertainties in evaluating and applying dietborne toxicity studies are also addressed.

2. METHODS AND MATERIALS

2.1. Study design

Two experiments were conducted regarding dietborne effects of organoarsenicals on the growth and survival of rainbow trout, one using live diets of oligochaete worms (*Lumbriculus variegatus*) and the other using pellet diets.

The “live diet experiment” was patterned after our previous work (Erickson et al. 2010, 2011) and consisted of 28-d exposures of juvenile trout (40-d post-hatch at start) to worms contaminated via waterborne exposures to the As compounds and concentrations specified in Table 1. This approach to contaminating diets was intended to provide more biologically-realistic incorporation of different As species into the diet.

For iAsV_{Live} and iAsIII_{Live}, the selected exposure concentrations (Table 1) were expected to produce As accumulations of $\approx 50 \mu\text{g As/g dry weight (dwt)}$ in the worms and to reduce growth of the trout by $\approx 30\%$ based on previous work (Erickson et al. 2010). Although these water exposures to the worms are exceptionally high, the accumulations are realistic for invertebrates from contaminated systems, with the high water concentrations being needed to achieve these accumulations within a limited exposure duration and in the absence of sediment/dietary exposures to the worms.

For the organoarsenicals, preliminary water exposures to worms were conducted to assess the maximum water concentrations tolerated by the worms and the As accumulations expected at these concentrations. DMA was toxic to the worms at 1 mg As/L , but the selected exposure concentration for DMA_{Live} of 0.5 mg As/L (Table 1) was still expected to result in an accumulation of $\approx 50 \mu\text{g As/g dwt worm}$, comparable to that expected in the iAsV_{Live} and iAsIII_{Live} exposures. For MMA, a 30 mg As/L water exposure was tolerated by the worms in these preliminary exposures, and resulted in accumulation of $210 \mu\text{g As/g dwt}$; based on this, a water exposure of 20 mg As/L (Table 1) was chosen for MMA_{Live}, with an expected accumulation $>100 \mu\text{g As/g dwt}$. For AsB_{Live}, the selected exposure of 10 mg/L (Table 1) caused an accumulation of $110 \mu\text{g As/g dwt}$ in worms. However, during the actual live diet experiment, 10 mg As/L of AsB was not tolerated by the worms, requiring exposures to be reduced (Table 1). This toxicity of AsB to worms is likely attributable to conversion of AsB to DMA in the exposure system (see Section 3.1.1).

Although the live diet experiment provided evidence for less toxicity from MMA and AsB than from iAsIII and iAsV (see Section 3.1), organoarsenical toxicity to the worms and other experimental constraints precluded testing dietary exposures high enough to adequately document relative toxicities, especially the order-of-magnitude differences reported by Cockell et al. (1988). Therefore, a second experiment (“pellet diet experiment”) evaluated growth and survival of juvenile ($\approx 100 \text{ d post-hatch at start}$) rainbow trout in 56-d exposures to pellet diets containing the As compounds and concentrations specified in Table 1. This experiment used older fish that would accept pellets large enough to avoid too-rapid leaching of arsenic (see Section 2.4.2).

For the pellet experiment, the two iAsIII treatment concentrations (Table 1) were chosen to produce moderate-to-large reductions in rainbow trout growth based on Erickson et al. (2010, 2011). For MMA and DMA, concentrations were set equal to the higher treatment for iAsIII and to 4-fold higher than this. Because previous work indicated As-contaminated diets can affect consumption rates (Erickson et al., 2010, 2011), this experiment also included 4 different control diet rations (Table 1) to document how conversion efficiency varies with consumption, and thereby support better interpretation of any As effects. In addition to experimental capacity reasons, AsB was not included in this experiment because the live diet experiment had sufficiently confirmed expectations for its low toxicity, and iAsV was not tested because iAsIII was considered the more relevant inorganic form for dietborne exposure.

Due to a freezer failure, diet and fish samples from the pellet diet experiment were compromised and could not be used to determine As speciation. To provide new samples for this purpose, an ancillary exposure was conducted using the same general procedures, but which had only the highest control feeding rate (Table 1), only the lower treatment for each As compound (Table 1), half the exposure duration (28 d), and only 2 replicates rather than 4 replicates; these ancillary treatments are designated iAsIII-60a, MMA-120a, and DMA-120a. This ancillary experiment was used only to provide analytical samples for arsenic speciation that would be informative of the primary pellet experiment, which was the sole source for actual effects information and for total As concentrations in both the diet and fish.

2.2. Test organisms and chemicals

Rainbow trout were obtained as eggs from the U.S. Geological Survey Upper Midwest Environmental Sciences Center (LaCrosse, WI, USA). Fish were hatched and maintained at the U.S. Environmental Protection Agency Mid-Continent Ecology Division (MED, Duluth, MN, USA) in flow-through filtered and aerated Lake Superior water (LSW) at 11°C. This water has a typical pH of ≈ 7.5 , a hardness of ≈ 48 mg CaCO_3/L , and an alkalinity of ≈ 44 mg CaCO_3/L . Worms were cultured at MED in LSW on a substrate of shredded paper towels and a diet of Tetramin® flake fish food; concentrations of arsenic and various metals in these culture worms were documented to be well below effect levels by Erickson et al. (2010).

For the live diet experiment, fish were fed starter trout chow (Silver Cup Fish Feed, Nelson and Sons, Murray, UT, USA, minimum protein 52%, minimum lipid 16%) *ad libitum* beginning at swim-up. After 2 wk, this was supplemented with non-contaminated worms from culture to introduce them to this source of food, with the experiment starting 2 wk later. For the pellet diet experiment, fish were also initially fed the starter trout chow *ad libitum* and then fed a progression of commercial trout chow pellets of increasing size at a daily rate of 0.02–0.03 g food/g wwt fish. One wk before the experiment start, fish were switched to the control pellet diet (see section 2.3.2) at a rate of 0.03 g food/g wwt fish/d.

Chemicals used for iAsV, iAsIII, MMA, DMA, and AsB exposures were, respectively, $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ (CAS#10048-95-0, purity >99%, Sigma-Aldrich, St. Louis, MO, USA), NaAsO_2 (CAS#7784-46-5, purity >99.0%, Fluka Analytical/Sigma-Aldrich Chemie, Buchs,

Switzerland), $\text{NaCH}_3\text{AsO}_3 \cdot 6\text{H}_2\text{O}$ (CAS#2163-80-6, purity 98.5%, Chem Service, West Chester, PA, USA), $\text{C}_2\text{H}_7\text{AsO}_2$ (CAS#75-60-5, purity ~98%, Sigma-Aldrich), and $\text{C}_5\text{H}_{11}\text{AsO}_2$ (CAS#64436-13-1, purity 95%, Fluka). For total As analyses, analytical standards and spiking solutions were prepared using certified As standard CGAS1 from Inorganic Ventures (Lakewood, NJ, USA). A separate certified standard, AccuTrace Reference Standard AA03-N-1 (AccuStandard, Inc., New Haven, CT, USA), was used to prepare independent quality control solutions. For inductively coupled plasma mass spectrometry (ICP/MS) analyses, standards were prepared in 10% methanol using high-performance liquid chromatography (HPLC) grade methanol (Fisher Scientific, Pittsburgh, PA, USA) and high-purity de-ionized water (Super-Q, Millipore Corporation, Bedford, MA, USA). Trace metal grade concentration HNO_3 (Fisher Scientific) was used in the preparation of HPLC eluents. All other chemicals used in analyses were of reagent grade.

2.3. Diet preparation and characterization

2.3.1. Live diet experiment.—The standard approach for creating the live diets was to expose worms under static conditions for 7 d to the treatment solutions specified in Table 1. After separating worms from the culture substrate, ~3 g wwt were placed in 300-ml beakers containing 250 ml of the treatment solution and held (with aeration) in a 23°C water bath. One or two beakers for each treatment were prepared each day as needed to provide sufficient masses of worms for the fish rations and analytical samples 7 d later.

On one occasion for the iAsIII treatment, two occasions for DMA, and multiple occasions for AsB, poor condition of the batch of worms earmarked for feeding fish on a day necessitated deviations from this standard approach, resulting in some worms being used with less than 7 d exposure; these cases are detailed in the Supplemental Information. However, although these shorter exposures to the worms would reduce As accumulations, measured concentrations in diet form the basis for the fish exposure characterizations.

Temperature was measured daily in one beaker from each treatment, and averaged 23.1°C (range 22.4°C to 23.7°C across all individual measurements). Dissolved oxygen was measured once per week in two beakers from each treatment, and pH and ammonia were measured once per week in one beaker from each treatment. The pH averaged 7.6 (range 7.3–7.8). Excluding measurements in one beaker that was discarded due to poor organism condition, ammonia ranged from 3 to 12 mg N/L and dissolved oxygen from 2.8 mg O_2 /L to 7.9 mg O_2 /L. Analytical methods were as described in Erickson et al. (2010, 2011).

Water samples for total As analysis were collected at the beginning and end of the 7-d exposure for 4 batches of worms from each treatment. Samples (0.3 g wwt) of the worms being fed to the fish were collected for total As analysis three times weekly from each treatment (with one-third of the treatments being sampled in duplicate), and additional 0.1 g wwt samples were collected once weekly for determination of As speciation (with one-sixth of the treatments being sampled in duplicate). Samples of 20 worms were collected once per week from each treatment (with one-sixth of the treatments being sampled in duplicate) for analysis of percent dry weight and lipid content as described in Erickson et al. (2010, 2011). Some of these scheduled samples were not collected due to the available mass of worms

being insufficient, and some samples were not analyzed as warranted by data uncertainty; the actual number of samples analyzed are provided in Section 3.

2.3.2. Pellet diet experiment.—Pellet diets were prepared in two batches for the primary pellet diet experiment, one prior to the start of the exposure and the other midway through, and a separate batch was prepared for the ancillary pellet exposure. Trout starter chow was combined in a 5:4 ratio (by weight) with filtered LSW containing the concentration of test chemical needed to achieve the nominal dietborne As concentrations specified in Table 1, and then processed into a dough using a kitchen mixer; gelatin was not used. This dough was extruded through a pasta-making mixer attachment with ≈ 2 mm diameter round holes, and the extruded strands were placed on aluminum foil and air-dried. Dried strands were broken into smaller pieces by hand and then ground with a wooden pestle against a #10 stainless steel sieve nested over #16 and #25 sieves. Particles retained by the #25 sieve were used, having a size similar to a commercial #2 crumble, with average pellet weights for different batch varying from 2.0–2.3 mg and moisture content varying from 8–13%. Food was refrigerated until used.

For the primary pellet experiment, duplicate 1 g samples of each diet were collected and frozen for total As analysis at the start, middle, and end of the use period for each batch of food. At the same times, duplicate 0.2 g samples were collected for measurement of the fresh weight per pellet and of fraction dry weight. For the ancillary experiment, samples of the diets were collected and analyzed for both total As and As speciation.

2.4. Fish exposures.

2.4.1. Live diet experiment.—Juvenile rainbow trout were exposed at a nominal temperature of 12°C in 24 rectangular glass chambers (6 treatments, 4 replicate chambers per treatment) holding 4.6 L and receiving 1.6 L/h of LSW. At the start of the experiment, 8 fish were randomly assigned to each experimental chamber. A separate sample of 20 fish were individually weighed for initial wet weight (mean=184 mg/fish, standard deviation=43 mg/fish), and an aggregate dry weight for this sample was determined (28.8 mg/fish, 15.7% dry weight).

Each chamber received a once-daily ration of worms. Before each feeding, worms left from the previous day were removed, blotted, and weighed to document uneaten food. Worm exposure beakers earmarked for that day's use were removed from the water bath and their contents were transferred to a shallow tray (pooling worms from any replicate beakers). The mass of worms was stirred gently to separate live worms from detritus, and clumps of live worms were then transferred to clean filter paper in a Buchner funnel, applying a gentle vacuum to remove excess water. Rations were then allocated by wet weight. The ration for the first week was set at 35 mg wwt/fish/d, corresponding to $\approx 20\%$ wwt worms/initial wwt fish/d ($\approx 3.5\%$ dwt worms/wwt fish/d). Based on control fish growth rates from previous experiments (Erickson et al. 2010, 2011) this ration was increased to 55 mg wwt/fish/d for the second week, and was scheduled to be 80 mg wwt/fish/d and 120 mg wwt/fish/d, respectively, for the third and fourth weeks. However, due to incomplete consumption of the worms and limited food supplies for some treatments, the third week ration was reduced

after 2 d (day 16 of the experiment) to 72 mg wwt/fish/d and the fourth week ration was only increased to 90 mg wwt/fish/d. Eight times during the experiment (5% of the total treatment/day combinations), a treatment had insufficient worm mass to provide the full rations, so that the ration for that treatment was either reduced or supplemented with culture worms on that day; further details are provided in the Supplemental Information, but the aggregate impacts on total consumption and As exposure were <1%.

Temperature was measured daily in one chamber for each treatment and all chambers weekly, averaging 12.2°C (range 11.7°C to 12.6°C across all individual measurements). Dissolved oxygen and pH were measured three times per week and ammonia once per week in one chamber for each treatment. Dissolved oxygen ranged from 8.4 mg O₂/L to 10.3 mg O₂/L, pH ranged from 7.2 to 7.5, and ammonia was not detectible (<1 mg N/L). Hardness and alkalinity were measured once per week in the influent water and averaged 47 mg CaCO₃/L and 44 mg CaCO₃/L respectively. Analytical methods were as described in Erickson et al. (2010, 2011).

Fish survival was monitored daily. Dead fish were removed, blotted and wet-weighted, and individually frozen at -20°C in a 6-ml, acid-cleaned HDPE vial. After a mortality, the total ration of worms to a chamber was reduced proportionately to the number of fish. At the end of the experiment, fish were euthanized; the standard lengths (nearest mm) and wet weights (nearest 0.1 mg) of each fish were measured; 3 randomly selected fish from each chamber were individually frozen for total As analyses as needed; and the remaining fish were combined into a single sample for percent dry weight determination.

2.4.2. Pellet diet experiment.—Juvenile rainbow trout were exposed at a nominal temperature of 12°C in 40 rectangular glass chambers (10 treatments, 4 replicate chambers per treatment) holding 13 L and receiving 17 L/h of LSW (for the ancillary exposure, 8 chambers were used for 2 replicates of each of the 4 treatments). Eighteen days before the start of the experiment, 5 fish were randomly assigned to each experimental chamber and their rearing continued as described in section 2.3.2. One day before the start of exposure (24 h after feeding so that weights were with an empty gastrointestinal tract), the aggregate unblotted wet weight of fish in each chamber was determined by netting the fish and transferring them to a tared beaker of water. These starting unblotted wet weights for each chamber (1.7–2.8 g/fish) were used to calculate the initial daily ration for the chamber, using the feeding rates specified in Table 1. A separate sample of 10 fish from the cohort of fish used to stock the experimental chambers was collected at the same time; the aggregate unblotted wet weight of this sample was measured by the same method, and the blotted wet weight of each individual fish and the aggregate dry weight of the total sample were then measured, thereby providing an unblotted:blotted wet weight ratio and a fraction dry weight for the fish at test start.

During the experiment, the daily ration for each chamber was divided into two feedings ≈4 hours apart. Prior to each feeding, chambers were siphoned to remove feces, and the standpipe of each chamber was raised to temporarily interrupt the overflow of water (and thus prevent loss of floating food pellets). The pellets were then added to the chamber and feeding was allowed for 10 min, after which any uneaten pellets were removed by siphon

and counted, and the standpipe restored to its original position. Once per week, water samples were also taken 30 min after the feeding period from one chamber for each treatment to document the extent of As contamination of the water from the food, which was always $<3 \mu\text{g As/L}$.

The short (10 min) feeding time was used to limit leaching of As from the pellets, which would reduce the dietborne As exposure from any pellets not consumed quickly. Separate leaching experiments for each diet (0.1 g of pellets in 15 ml of LSW) showed losses of As from the pellets of 10–12% after 2 min and 20–28% after 10 min for iAsIII, and 18–21% after 2 min and 41–44% after 10 min for MMA and DMA (losses were not appreciably different between diet concentrations and between MMA and DMA). The time-averaged concentrations in the pellets over 10 min were estimated to be 85% of nominal for iAsIII, 72% of nominal for MMA, and 71% of nominal for DMA. Because most consumption during the actual experiment was in the first few minutes, the average exposures during the experiment would be higher than these average concentrations, closer to the 2 min results but to an uncertain degree. Because of this uncertainty, results reported in Section 3 are not corrected for this loss, so that actual effect concentrations are likely 10–20% smaller than reported. This loss of arsenic is in contrast to the good retention of As in worms reported by Erickson et al. (2010), warranting consideration of this leaching issue not only in the interpretation of the current experiment, but also of other dietborne exposure studies with aquatic organisms, and the effect on leaching of using gelatin as a binding agent in food pellets (Oladimeji et al., 1984; Cockell et al., 1988, 1991).

Fish survival was monitored daily. Dead organisms were removed, blotted and wet-weighted, and individually frozen at -20°C in a 6-ml, acid-cleaned HDPE vial for possible analyses. Although the chambers were covered, 6 fish escaped during the exposure (see Section 2.7.2). Rations to the chamber following death and other loss were reduced in proportion to the change in number of fish. The unblotted aggregate fish wet weights in each chamber were determined every two weeks by the same method as at test start, and rations for the subsequent two weeks were recalculated based on these weights and target ration (Table 1). Fish were not fed during the last 40 hours of the experiment and the experiment was terminated at 56 d (28 d for the ancillary exposure) with a final determination of the aggregate unblotted weight for each chamber. After euthanasia, the standard length (nearest mm) and blotted wet weight (nearest 0.1 mg) of each fish was measured. One randomly chosen fish from each chamber was selected for dry weight analysis and the rest were individually frozen for analysis of total As analysis and As speciation.

Temperature was measured daily in one chamber for each treatment and all chambers weekly, averaging 11.8°C (range 11.2°C to 12.3°C across all individual measurements). Dissolved oxygen and pH were measured three times per week and ammonia once every two weeks in one chamber for each treatment. Dissolved oxygen ranged from $7.6 \text{ mg O}_2/\text{L}$ to $10.7 \text{ mg O}_2/\text{L}$, pH ranged from 7.1 to 7.6, and ammonia was not detectible ($<1 \text{ mg N/L}$). Hardness and alkalinity were measured once every two weeks in the influent water and averaged $46 \text{ mg CaCO}_3/\text{L}$ and $43 \text{ mg CaCO}_3/\text{L}$ respectively.

2.5. Field Invertebrate Samples

The practical significance of the results from the laboratory experiments of the present study will depend on the nature of As contamination in natural systems to which these results might be applied. To provide some information on this issue, total As and As speciation were analyzed in benthic invertebrates from Panther Creek (Idaho, US), a stream with a history of contamination by mining activities (Mebane et al. 2015). These samples, provided by Christopher Mebane of the U. S. Geological Service (Boise, ID, USA), included 3 or 4 samples (1–3 g) each of *Arctopysche grandis* (a filter-feeding caddisfly), *Hesperoperla pacifica* (a predatory stonefly), and *Pteronarcys californica* (a detritus-grazing stonefly). Although rinsed, these organisms were not allowed to void their gut contents, which should better reflect dietborne exposures to their consumers.

2.6. Arsenic Analyses

As described in Erickson et al. (2010, 2011), total As in water samples was determined by flameless atomic absorption spectrophotometry (FAAS) and total As in diet and fish were determined by FAAS after microwave-assisted acid digestion. For the smaller fish of the live diet experiment, digestion was of the whole fish as in Erickson et al. (2011); for the larger fish of the pellet diet experiment, each fish was homogenized with an equal weight of water, and 1 g of this homogenate was digested. Due to matrix effects, FAAS analysis of diet samples used standard curves prepared in digestates of uncontaminated diet (with calculated concentrations adjusted for the estimated As in the uncontaminated diet); this was possible because all diet samples were of nearly the same size so that matrix effects would be similar across samples. This was also true for the analyses of fish homogenate from the pellet diet experiment, but for the live diet experiment, FAAS analyses of fish digestates were by standard addition due to the different weights of fish that were digested. Blanks were <5% of the concentrations in samples from As treatments (<20% for control food and fish samples) and relative standard deviations of replicate analyses were <5%. Spiked samples and QA standards were within 10% of nominal.

Arsenic speciation in the worms was determined by methods described in Erickson et al. (2011; also provided in the Supplemental Information). Methods for As speciation in food pellets, fish, and stream invertebrates were slightly modified from that for the worms and are also described in the Supplemental Information. It was assumed that the relative concentrations of each As species in the food and fish from the ancillary pellet exposure applied to the primary pellet diet experiment, so that the concentrations of the As species in the food and fish in the primary experiment could be estimated by multiplying their total As concentrations by the relative concentrations from the ancillary experiment.

2.7. Data analysis

2.7.1 Fish Growth Metrics—For evaluating growth, chambers were used as the experimental unit, because using fixed rations per chamber (not feeding *ad libitum*) eliminated any statistical independence of individual fish. The primary metric evaluated was the cumulative percentage growth (G) on a dry weight basis:

$$G = 100 \times \left(\frac{W_F - W_I}{W_I} \right)$$

(1)

where W_i and W_F are, respectively, the initial and final total dry weights of fish for the experimental period.

However, to more thoroughly describe how growth varies with treatment and time, three other metrics were examined. The average growth rate (R) over the experimental period, as percentage dry weight gain/day, was calculated as:

$$R = 100 \times \ln(W_F/W_I)/t$$

(2)

where t is the duration of the period.

Effects on growth rates can result either from reduced food consumption or reduced conversion of consumed food to biomass, and we previously reported dietborne As can affect both processes (Erickson et al. 2010). Because fish were fed roughly in proportion to weight, the average weight-normalized food consumption (C) over an experimental period, as a percentage per day on a dry weight basis, was calculated as:

$$C = 100 \times \left(\frac{(F - U)/t}{(W_F - W_I)/\ln(W_F/W_I)} \right)$$

(3)

where F is the total dry weight of food provided to the chamber over the period and U is the total dry weight of food uneaten. The denominator in this expression is the average dry weight of fish in the chamber over the period, assuming constant growth rate during the period.

Finally, the percent efficiency of conversion of food to fish weight (E) was calculated simply as:

$$E = 100 \times \frac{W_F - W_I}{F - U}$$

(4)

2.7.2 Treatment of lost fish in calculations—Equations 1–4 can only be rigorously applied if there are no deaths or other losses of fish during the experimental period. For example, if a fish dies during the experiment, this creates uncertainty in the initial weight of the survivors for growth calculations, even if both the dead fish's weight and the initial aggregate fish weight are known. Furthermore, how should food consumption for the chamber be adjusted to account for what was eaten by the fish prior to death? If a fish escapes and has an unknown weight, the situation is even less clear.

In some cases, reasonable assumptions might allow some approximations of growth metrics using Equations 1–4, but to avoid ambiguities, replicates with lost fish for an experimental period were simply excluded from statistical analyses, although measured weights are still reported. This resulted in excluding from the statistical analyses 2 of 24 replicates (8%) in the live diet experiment and 10 of 160 replicate×period combinations (6%) in the pellet diet experiment; this low level of censoring did not materially affect conclusions regarding effects.

2.7.3 Weight conversions—For the live diet experiment, the ration provided to each chamber and the amount of uneaten worms were measured daily as wet weight of worms. These measured wet weights were multiplied by the average fraction dry weight for worms across all treatments and sampling times to calculate F and U. In the pellet diet experiment, the ration to each chamber was measured daily as weight of air-dried pellets and the uneaten food as the number of pellets. The weight of uneaten food was estimated as the product of the number of uneaten pellets and the average air-dried pellet weight. These air-dried weights were multiplied by the fraction dry weight for the respective batch of pellets to calculate F and U.

Dry weight conversion for the fish was more complicated because fraction dry weight varies with fish size (Erickson et al. 2010) and only a subset of the fish were allocated to dry weight determinations (some of which were not conducted due to the freezer failure). To provide an estimator for the dry weight of an individual fish or group of fish, the fraction dry weights that were measured for the present study and by Erickson et al. (2010) were subject to least-squares linear regression analysis (Sigmaplot Version 13, Systat Software, San Jose, CA, USA) of fraction dry weight versus wet weight (Figure 1), yielding the estimator:

$$\text{fraction dry weight} = 0.1934 + 0.0809 \times \log_{10}(\text{wet weight(g)})$$

(5)

There is some suggestion of curvature at the lower end of this relationship (Figure 1); however, only the fish at the start of the live diet experiment would be in this size range, and their fraction dry weight was measured and did not need this estimator.

For the pellet diet experiment, an analysis of the aggregate unblotted wet weights versus blotted wet weights was also conducted using the measurements on the set of 10 fish measured at test start and the final measurements of weight from each chamber. The ratio of the total blotted wet weights of these sets of fish to their aggregate unblotted wet weights varied from 0.957 to 1.001, with a mean of 0.976% (n=40) (not shown). Although this range was small, there was a weak but statistically significant ($p<0.01$) relationship of this ratio with the average wet weight of the fish, so a linear regression for these data was also conducted:

$$\frac{\text{unblotted wet weight}}{\text{blotted wet weight}} = 0.955 + 0.00332 \times \text{unblotted wet weight (g)}$$

(6)

This resulted in adjusting measured aggregate unblotted wet weights by a factor of 0.960 to 0.985 to produce estimated blotted wet weights, although this only had a maximum impact of 1.5% on growth rate estimates compared to using the overall average blotted:unblotted wet weight.

Consequently, for the live diet experiment, the initial fish dry weight was based on the separate sample of fish collected at test start and the final fish dry weights were estimated by applying Equation 5 to the measured final wet weights. For the pellet diet experiment, all fish dry weights were based on applying both Equations 6 and 5 to the measured unblotted wet weights.

2.7.4 Statistical analyses—Significant treatment effects on all the growth metrics over the entire duration of each experiment were tested by one-way analysis of variance (ANOVA) and by correlation coefficients between exposure and effect variables, using Statistica Version 13 (TIBCO, Palo Alto, CA, USA). For the ANOVA analyses, Dunnett's test was used to evaluate significant effects of specific treatments relative to the control; these Dunnett's results are reported for informational purposes regarding possible treatment effects even when overall ANOVA results are not significant.

In the pellet diet experiment, values for growth rate, food consumption, and conversion efficiency are reported for each biweekly period to show certain trends, but were not separately subject to ANOVAs because the values in different periods are for the same fish and therefore not statistically independent. Due to the limited number of concentrations tested for each chemical, exposure-effects curves could not be computed for the present study, but effects on growth rate in the present study were compared to exposure-effects relationships for the more extensive data for inorganic As from Erickson et al. (2010, 2011).

3. RESULTS AND DISCUSSION

3.1 Live diet experiment

3.1.1 Exposure characterization—Measured As accumulations, fraction dry weights, and lipid contents for the worms in the live diet experiment are reported in Table 2. For lipid content and fraction dry weight, ANOVA indicated no significant differences among the treatments ($p>0.3$), the smaller values for AsB_{Live} being within data variability. Therefore, the overall average fraction worm dry weight (0.183) was used in the calculations for worm As content, and diet quality was not considered a factor in interpreting effects on fish growth.

Observed As accumulations in worms were less than expected (Section 2.1 vs. Table 2) and were also quite variable (relative standard deviations in Table 2). For the AsB_{Live} treatment, changes to the exposure concentration and duration (Section 2.4.1, Supplemental Information) contributed to this result, but another source of both variability and reduced accumulation for all treatments likely was the static exposures in the present study versus the flow-through exposures of Erickson et al. (2010, 2011). This difference could alter bioavailability via changes in water chemistry (e.g., dissolved organic carbon) and/or the degree to which water penetrates the clumps of aggregated worms. Depletion of As in exposure water did not contribute to the lower accumulation because measurements of total As in the water indicated concentrations were within 10% of nominal throughout the exposures.

Figure 2 summarizes As speciation in the worms, including the As species that were quantified and the fraction of total As in the sample for which speciation was not determined, either due to not being extracted or not being detected in the HPLC-ICP/MS analysis of the extract. The sum of the measured As species as a fraction of the total measured As in the worms was 74% for iAsIII_{Live}, 86% for iAsV_{Live}, 75% for MMA_{Live}, 91 % for DMA_{Live}, and 92% for AsB_{Live}. For the MMA_{Live} and DMA_{Live} treatments, the measured As species in the worms were entirely as those species. For the iAsIII_{Live} treatment, a small fraction of the measured As species (9%) was iAsV, possibly due to oxidation during sample storage and processing. For the iAsV_{Live} treatment, about 75% of measured As species was iAsIII, presumably due to the reducing environment within the organisms. This preponderance of iAsIII within worms whether exposure is to iAsIII or iAsV was previously noted by Erickson et al. (2011) as likely being responsible for iAsIII and iAsV exposures having similar toxicity on the basis of total As concentration in the worms. For the AsB_{Live} treatment, there was considerable but variable conversion to DMA, this being only 20% of the measured As species in the day 1 sample, but averaging 74% for

the later samples (Figure 2). Given the observed sensitivity of worms to DMA in water, this conversion of AsB to DMA likely accounts for the observed toxicity of the AsB_{Live} treatment to the worms, as well as the variability of this toxicity. For control worms (not shown), only 15% of the As was extracted and identified, comprising 40% inorganic As, 15% DMA, and 45% AsB, but the total As in these worms (<2 µg As/g dwt) would be of little toxicological importance.

As noted in Section 2.1, toxicity to the worms limited the DMA water concentration to which they could be exposed. Furthermore, DMA was found to be more toxic to the worms than iAsIII. Although toxicity was not quantified at each water concentration tested in the preliminary worm exposures, mortality was 100% within 3 d at a DMA concentration of 3 mg As/L and some toxicity (indicated by cloudiness in the exposure beaker) was evident at 1 mg As/L, whereas exposures of worms to 3 mg As/L as iAsIII showed no apparent effects. DMA has previously been reported to be much less toxic than inorganic As in dietborne exposures to rainbow trout (Cockell et al., 1988) and mice (Kaise et al. 1989), in waterborne exposures to cladocerans (Shaw et al., 2007; Rahman et al., 2014), and in cytotoxicity tests with human cell lines (Leffers et al., 2013; Moe et al., 2016), and we know of no prior animal studies to the contrary. The cytotoxicity of the trivalent analog of DMA has been reported to be greater than iAsIII (Moe et al. 2016), so one possibility for the observed toxicity in the present study would be a high degree of reduction of DMA within the worms.

3.1.2 Effects on fish—For each replicate chamber, Supplemental Table S1 provides fish survival, fish weights, the weights of worms fed to the fish, and the weights of worms not consumed. For each chamber without mortality, it also provides the calculated cumulative percentage growth (Equation 1), the average growth rate (Equation 2), the consumption rate (Equation 3), and the conversion efficiency (Equation 4) over the 28 d duration.

Table 3 provides the accumulated total As in the fish at test termination, and means and relative standard deviations across replicates for the four growth metrics. ANOVA indicated no significant ($p>0.3$) effects of any As species on any growth metric. In fact, the greatest growth was in the AsB_{Live} treatment, despite the high As accumulation, lower lipid content, and lower fraction dry weight in the diet for this treatment (Table 2). Despite this absence of effects, these results still provide some information on the relative toxicity of arsenic species, as follows.

Figure 3A compares growth rates observed in the present study to those from inorganic As exposures reported by Erickson et al. (2010, 2011) on the basis of As accumulation in the worms, while Figure 3B makes this comparison on the basis of As accumulation in the fish. For the iAsIII_{Live} and iAsV_{Live} treatments, because accumulations in the worms were less than their targets, the absence of growth effects for these treatments is consistent with the earlier data – at the upper end of variability for the absence of effects on the basis of accumulation in the food (Figure 3A) and very near the apparent threshold for effects on the basis of accumulation in the fish (Figure 3B).

For the DMA_{Live} treatment, As accumulations in both the diet and fish were similar to the inorganic As treatments (Figure 3). Because these accumulations were near the threshold for effects from the inorganic As treatments and because As in the food was >90% DMA (Figure 2), the absence of effects in the DMA_{Live} treatment allows the conclusion that DMA is not substantially more toxic than inorganic As on the basis of diet concentrations, but not whether it is less toxic. This illustrates a limitation for live diets in dietborne toxicity studies; although they have the advantage of the toxicant being in a more relevant matrix, toxicity to the organism used for the diet is a possibility, especially when contamination is via just water over a short time. This limitation for the DMA_{Live} treatment in large part motivated the pellet diet experiment.

For the MMA_{Live} treatment, Figure 3A shows absence of effects on growth for an As accumulation in the diet about 3-fold higher than for the highest inorganic exposure without effect and at which the previous experiments with inorganic As indicated reductions on growth should be 30% or more. However, Figure 3B shows an accumulation in the fish for MMA_{Live} similar to inorganic As and consistent with the absence of effects, indicating the apparent low MMA toxicity on the basis of diet could simply be a matter of low bioaccumulation by the fish, such low uptake also being evident in the high water concentrations needed to contaminate the worms. Although this finding that MMA in the diet is at least 3-fold less toxic than inorganic As would still be useful in assessments, especially for low MMA concentrations, further definition of MMA's relative toxicity was addressed in the pellet diet experiment using higher diet concentrations and evaluating As speciation in the fish.

For the AsB_{Live} treatment, Figure 3A also shows an absence of effects on growth for an As accumulation in the diet substantially (about 2-fold) above the highest inorganic exposure without effect and at which inorganic As effects were substantial (15–30%) in the previous experiments. In contrast to MMA, Figure 3B shows that lack of growth effects in the AsB_{Live} treatment is associated with high accumulations in the fish (20 µg As/g dwt fish), more than 4-fold higher than the apparent threshold for effects of inorganic exposures and at which inorganic exposures caused reductions in growth of over 20%.

However, as shown in Figure 2, most of the As in the AsB_{Live} diet past exposure day 9 was DMA rather than AsB. In fact, on the basis of accumulation in the diet, the AsB_{Live} treatment provides stronger evidence for dietborne toxicity of DMA being less than inorganic As than it does for AsB. This is another possible uncertainty in the live diet approach – metabolism by the diet organism altering the toxicant from the form of interest. Nevertheless, low toxicity for AsB can be inferred from these data. This is done by applying a fish:food accumulation factor for the DMA_{Live} treatment ($0.127 = 3.4 \mu\text{g As/g dwt fish} / 26.7 \mu\text{g As/g dwt food}$, Tables 2 and 3) to the concentration of DMA in the AsB_{Live} diet ($44 \mu\text{g As/g dwt} = 68\% \times 65 \mu\text{g As/g dwt}$, Figure 2, Table 2), which provides an estimated total accumulation in the fish due to the DMA in the food of $5.3 \mu\text{g As/g dwt}$. Subtracting this from the $20.3 \mu\text{g As/g dwt}$ in the fish from the AsB_{Live} treatment (Table 3) results in an accumulation in the fish attributable to AsB in the food of $15 \mu\text{g As/g dwt}$, which would be expected based on Figure 3B to reduce the growth rate by about 25%, and is about 3-fold

higher than the effects threshold for inorganic As treatments. Although not as direct as intended, this calculation supports expectations from the literature that AsB has low toxicity.

3.2 Pellet diet experiment

3.2.1 Exposure characterization—Measured As concentrations in the pellets, individual pellet weights, and pellet fraction dry weights for the pellet diet experiment are provided in Table 2. Pellet weights and fraction dry weights varied among treatments and batches, so batch-specific values were used in growth calculations. Lipid measurements of the pellets averaged 18% (range 15% to 20%, n=32) on an air-dried weight basis, consistent with manufacturer specifications, and did not vary significantly among pellet batches. With one exception, measured total As concentrations in the different batches exceed the nominal concentrations in Table 1 by 10–15%, this being expected because the measurements are on an oven-dried weight basis, whereas addition of chemical was based on the as-sold weight of the commercial fish food (with some water content). The one exception to this is for the second pellet batch for treatment iAsIII-120, for which the As concentration was less than nominal and 25% less than for the first batch; this indicates a preparation error that is considered in the interpretation of growth rates for this treatment in Section 3.2.2.

Figure 4 summarizes As speciation measured in the food pellets from the ancillary experiment, including the As that was not extracted, the identified As species in the final supernatant, and the total As in the final supernatant not accounted for by the measured species. This last number is the difference between the sum of the measured species and the total As separately measured in the supernatant; this would include any unmeasured species, but also reflects the cumulative error in such a difference. The control pellets (not shown) had minor As contamination (2.5 µg As/g dwt), of which only 40% was extractable, consisting primarily (65%) of AsB. For the iAsIII treatment, the measured species accounted for 70% of the total As. Of the As species that were measured, 88% was iAsIII and 12% was iAsV, the latter again possibly from oxidation during storage and processing. For the MMA treatment, the measured species accounted for 89% of the total As. Of the As species that were measured, 91% was MMA and 9% was iAsV from demethylation of MMA during diet preparation, storage, and/or analysis; implications of this inorganic As in the food are considered in Section 3.2.6. For the DMA treatment, the measured species accounted for 84% of the total As. Of the As species that were measured, 100% was DMA. Despite the speciation of some of the total As not being established and despite some conversions of the As species, for all treatments the pellets contained a preponderance of the intended As species.

3.2.2 Effects on fish - general—For each replicate chamber and biweekly period, Supplemental Table S2 provides fish survival, fish weights, ration weights, and estimated weights of rations not consumed for each biweekly period. For each chamber without mortality, it also provides the cumulative percentage growth (Equation 1) at both 28 and 56 days, and the estimated average growth rate (Equation 2), consumption rate (Equation 3), and conversion efficiency (Equation 4) over each biweekly period and the entire experiment. Based on this information, Table 4 lists means and relative standard deviations across

replicates for all four growth metrics over the entire experiment, as well as accumulated total As concentrations in the fish at the end of the experiment.

Figure 5 shows the means and ranges across replicates for cumulative percentage growth at both 28 and 56 days, as well as the Dunnett's significance level (p) for the difference of each treatment from the control. As expected, restricted rations reduced cumulative growth (7%, 32%, and 65% at 28 d and 9%, 31%, and 65% at 56 d for Ctrl-Lim1, Ctrl-Lim2, and Ctrl-Lim3, respectively), but these reductions were statistically significant only for Ctrl-Lim2 and Ctrl-Lim3. These growth reductions are not in strict proportion to reductions in rations, especially for Ctrl-Lim1, as further discussed in Section 3.2.3.

For the iAsIII exposures, there were substantial and exposure-dependent reductions in cumulative growth (Figure 5) compared to the control (33% at 28 d and 27% at 56 day for iAsIII-60; 68% at 28 d and 57% at 56 day for iAsIII-120), which are statistically significant, although just marginally so at 56 d for iAsIII-60. For DMA, cumulative growth was not significantly affected, being within 4% of control for both treatments at 56 d. For MMA-120, cumulative growth was also within 4% of the control, but for MMA-480 cumulative growth was reduced 11% at 28 d and 19% at 56 d. Although the overall ANOVA for MMA is not significant ($p=0.19$), further consideration of MMA-480 as an effect level (see Section 3.2.6) was warranted given the substantial, progressive declines, the marginal significance ($p=0.08$) indicated by Dunnett's test (Figure 5), and a correlation coefficient of -0.55 for cumulative percentage growth to dietborne DMA that was also marginally significant ($p=0.06$).

To further evaluate these effects of arsenic on cumulative percentage growth, the following sections will evaluate effects on growth rate, consumption rate, and conversion efficiency, organized by treatment type. To support this evaluation, Figure 6 provides individual values of these growth metrics for each two-week experimental period.

3.2.3 Effects of reduced ration—As would be expected from reduced rations, the Ctrl-Lim1, Ctrl-Lim2, and Ctrl-Lim3 treatments (Table 4) produced monotonic and substantial reductions in consumption relative to the control (7%, 23%, and 48%, respectively, at 56 d). However, because one response to reduced rations was more complete consumption of the ration, these reductions in consumption were less than the ration reductions (23%, 43%, and 67%, respectively). The average growth rate was reduced roughly in proportion to consumption - by 5% in Ctrl-Lim1, 19% in Ctrl-Lim2, and 48% in Ctrl-Lim3 (Table 4). As for cumulative growth, the small decrease in growth rate for Ctrl-Lim1 was not statistically significant (Dunnett $p=0.18$) because more complete consumption largely compensated for the reduced ration.

Because changes in growth closely tracked changes in consumption, the conversion efficiencies (Table 4) did not vary significantly among the control and the reduced ration treatments (ANOVA $p=0.6$), varying just from 35% to 37%. Therefore, any changes in conversion efficiency from the As treatments should not be ascribed to differences in consumption.

For the control treatment, the individual biweekly values for growth rate and consumption showed substantial declines with time (Figure 6A, 6B), both dropping by 35% between the 1st and 4th experimental periods. Because consumption of the control ration was not complete (Figure 6B), these decreases cannot be attributed to restricted rations, but must rather reflect increasing fish size. These decreases with time became less pronounced as rations were progressively reduced (Figure 6A, 6B), because consumption and growth became more a function of food limitations than of fish size. In contrast, conversion efficiencies remained constant with time as well as with ration size (Figure 6C).

This decrease in consumption and growth rate with time (fish size) in the control treatment can be important for interpreting the effects of As. If As-exposed fish are smaller than controls in the latter part of a test, their consumption and growth rates should be compared to that of control fish of comparable size earlier in the tests. Effects will be confounded by fish size if smaller As-affected fish are compared to the larger control fish collected at the same time. To some degree, this issue might also make the effects in longer tests appear to be less than in shorter tests, and can create a false appearance of recovery in latter parts of tests. Care is thus needed in the selection of metrics for evaluating growth. For the present study, conversion efficiency would be less sensitive to these confounding influences because it is less sensitive to fish size.

3.2.4 Effects of arsenite—The control, iAsIII-60, and iAsIII-120 treatment results (Table 4) show significant dose-dependent reductions in growth rate (ANOVA $p=0.02$) and conversion efficiency (ANOVA $p=0.04$). For consumption, marginally significant effects (ANOVA $p=0.07$) were indicated, but these were not monotonic with dose (i.e., a 10% increase for iAsIII-60 and 4% decrease for iAsIII-120 relative to the control), with neither iAsIII treatment being significantly different than the control by Dunnett's test ($p>0.3$).

For the iAsIII-120 treatment (Table 4), growth rate was reduced by 41% (Dunnett $p=0.006$) and conversion efficiency by 34% (Dunnett $p=0.014$), despite the pellet preparation error that caused lower contamination (Table 2) for the last 3 weeks of the experiment. Some reduction of effects apparently occurred during this time, as both growth rate and food consumption improved in the latter part of the test (Figure 6). A separate ANOVA for the first half of the experiment (prior to the reduction in exposure) showed even greater reductions in growth rate (49%, Dunnett $p=0.001$) and conversion efficiency (43%, Dunnett $p=0.002$).

For the iAsIII-60 treatment (Table 4), growth rate was reduced by 18% (Dunnett $p=0.13$) and conversion efficiency by 24% (Dunnett $p=0.09$). The ANOVA for the first half of the test indicated slightly greater effects, with growth rate reduced by 26% (Dunnett $p=0.06$) and conversion efficiency by 28% (Dunnett $p=0.06$). Despite these borderline statistical significances, we consider these growth effects to indicate true effects, as part of a larger weight-of-evidence including the significant effects on cumulative percentage growth, the monotonic trend with increasing As, and consistency with the earlier results from Erickson et al. (2010, 2011); this consistency is shown in Figure 7, which adds the pellet diet experiment growth rates to the results already shown in Figure 3. Although most of the earlier data in Figure 7 is for iAsV exposures to the worms, the conversion of iAsV to iAsIII

within the worms allows this earlier data to be compared to the iAsIII pellet treatment data in the present study.

3.2.5 Effects of DMA—Like cumulative growth (Figure 5), growth rate was not affected by either DMA treatment (ANOVA $p=0.97$), differing by less than 2% from the control (Table 4, Figure 6). Consumption rates and conversion efficiencies were more variable (Table 4), but were also not significantly affected (ANOVA $p=0.18$ for conversion efficiency and 0.14 for consumption) and were not correlated with dose. Variations of growth rates, consumption, and conversion efficiencies with time (Figure 6A, 6B, 6C) were similar to the control treatment, further supporting a conclusion that these DMA treatments represent no perturbations from the control.

The absence of effects for treatment DMA-480 with food concentrations of 556 $\mu\text{g As/g dwt}$ compared to the substantial effects for treatment iAsIII-60 with 72 $\mu\text{g As/g dwt}$ (Table 2) indicates DMA to be at least 8-fold less toxic than inorganic As on a food concentration basis. Further comparing this dietborne NOEC for DMA to an apparent threshold of $<30 \mu\text{g As/g dwt}$ for inorganic As (Figure 7) suggests DMA to be at least 18-fold less toxic than inorganic As.

3.2.6 Effects of MMA—Like cumulative percentage growth, the overall MMA ANOVA for growth rate was not significant, but the MMA-480 did have an 11% decrease in growth rate and the correlation coefficient was marginally significant ($p=0.06$). That MMA-480 is an effect level is supported by significant effects of MMA on conversion efficiencies (ANOVA $p=0.02$), due to a 19% decrease of efficiency in the MMA-480 treatment (Dunnett's $p=0.007$). The effects of MMA-480 are more clear using conversion efficiency because weight-normalized consumption is about 10% higher in this treatment (Table 4, Figure 6B). This higher relative consumption would mitigate growth reductions to some degree, but would not affect conversion efficiencies, which more clearly show the toxic effect.

Based on the MMA-480 treatment eliciting an 11% reduction in growth rate at 568 $\mu\text{g As/g dwt}$ diet versus the iAsIII-60 treatment eliciting a 19% reduction at 72 $\mu\text{g As/g dwt}$ diet, MMA is at least 8-fold less toxic than inorganic As on a food concentration basis. Furthermore, based on the aggregate data for inorganic As effects (Figure 7A), the same 11% growth reduction would require $<50 \mu\text{g As/g dwt}$ diet, suggesting dietborne MMA is at least 12-fold less toxic than inorganic As.

However, the cause of the toxicity in the MMA-480 treatment is made uncertain by the MMA diet (Figure 4) containing 9.4% inorganic arsenic. This would represent about 50 $\mu\text{g As/g dwt}$ of inorganic As in the food, enough to explain the observed effects on growth; therefore, MMA might be less toxic than suggested by the comparisons above. This issue will be further considered in Section 3.2.7 and warrants future attention, especially whether the inorganic As measured in the pellet is an artifact of sample storage/processing or was actually in the diet when used.

3.2.7 Arsenic accumulation and speciation in the fish—Consideration of the speciation of arsenic accumulated by the fish can help the interpretation of the relative toxicities of these various As species. For all three ancillary pellet diet treatments, the measured As species in the fish accounted for about 80% of the total As (left side of Figure 8), although the treatments did vary regarding how much As was not extracted and how much was not accounted for in the final extract by HPLC-ICP/MS analysis. Although the measured As species in the iAsIII-treated pellets was >99% inorganic (Figure 4), much of this was converted to organic forms in the fish, including MMA, DMA, AsB, and an unidentified organoarsenical AsX, so that only 37% of the measured speciation in the fish was inorganic (Figure 8). The unknown AsX had a longer HPLC retention time than AsB and was suspected to be AsC, but had a very slightly different retention time than AsC and could not be further characterized within project resources. Oladimeji et al. (1979) also reported two unidentified organoarsenicals when rainbow trout were exposed to dietborne iAsV, the predominant of these also having a retention time longer than AsB. For the MMA-120 treatment, 67% of the measured species in fish remained as MMA, with just 2% iAsV and the remainder including AsX (18%), AsB (11%) and DMA (2%). Similarly, for the DMA-120 treatment, 58% remained as DMA, with just 2% was iAsV and the remainder including AsX (35%), AsB (5%), and MMA (1%).

Because of the mix of As species in the fish for the iAsIII-60 treatment of the ancillary exposure, a definitive conclusion cannot be made, based just on this speciation information, regarding which As species within the fish caused the observed toxicity in iAsIII treatments of the primary pellet data experiment, although the expectation would be that the iAsIII would be the principal cause based on past work (Section 1). However, by using the relative speciation from the ancillary experiment (left side of Figure 8) to estimate absolute concentrations of different species for the fish in the primary pellet diet experiment (right side of Figure 8), some conclusions are possible. For *every* measured organoarsenical and for the total concentration of these organoarsenicals, either the MMA-480 or the DMA-480 treatment, or both, had much higher accumulation, but less effect, than the iAsIII-60 treatment. In contrast, inorganic As in the iAsIII-60 treatment, especially the more toxic iAsIII, was much higher than in the MMA-480 and DMA-480 treatments. Therefore, inorganic As in the fish discriminates the effect in the iAsIII-60 treatment from the lesser effects in the MMA-480 and DMA-480 treatments.

The accumulation data in Figure 8 also has relevance to the possible contribution of iAsV in the MMA-treated pellets to the observed effects in the MMA-480 treatment. Based on 9.4% iAsV in the MMA pellets from the ancillary exposure, the pellets in the MMA-480 treatment of the primary exposure would be calculated to contain 55 µg As/g dwt of inorganic As, about the same as the inorganic As in the iAsIII-60 treatment. However, the estimated accumulation of inorganic As in the MMA-480 treatment is about 5-fold less than in the iAsIII-60 treatment (right side of Figure 8), too low to attribute the effects from the MMA-480 treatment to inorganic As; this low accumulation could be due to the demethylation to iAsV occurring after the use of the pellets or because iAsV accumulates less than iAsIII. Thus the observed effect in MMA-480 is more likely actually due to MMA,

although this is of little practical significance to realistic environmental exposures given the high levels of MMA in diet needed to elicit these effects.

It is thus apparent that assessments of arsenic risk to salmonid fish need to include characterization of speciation in the water and diet and to focus on the inorganic fraction. Expressing Figure 7 on the basis of inorganic As would move all the organoarsenical data to the no-effect plateau region and provide consistency across all data, except for the minor effects of MMA-480. However, it should be noted that the speciation measurements in the present study encompass a limited scope of exposure conditions and tissues, so that whether inorganic arsenic is a sufficient exposure metric in diet or fish warrants further investigation.

3.3 Panther Creek invertebrate analysis

Figure 9 shows As species measured within macroinvertebrates from Panther Creek on both a percentage and absolute concentration basis. These As species include three unidentified organoarsenicals (AsX1, AsX2, AsX3), distinct from the AsX species found in the laboratory fish extracts. The As in these three insect species shows considerable variability, both in the absolute concentrations and the relative amounts of different As species.

The detritivorous stonefly (*P. californica*) had the highest total accumulation (3.0 µg As/g dwt), of which about 60% was extractable. The measured As species accounted for all of the total extracted As. Of the species measured, 18% was inorganic As, with 78% being the unidentified organoarsenicals and 4% being DMA.

The filter-feeding caddisfly (*A. grandis*) had half as much total accumulation (1.5 µg As/g dwt), of which only 40% was extractable. The measured As species accounted for 94% of the total extracted As, and included proportionately more inorganic As (44%), more MMA, DMA, and AsB (18% total) and only about half as much (38%) of the unidentified organoarsenicals as *P. californica*. Despite the lower total concentration and extraction efficiency, the absolute concentration of inorganic As measured in *A. grandis* (0.25 µg As/g dwt) was nearly as high as in *P. californica* (0.33 µg As/g dwt).

The predatory stonefly (*H. pacifica*) had much less total accumulation than the other two insect species (0.35 µg As/g dwt). Lower concentrations of As in organisms higher in the food chain have been previously reported, including in natural ecosystems (Kiser et al., 2010; Rahman et al., 2012; Dovick et al., 2015) and experimental ecosystems (review by Kuehnelt and Goessler, 2003). *H. pacifica* also had less extractable As than the other species, and the highest amount (86%) of inorganic As in the measured species, with only minor amounts of DMA (9%) and AsX3 (5%). Because of the low total As content and the low extraction efficiency, the measured inorganic As was much lower (0.07 µg As/g dwt) than the other two species.

It should be noted that the concentrations of inorganic As in these macroinvertebrates (Figure 9) are too low to cause the effects on rainbow trout growth reported in the present study (Figures 7, 8), so that this part of Panther Creek should not pose risks in this regard. Furthermore, given the low total As concentrations in these insects, the distribution of As species might not be representative of what would occur at more highly contaminated sites.

The filter feeding caddisfly *Stenopsyche marmorata* from a As-contaminated river has been reported (Miyashita et al., 2009) to have total As of 18 µg As/g dwt and a larger proportion of inorganic As (74% of extracted arsenic, with only 35% extracted) than the results for *A. grandis* here. The arsenic extracted from a polychaete *Nereis diversicolor* from an arsenic-contaminated estuary (Boyle et al., 2008) was also highly inorganic (83% inorganic of 71% extracted from a total As of 136 µg As/g dwt), in contrast to organisms from less contaminated estuary (2% inorganic of 38% extracted from a total of 15 µg As/g dwt). Such variability in both the absolute and relative concentrations of different As species emphasizes the need to characterize diet composition when assessing potential risks to fish from dietborne As exposure.

4. SUMMARY AND IMPLICATIONS

The experiments in the present study confirmed expectations from previous work that organoarsenicals should be less toxic than inorganic As for dietborne exposures to salmonid fish. For the live diet experiment, practical problems in contaminating a live diet to high concentrations limited conclusions, but did indicate MMA to be at least 3-fold less toxic than inorganic As on the basis of accumulation in the diet and AsB to be at least 4-fold less toxic on the basis of accumulation in the fish.

For the pellet diet experiment, MMA showed limited toxicity and DMA showed no toxicity at about 550 µg As/g dwt in the food, and are at least an order of magnitude less toxic than inorganic As based on concentrations in food. Analysis of As speciation in fish indicated that MMA and DMA are also much less toxic to fish than inorganic As based on internal exposure.

Accordingly, risk assessments addressing salmonid growth will need to consider speciation of As; assessing both dietary exposure and accumulated dose on the basis of inorganic As might be a useful part of this. Arsenic speciation in macroinvertebrates from As-contaminated systems indicated that both absolute concentrations and the relative amounts of different As species can be quite variable, and that estimating dietborne exposures to inorganic As will require considerable effort.

While not part of the intent of the present study, it should also be noted that DMA was more toxic than iAsIII in the waterborne exposures of *L. variegatus* during the live diet experiment. Thus, the expectations and findings in the present study regarding the relative nontoxicity of organoarsenicals in diet do not necessarily apply broadly to different aquatic taxa and endpoints, potentially further complicating ecological risk assessments involving arsenic.

Finally, the present study illustrated several general issues warranting better consideration in dietborne exposures and fish growth evaluations. Although live diets arguably provide a more relevant exposure medium for dietborne toxicants, their utility can be affected by toxicity to the diet organisms and biotransformation of the toxicants. Pellet diets can be more susceptible to leaching of the toxicant, and can also involve changes in the toxicant form and bioavailability that warrant characterization. Regarding fish growth evaluations,

better attention is needed regarding how much food is offered and consumed, and to the choice and application of growth metrics; in particular, the influence of size-dependent growth rates on data interpretation warrants more care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Fraction dry weight versus blotted wet weight per fish for pellet diet experiment (red), live diet experiment (blue), and Erickson et al. [2010] (green). Line denotes linear least-squares regression of these data.

FIGURE 2.

Arsenic species measured in worms exposed to different waterborne arsenic compounds in the live diet experiment; separate bars for AsB denote measurement on day 1 of fish exposure versus at day 7 and later. iAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, AsB denotes arsenobetaine, and NM denotes total arsenic not measured as a species (either not extracted or not detected in chromatogram).

FIGURE 3.

Control-normalized growth rates of juvenile rainbow trout versus (A) total arsenic in diet and (B) total arsenic accumulated by the fish. Colored circles denote means from the live diet experiment in the present study, with error limits denoting range for replicate chambers. Black dots are growth rates calculated from data of Erickson et al. (2010, 2011) for both waterborne and dietborne exposures to arsenate or arsenite.

FIGURE 4.

Arsenic species measured in food versus arsenic species added to food for ancillary pellet diet exposure. iAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, NE denotes As not extracted, and NM denotes As in the final extract not accounted for in the measured species.

FIGURE 5.

Percentage growth of juvenile rainbow trout, relative to initial weights, in the pellet diet experiment at 28 d (circles) and 56 d (stars). Error limits denote range across replicate chambers and “p” denotes the Dunnett’s test significance level for effects relative to the control treatment. See text for definition of treatment codes.

FIGURE 6.

Growth rate (panel A), food consumption (panel B), and conversion efficiency (panel C) in the pellet diet experiment. Stars denote overall value across the entire 56-d exposure and circles denote values for individual two-week periods (1–4). Error limits denote range across replicate chambers. In panel B, x's denote total ration. See text for definitions of treatment codes.

FIGURE 7.

Control-normalized growth rates of juvenile rainbow trout versus (A) total arsenic in diet and (B) total arsenic accumulated by the fish. Colored circles denote means from the live diet experiment and colored squares denote means from the pellet diet experiment in the present study, with error limits denoting range for replicate chambers. Black dots are growth rates calculated from data of Erickson et al. (2010, 2011) for dietborne exposures using worms exposed to arsenate or arsenite and for waterborne exposures of fish to arsenate.

FIGURE 8.

Arsenic species in fish versus arsenic species added to food. Left panel denotes relative concentrations of species in ancillary pellet diet experiment. Right panel denotes estimated absolute concentrations of species in primary pellet diet experiment. iAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, AsB denotes arsenobetaine, AsX denotes an uncharacterized organoarsenical, NE denotes As not extracted, and NM denotes As in the final extract not accounted for in the measured species.

FIGURE 9.

Arsenic species measured in three macroinvertebrate species from Panther Creek, ID, USA on the basis of relative concentrations (left) and absolute concentrations (right). iAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, AsB denotes arsenobetaine, AsX1/AsX2/AsX3 denote uncharacterized organoarsenicals, NE denotes As not extracted, and NM denotes As in the final extract not accounted for in the measured species.

TABLE 1.

Experimental treatments.

Live Diet Experiment		Pellet Diet Experiment		
Treatment ¹	Worm water exposure (mg As/L)	Treatment ¹	Fish diet exposure (µg As/g dwt)	Feeding rate (%/d) (fish wwt basis)
Control	<0.1	Control	<1	3.0, 2.5 ³
iAsIII _{Live}	2.4	Ctrl-Lim1	<1	2.3, 2.0
iAsV _{Live}	6.0	Ctrl-Lim2	<1	1.6, 1.5
MMA _{Live}	20	Ctrl-Lim3	<1	1.0, 1.0
DMA _{Live}	0.5	iAsIII-60	60	3.0, 2.5
	10 (d0-1) ²	iAsIII-120	120	3.0, 2.5
AsB _{Live}	6 (d2-3)			
	4 (d4-12)			
	2(d13+)	MM A-120	120	3.0, 2.5
		MMA-480	480	3.0, 2.5
		DMA-120	120	3.0, 2.5
		DMA-480	480	3.0, 2.5

¹ iAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, and AsB denotes arsenobetaine.

² "d" denotes the designated day for feeding the worms to the fish, so that the water exposure started 7 days previously.

³ Two numbers are for first and second half of the test.

TABLE 2

Diet characteristics. Parentheses denote relative standard deviation (%) and the number of samples analyzed.

Treatment ^I	Live Diet Experiment			Pellet Diet Experiment					
	Total As (µg/g dwt)	Percent Dry Wt	Percent Lipid	Treatment ^I	Total As (µg/g dwt)	Percent Dry Weight	Percent Dry Weight	Pellet Weight (mg)	Pellet Weight (mg)
					Batch 1	Batch 2	Batch 1	Batch 2	Batch 2
Control	1.7 (20%, n=8)	18.6 (7.9%, n=4)	20.9 (21%, n=4)	Control	3.2 (4.6%, n=4)	3.0 (11%, n=4)	90.6 (0.8%, n=9)	92.1 (0.2%, n=6)	2.37 (12%, n=9)
iAsIII _{Live}	32.6 (20%, n=10)	18.7 (1.7%, n=4)	18.2 (19%, n=3)	iAsIII-60	72 (4.6%, n=6)	69 (9.8%, n=6)	87.8 (0.7%, n=9)	91.8 (0.4%, n=6)	1.99 (12%, n=9)
iAsV _{Live}	37.9 (31%, n=9)	18.7 (6.4%, n=5)	19.3 (12%, n=5)	iAsIII-120	134 (6.6%, n=6)	100 (7.3%, n=6)	87.1 (0.5%, n=9)	92.8 (0.3%, n=6)	1.98 (12%, n=9)
MMA _{Live}	108 (10%, n=8)	18.0 (1.6%, n=3)	20.2 (11%, n=3)	MMA-120	143 (6.5%, n=6)	151 (3.0%, n=6)	85.4 (0.5%, n=9)	92.5 (0.6%, n=6)	2.08 (9.4%, n=9)
DMA _{Live}	26.7 (32%, n=8)	18.2 (9.5%, n=6)	19.1 (73%, n=5)	MMA-480	556 (1.2%, n=6)	580 (12%, n=6)	89.9 (0.2%, n=9)	92.6 (0.4%, n=6)	2.58 (10%, n=9)
AsB _{Live}	88 (41%, n=9, d 9)	16.2 (15%, n=2)	16.4 (7.0%, n=4)	DMA-120	142 (2.1%, n=6)	139 (7.7%, n=6)	87.8 (0.3%, n=9)	92.7 (0.4%, n=6)	2.47 (12%, n=9)
	65 (35%, n=12, d>9)			DMA-480	555 (4.4%, n=6)	558 (1.5%, n=6)	87.5 (0.5%, n=9)	92.9 (0.2%, n=6)	2.01 (27%, n=9)

^I iAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, and AsB denotes arsenobetaine.

TABLE 3

Fish arsenic accumulation and growth metrics for 28-d live diet experiment. Parentheses provide percent standard deviation among replicates and the number of replicates.

Treatment^I	Total As (µg As/g dwt)	Cumulative Growth (%, dwt basis)	Growth Rate (dwt basis) (%/d)	Consumption (dwt basis) (%/d)	Conversion Efficiency (%, dwt basis)
Control	0.2 (<i>n</i> =6)	215 (16%, <i>n</i> =4)	4.1 (9.8%, <i>n</i> =4)	19.1 (3.9%, <i>n</i> =4)	21.5 (12%, <i>n</i> =4)
iAsIII _{Live}	4.2 (14%, <i>n</i> =8)	230 (11%, <i>n</i> =4)	4.3 (6.2%, <i>n</i> =4)	18.9 (4.0%, <i>n</i> =4)	22.6 (9.8%, <i>n</i> =4)
iAsV _{Live}	4.2 (24%, <i>n</i> =8)	242 (16%, <i>n</i> =3)	4.4 (9.5%, <i>n</i> =3)	17.9 (2.3%, <i>n</i> =3)	24.5 (11%, <i>n</i> =3)
MMA _{Live}	5.5 (36%, <i>n</i> =8)	254 (10%, <i>n</i> =3)	4.5 (5.8%, <i>n</i> =3)	18.4 (3.7%, <i>n</i> =3)	24.6 (9.4%, <i>n</i> =3)
DMA _{Live}	3.4 (13%, <i>n</i> =8)	254 (10%, <i>n</i> =4)	4.5 (6.7%, <i>n</i> =4)	18.0 (4.6%, <i>n</i> =4)	25.0 (11%, <i>n</i> =4)
AsB _{Live}	20.3 (22%, <i>n</i> =8)	265 (16%, <i>n</i> =4)	4.6 (9.2%, <i>n</i> =4)	18.5 (6.3%, <i>n</i> =4)	25.1 (16%, <i>n</i> =4)

^IiAsIII denotes arsenite, iAsV denotes arsenate, MMA denotes monomethylarsonate, DMA denotes dimethylarsinate, and AsB denotes arsenobetaine.

TABLE 4

Fish As accumulation and growth parameters over 56-d in pellet diet experiment. Parentheses provide percent standard deviation among replicates and the number of replicates.

Treatment ^I	Total As ($\mu\text{g As/g dwt}$)	Cumulative Growth (%, dwt basis)	Growth Rate (dwt basis) (%/d)	Consumption (dwt basis) (%/d)	Conversion Efficiency (%,dwt basis)
Control	2.3 (13%,n=4)	312 (14%,n=4)	2.52 (10%,n=4)	7.21 (7.0%,n=4)	34.8 (7.1%,n=4)
Ctrl-Lim1		284 (3.2%,n=2)	2.40 (2.4%,n=2)	6.67 (0.7%,n=2)	36.2 (3.8%,n=2)
Ctrl-Lim2		214 (7.5%,n=3)	2.04 (6.7%, n=3)	5.51 (3.4%,n=3)	37.0 (6.2%,n=3)
Ctrl-Lim3		110 (4.8%,n=2)	1.32 (6.5%,n=2)	3.77 (1.1%,n=2)	35.2 (5.2%,n=2)
MMA-120	9.4 (29%,n=4)	201 (6.1%,n=4)	2.48 (4.5%,n=4)	7.59 (6.9%,n=4)	32.9 (7.1,n=4)
MMA-480	24.2 (15%,n=4)	253 (13%,n=4)	2.24 (10%,n=4)	7.94 (6.2%,n=4)	28.1 (13%,n=4)
DMA-120	12.3 (19%,n=4)	305 (18%,n=3)	2.48 (13%,n=3)	6.65 (7.6%,n=3)	37.2 (4.9%,n=3)
DMA-480	32.0 (33%,n=4)	302 (76%,n=4)	2.47 (12%,n=4)	7.56 (7.3%,n=4)	32.8 (11%,n=4)
iAsIII-60	8.6 (20%,n=8)	227 (27%,n=3)	2.06 (26%,n=3)	7.95 (6.6%,n=3)	26.4 (32%,n=3)
iAsIII-120	14.9 (24%,n=8)	134 (26%,n=4)	1.49 (26%,n=4)	6.88 (7.7%,n=4)	21.7 (31%,n=4)

^I iAsIII denotes arsenite, MMA denotes monomethylarsonate, and DMA denotes dimethylarsinate.