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Anaerobic Biotransformation of Organoarsenical Pesticides Monomethylarsonic Acid and Dimethylarsinic Acid

REYES SIERRA-ALVAREZ,[†] UMUR YENAL,[†] JIM A. FIELD,*[,†] MIKE KOPPLIN,[‡] A. JAY GANDOLFI,[‡] AND JOHN R. GARBARINO[§]

Department of Chemical and Environmental Engineering, University of Arizona, Arizona, P. O. Box 210011, Tucson, Arizona 85721, Department of Pharmacology and Toxicology, University of Arizona, Tucson, Arizona 85721, and U.S. Geological Survey, National Water Quality Laboratory, P.O. Box 25046, MS407, Building 95, Denver Federal Center, Denver, Colorado 80225-0046

Monomethylarsonic acid (MMAV) and dimethylarsinic acid (DMAV) are extensively utilized as pesticides, introducing large quantities of arsenic into the environment. Once released into the environment, these organoarsenicals are subject to microbial reactions. Aerobic biodegradation of MMAV and DMAV has been evaluated, but little is known about their fate in anaerobic environments. The objective of this study was to evaluate the biotransformation of MMAV and DMAV in anaerobic sludge. Biologically mediated conversion occurred under methanogenic or sulfate-reducing conditions but not in the presence of nitrate. Monomethylarsonous acid (MMAIII) was consistently observed as an important metabolite of MMAV degradation, and it was recovered in molar yields ranging from 5 to 47%. The main biotransformation product identified from DMAV metabolism was MMAV, which was recovered in molar yields ranging from 8 to 65%. The metabolites indicate that reduction and demethylation are important steps in the anaerobic bioconversion of MMAV and DMAV, respectively.

KEYWORDS: Arsenic; methanogenic; sulfate reduction; redox transformations; monomethylarsonous acid

INTRODUCTION

The newly enacted United States Environmental Protection Agency (US-EPA) maximum contaminant level for drinking water for arsenic of 10 ppb reflects a growing national concern on the public health effects of low-level exposure to arsenic (*1*). The release of arsenic from weathering rocks has widely been recognized as a major source of arsenic in groundwater (*2*). Arsenic-containing chemicals are also used on a massive scale as pesticides in agriculture. In the United States, large quantities of sodium salts of monomethylarsonic acid (MMAV) are utilized as herbicides in cotton production (*3*). The monosodium salt of MMAV is among the top pesticides utilized in the United States with approximately 1.2 million kg of arsenic applied annually to 3.8 million acres (*4*). In addition, approximately 35 000 kg of arsenic from dimethylarsinic acid (DMAV) is applied annually as defoliant (*3*).

These organoarsenical pesticides have been measured in the environment. In cotton-producing areas, MMAV and DMAV are detected in surface water, groundwater, and rivers (*3*, *5*). The highest levels were observed in stagnant surface water, where concentrations reached as high as 10 and 100 ppb DMAV and MMAV, respectively. These pesticides were also detected in soil 1−1.5 years after their application to experimental field plots (*6*, *7*). MMAV applied to golf course greens was detected in percolate water (*8*).

Once these compounds are released into the environment, they are subject to transformation into other arsenic species. The structures of the major species considered in this study are shown in **Figure 1**. Environmental samples obtained in cotton-growing areas indicate that arsenate (AsV) is a predominant species in areas historically subjected to DMAV and MMAV applications (*3*). AsV was also the main species formed in soil from experimental field plots exposed to high application rates of either DMAV or MMAV (*6*, *7*). Arsenite (AsIII) was observed as an important species in samples collected from an irrigation ditch adjacent to cotton fields (*3*). MMAV applied to golf course greens was converted to two major products, AsV and DMAV (*8*).

The speciation observed in the field studies is in agreement with laboratory studies conducted under aerobic conditions. MMAV incubated with soil results in the formation of AsV. Forty-three percent of the MMAV applied at 100 mg of As kg^{-1} of soil was converted to AsV in 70 days (*9*). Ten percent of labeled [^{14}C]MMAV applied at 10 mg of As kg^{-1} of soil was mineralized $^{14}CO_2$ in 21 days, and the presence of AsV was demonstrated with thin-layer chromatography of soil

* Corresponding author: Telephone: (520) 626-5858. Fax. (520) 621-6048. E-mail: jimfield@email.arizona.edu.
[†] Department of Chemical and Environmental Engineering, University of Arizona.
[‡] Department of Pharmacology and Toxicology, University of Arizona.
[§] U.S. Geological Survey.

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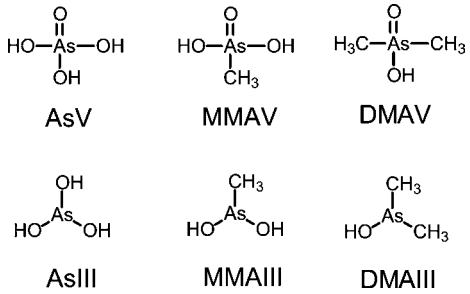

Figure 1. Arsenic compounds monitored in this study: arsenate (AsV); monomethylarsonic acid (MMAV); dimethylarsenic acid (DMAV); arsenite (AsIII); monomethylarsonous acid (MMAIII); dimethylarsinous acid (DMAIII).

extracts. In similar experiments, 16% of labeled [^{14}C]MMAV applied at 210 mg of As kg^{-1} of soil was converted to $^{14}CO_2$ after 30 days (*10*).

Several laboratory studies also evaluated the speciation of DMAV under aerobic conditions. Seventy-three percent and 0.8% of DMAV applied at 100 mg of As kg^{-1} of soil were converted to AsV and MMAV, respectively, in 70 days (*9*). Forty-five percent of labeled [^{14}C]DMAV applied at 10 mg of As kg^{-1} of soil was mineralized to $^{14}CO_2$ with concomitant conversion of the arsenic to AsV (38%) and MMAV (8%) in 60 days (*6*). Low concentration of DMAV (10 μg of As L^{-1}) incubated for 1 day in estuarine water was converted to AsV (12.7%) (*11*).

In addition to AsV, DMAV, and MMAV as the major speciation products, several studies have demonstrated minor formation of volatile arsine and methylarsines. The yield of arsines from soils incubated with MMAV or DMAV ranged from 0.001 to 1.4% (*6*, *9*). The highest arsine yields correspond to soils receiving organic amendments.

The fate of MMAV and DMAV in anaerobic environments is not well understood. Only a few experiments have examined the speciation of DMAV under conditions of low elemental oxygen. An experiment comparing [^{14}C]DMAV conversion in water-unsaturated and water-saturated soils indicated a marked decrease in the mineralization to $^{14}CO_2$ and AsV (*6*). Instead, a large accumulation of MMAV (36% of added As) was observed under water-saturated conditions.

The goal of this study was to evaluate the biotransformation of MMAV and DMAV under strict anaerobic conditions. These organoarsenicals were incubated with anaerobic sludge as a representative anaerobic microbial consortium under methanogenic, sulfate-reducing, and denitrifying conditions to evaluate the conversion in the absence of elemental oxygen.

MATERIALS AND METHODS

Microorganisms. Anaerobic methanogenic sludge was used as inoculum in the assays. It was obtained from an industrial upward-flow anaerobic sludge blanket (UASB) treatment plant treating recycled paper wastewater (Industriewater, Eerbeek, The Netherlands) The sludge was washed and sieved to remove fine particles before use in the tests. The content of volatile suspended solids (VSS) in the Eerbeek wastewater was 12.9%. The anaerobic sludge was stored under nitrogen gas at 4 °C.

Batch Bioassay. Anaerobic biotransformation of MMAV and DMAV was performed in batch bioassay flasks that were incubated in a climate-controlled chamber at 30 ± 2 °C in an orbital shaker (75 rpm). Serum flasks (160 mL) were supplied with 75 mL of a basal mineral medium (pH 7.0−7.2) containing (in mg L^{-1}) NH_4Cl (280), $NaHCO_3$ (2000), $CaCl_2 \cdot 2H_2O$ (*10*), $MgSO_4 \cdot 7H_2O$ (100), K_2HPO_4 (600), and $NaH_2PO_4 \cdot 2H_2O$ (795); yeast extract (*20*); and 1 mL L^{-1} trace element solution. The medium was also supplemented with MMAV or DMAV (concentrations indicated in the tables and figures). The sludge inoculum was supplied at 1.5 g of VSS L^{-1}. A mixture of volatile fatty acids (VFA) (concentration (as mM): acetate (7.5), propionate (6.1), butyrate (5.1)) equivalent to 2.5 g of chemical oxygen demand (COD) L^{-1} was provided as a cosubstrate in one experiment. Selected assays also received 2 g of SO_4^{2-} L^{-1} or 2 g of NO_3^- L^{-1} to support the sulfate-reducing or nitrate-reducing bacteria, respectively. Those assays also received the methanogenic inhibitor, 2-bromoethanesulfonate (30 mM final concentration). The final pH value of all media was adjusted to 7.0−7.2 with NaOH or HCl, as needed. Each experiment included several controls. Abiotic controls were prepared without adding microbial inoculum. Killed sludge controls were prepared by adding inoculum and subsequently placing the bottles in an autoclave for 1 h at 120 °C; the contents were subsequently allowed to cool and then sealed aseptically. All flasks were sealed with butyl rubber stoppers and aluminum crimp seals. The liquid contents and headspace were flushed with N_2:CO_2 gas (80:20, v/v) to exclude oxygen from the assay. All assays were conducted in triplicate.

Analytical Methods. Inorganic arsenic species (AsIII and AsV) and organic arsenic species (MMAV, DMAV, MMAIII, and DMAIII) in liquid samples were analyzed by high-performance liquid chromatography−inductively coupled plasma−mass spectrometry (HPLC−ICP−MS) using a method adapted from Gong et al. (*12*). The stability of all species except for DMAIII is well documented under the storage conditions used in this study (*12*, *13*). DMAIII is not stable under storage. The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies, Inc., Palo Alto, CA) with a reverse-phase C18 column (Prodigy 3u ODS^3, 150 × 4.60 mm, Phenomenex, Torrance, CA). The mobile phase (pH 5.85) contained 4.7 mM tetrabutylammonium hydroxide, 2 mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 mL min^{-1}. The column temperature was maintained at 50 °C. An Agilent 7500a ICP−MS with a Babington nebulizer was used as the detector. The operating parameters were as follows: radio frequency power 1500 W, plasma gas flow 15 L min^{-1}, and carrier flow 1.2 L min^{-1}; arsenic was measured at *m*/*z* 75 and terbium (IS) was measured at *m*/*z* 159. The injection volume was 10 μL. The detection limit for the various arsenic species was 0.1 μg L^{-1}. Total arsenic concentration in liquid samples was determined by direct injection into the ICP−MS. Total arsenic was calibrated with arsenic trioxide (As_2O_3) reductometric standard (Aldrich).

All liquid samples were centrifuged and membrane filtered (0.45 μm) immediately after sampling and stored in polypropylene vials (2 mL). The filtered samples were then stored at −20 °C until the analysis was performed in order to reduce changes in arsenic speciation.

Volatile arsenic species were determined by flushing headspace of the biological assay bottles with N_2 gas for 20−24 h. The gas was bubbled through 20 mL of 2 M nitric acid used as scrubbing fluid. Samples of the scrubbing fluid were analyzed for total arsenic.

The sludge extraction was performed for the two of three replicated experimental assay bottles by using a sodium hydroxide (NaOH) extraction method (*14*). An aliquot of 20 mL of 1.0 M NaOH was added to sludge that was prewashed by decanting with demineralized water. The sealed serum flasks were shaken vigorously for 6 h in a water bath at 80 °C. A 1 mL sample of the extractant was diluted with 9 mL of 0.1 M HCl. After centrifuging of the diluted extractant in an Eppendorf tube, the sample was diluted to the proper concentration range for measurement with the HPLC−ICP−MS.

The methane content in the headspace of the serum flasks was determined by gas chromatography using an HP5290 Series II system (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (GC−FID). The GC was fitted with a Nukol fused silica capillary column (30 m length × 0.53 mm i.d., Supelco, St. Louis, MO). The temperature of the column, the injector port, and the detector was 140, 180, and 275 °C, respectively. The carrier gas was helium at a flow rate of 9.3 mL min^{-1} and a split flow of 32.4 mL min^{-1}. Samples for measuring methane content (100 μL) in the headspace were collected using a pressure-lock gas syringe.

Sulfate was determined by ion chromatography with suppressed conductivity using a Dionex system equipped with a Dionex AS11-HC4 column (Dionex, Sunnydale, CA) and a conductivity detector. The mobile phase was 15 mM KOH at a flow rate of 1.2 mL min^{-1}. The column temperature was maintained at room temperature. The

Table 1. Extent of Degradation, First-Order Rate Constants (k), and Molar Yield of Detected Intermediates during the Biotransformation of MMAV (A) and DMAV (B)

							intermediate			
expt no.	durati on (days)	concn (mM)	redox condition	added e^- donor	degradation[a] (%)	k ($days^{-1}$)	compound	concn (mM)	max molar recovery[b] (%)	time[c] (days)
					(A) MMAV as Substrate					
expt 1-1	235.5	2.85	methanogenic	no	49.3		MMA(III)[d]	0.258	28.9	111.8
expt 1-2	235.5	14.25	methanogenic	no	24.0		MMA(III)[d,e]	0.281	5.3	111.8
expt 3-3	216.7	1.50	methanogenic	no	49.4		MMA(III)	0.048	NA[f]	47.9
expt 3-4	216.7	1.50	methanogenic	VFA	26.5		MMA(III)	0.053	NA[f]	47.9
expt 4-2	136.8	0.70	methanogenic	no	80.0	0.0112	MMA(III)	0.045	13.5	80.8
expt 6-4	56.9	0.70	methanogenic	no	39.6	0.0155	MMA(III)	0.052	19.4	29.8
							DMA(V)	0.005	2.5	56.9
expt 6-5	56.9	0.70	SO_4^{2-} reduction	no	95.8	0.0408	MMA(III)	0.089	15.6	29.8
							DMA(V)	0.009	2.0	56.9
expt 7-2	142.8	0.70	SO_4^{2-} reduction	no	74.1	0.0106	MMA(III)	0.279	46.7	142.8
					(B) DMAV as Substrate					
expt 2-1	207.0	1.00	methanogenic	no	95.3	0.0126	MMA(V)	0.157	33.0	7.0
expt 2-2	81.1	5.00	methanogenic	no	32.6		MMA(V)	0.716	65.0	27.8
expt 3-1	216.7	1.00	methanogenic	no	95.4	0.0148	MMA(V)	0.113	21.7	17.9
expt 3-2	216.7	1.00	methanogenic	VFA	75.4		MMA(V)[d]	0.084	12.9	23.9
expt 4-1	44.0	0.50	methanogenic	no	97.8	0.0983	MMA(V)	0.001	0.2	44.0
expt 6-1	56.9	0.50	methanogenic	no	99.8		MMA(V)	0.038	12.3	56.9
expt 6-2	56.9	0.50	SO_4^{2-} reduction	no	100.0		MMA(V)	0.049	9.9	20.8
expt 7-1	46.8	0.50	SO_4^{2-} reduction	no	99.6	0.0881	As(V)[g]	0.034	7.8	21.0

[a] Percent degradation = 100(killed $control_{tx}$ − $treatment_{tx}$)/killed $control_{tx}$. [b] Maximum molar recovery = 100($intermediate_{tx}$)/(killed $control_{tx}$ − $treatment_{tx}$). [c] Time of maximum molar recovery. [d] As(III) is detected as an intermediate in trace amount. [e] As(V) is detected as an intermediate in trace amount. [f] Cannot calculate due to unreliable values of control. [g] MMA(V) is detected as an intermediate in trace amount.

injection volume was 25 μL. The pH was determined immediately with an Orion Model 310 PerpHecT pH meter with a PerpHecT ROSS glass combination electrode.

Volatile suspended solids were determined according to *Standard Methods for the Examination of Water and Wastewater* (*15*).

RESULTS

Anaerobic Biotransformation of MMAV under Methanogenic Conditions. Several experiments were conducted in which anaerobic sludge was incubated with MMAV under methanogenic conditions. In a typical experiment in which 2.7 mM MMAV was added with either anaerobic sludge or heat-killed sludge, there is no decline in MMAV concentration in the heat-killed control, while there is a significant decline of the MMAV concentration in the living treatment (**Figure 2A**). At the end of the incubation of 236 days, 49.3% of MMAV was removed. From day 112 onward, there was no further decline in the MMAV concentration, suggesting inhibition by a biotransformation product. MMAIII was identified as a biotransformation product with a maximum concentration of MMAIII observed after 112 days, corresponding to a molar yield of 28.9% of MMAV removed (**Figure 2B**). MMAIII was not detected in the heat-killed controls.

The biotransformation of MMAV was also tested at a higher concentration (14.3 mM). Even at this high concentration some removal was observed, albeit only 24.0% after 236 days (experiment 1-2 in **Table 1**). Removal of MMAV completely ceased at day 112. The data listed in **Table 1** show that MMAIII was observed as a biotransformation product. Due to suspect inhibition of biotransformation products, all further tests were conducted with lower initial concentrations of MMAV. The lower concentrations generally permitted greater extents of MMAV removal (experiments 4-2, 6-5, and 7-2 in **Table 1**). MMAIII was consistently observed as a biotransformation product of MMAV.

Anaerobic Biotransformation of MMAV under Different Redox Conditions. An experiment was conducted comparing the anoxic degradation of MMAV (0.7 mM) under methanogenic, sulfate-reducing, and denitrifying conditions (**Figure 3A**). The results indicate bioconversion of MMAV under the methanogenic and sulfate-reducing conditions and no conversion under denitrifying conditions. The results illustrate a significant decrease in the MMAV concentration in the active treatments

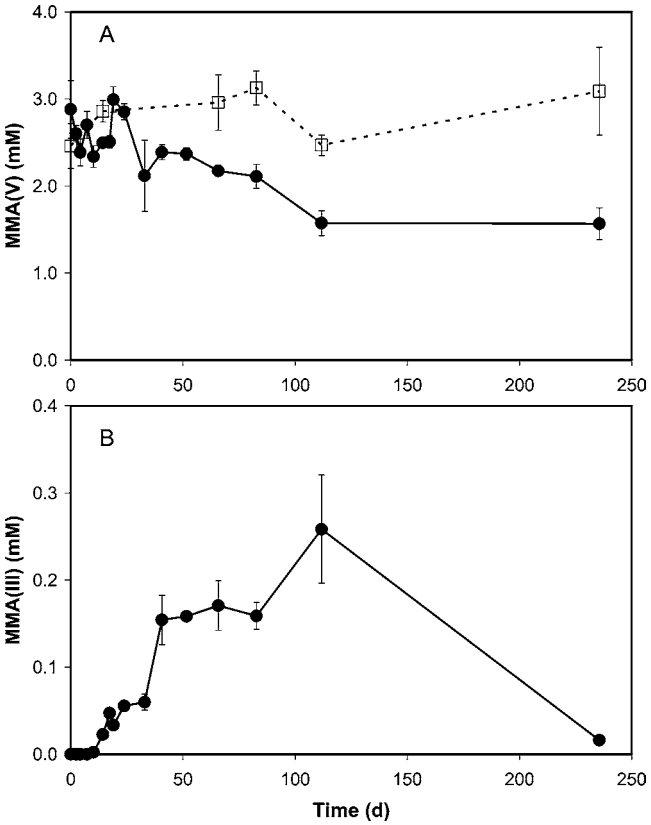

Figure 2. Biotransformation of MMAV (2.7 mM) during incubation with anaerobic sludge under methanogenic conditions. (**A**) Removal of MMAV; (**B**) formation of the intermediate, MMAIII. Open squares; heat-killed control; filled circles, biologically active.

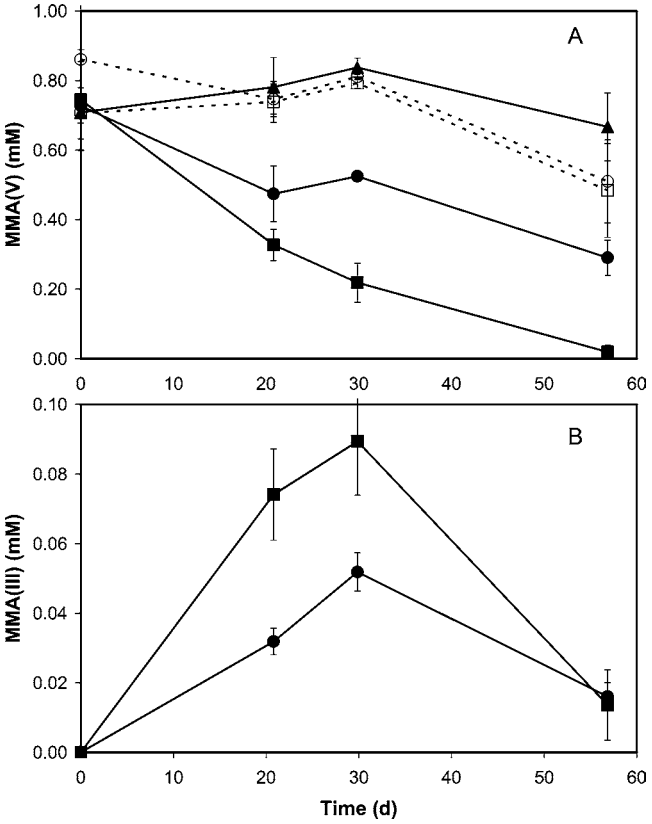

Figure 3. Biotransformation of MMAV (0.7 mM) during incubation with anaerobic sludge under methanogenic, sulfate-reducing, or denitrifying conditions. (**A**) Removal of MMAV; (**B**) formation of the intermediate, MMAIII. Open circles, noninoculated control; open squares, heat-killed control; filled circles, biologically active under methanogenic conditions; filled squares, biologically active under sulfate-reducing conditions; filled triangles, biologically active under denitrifying conditions.

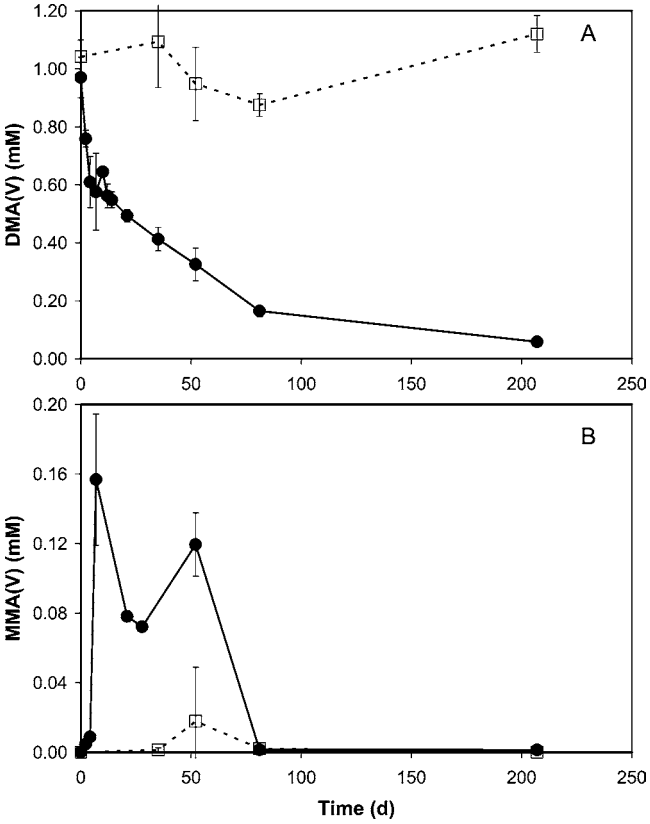

Figure 4. Biotransformation of DMAV (1.0 mM) during incubation with anaerobic sludge under methanogenic conditions. (**A**) Removal of DMAV; (**B**) formation of the intermediate, MMAV. Open squares, heat-killed control; filled circles, biologically active.

compared to noninoculated controls and controls with heat-killed inoculum. Sulfate-reducing conditions promoted a more extensive conversion of MMAV compared to methanogenic conditions. The sulfate-reducing assay removed 95.8% of the MMAV by the end of the experiment (day 57). MMAIII was the most important biotransformation product identified (**Figure 3B**). The maximum molar yields of MMAIII ranged from 15.6 to 19.4% (experiments 6-4 and 6-5 in **Table 1**). No MMAIII was observed in the controls or in the denitrifying culture. To a lesser extent DMAV was also observed as a metabolite of MMAV conversion at the end of the assay; however, its molar yield was only 2.0−2.5%. Some DMAV (12 μM) was also detected in the denitrifying culture.

A second experiment was conducted to confirm biotransformation of MMAV under sulfate-reducing conditions (experiment 7-2 in **Table 1**). Extensive biologically mediated removal of MMAV was again observed. Likewise, MMAIII was an important product of the biotransformation.

Anaerobic Biotransformation of DMAV under Methanogenic Conditions. The anaerobic biodegradability of DMAV by methanogenic sludge was also tested under a variety of experimental conditions. Results from a typical experiment are shown in **Figure 4A**, in which 1.0 mM DMAV was incubated with either anaerobic sludge or heat-killed sludge. There is no decline in DMAV concentration in the heat-killed control, while there is a rapid decline in the DMAV concentration in the living treatment (**Figure 4A**). At the end of the incubation of 207 days, 95.3% of DMAV was removed. MMAV was identified as an important biotransformation of DMAV (**Figure 4B**). The maximum accumulation of MMAV occurred on day 7, at which time the molar yield was 32.6% of the DMAV removed. Only minor amounts of MMAV were incidentally detected in the heat-killed control.

The biotransformation of DMAV was also tested at a higher concentration of 5 mM. Biologically mediated removal was also observed at the higher concentration, but only 32.6% was removed after 81 days (experiment 2-2 in **Table 1**). Removal of DMAV decreased after the first week and finally ceased at day 52, suggesting the occurrence of a toxic intermediate. All further tests were conducted with initial concentrations of DMAV of 1.0 mM or lower to avoid inhibition problems. Working at the lower concentrations permitted extensive degradation of DMAV (**Table 1**). MMAV was observed as the biotransformation intermediate of DMAV in most of the experiments conducted.

Effect of Cosubstrate on Anaerobic Biotransformation of DMAV Conditions. The impact of cosubstrate on the biotransformation of DMAV was evaluated by incubating the organoarsenical in the presence and absence of a mixture of volatile fatty acids (VFA). The addition of VFA had no major effect on DMAV removal during the first 48 days (**Figure 5A**). However, VFA decreased the long-term biodegradability of DMAV from day 48 onward. The formation of MMAV as biotransformation product was evident during the first 30 days of the experiment (**Figure 5B**). The presence of VFA lowered the maximum accumulation of MMAV to a small extent and corresponded to a lower molar yield compared to the treatment lacking VFA (experiments 3-1 and 3-2 in **Table 1**).

Anaerobic Biotransformation of DMAV under Different Redox Conditions. An experiment was conducted comparing

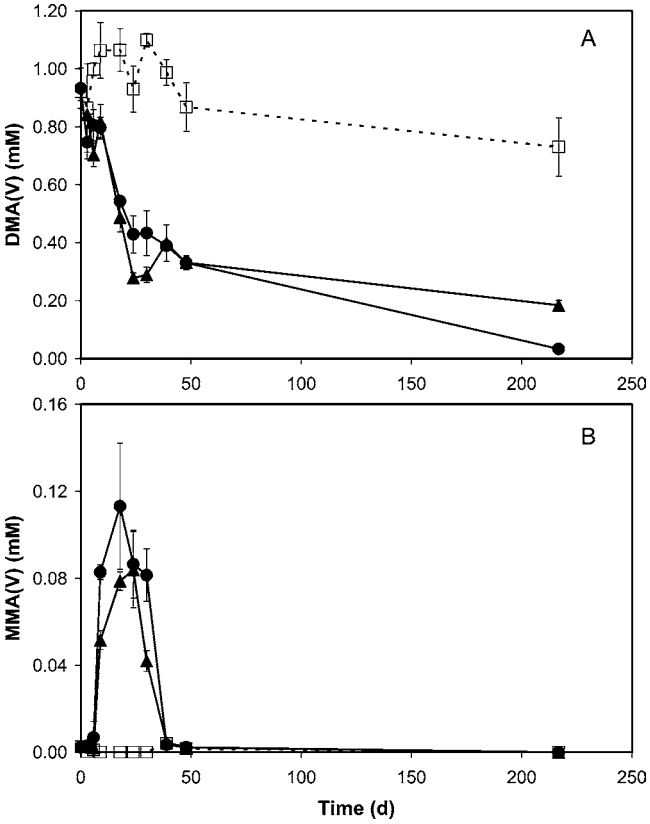

Figure 5. Biotransformation of DMAV (1.0 mM) during incubation with anaerobic sludge under methanogenic conditions in the presence and absence of added cosubstrate (volatile fatty acids). (**A**) Removal of DMAV; (**B**) formation of the intermediate, MMAV. Open squares, heat-killed control; filled circles, biologically active without cosubstrate; filled triangles, biologically active with cosubstrate addition.

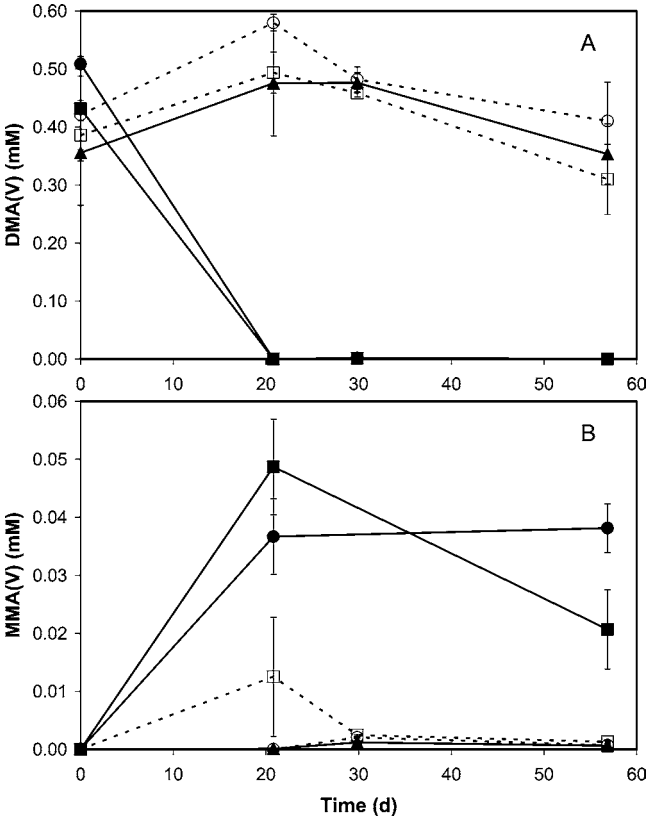

Figure 6. Biotransformation of DMAV (0.5 mM) during incubation with anaerobic sludge under methanogenic, sulfate-reducing, or denitrifying conditions. (**A**) Removal of DMAV; (**B**) formation of the intermediate, MMAV. Open circles, noninoculated control; open squares, heat-killed control; filled circles, biologically active under methanogenic conditions; filled squares, biologically active under sulfate-reducing conditions; filled triangles, biologically active under denitrifying conditions.

the anoxic degradation of DMAV (0.5 mM) under methanogenic, sulfate-reducing, and denitrifying conditions. The results indicate that complete removal of DMAV under the methanogenic and sulfate-reducing conditions had occurred already by the first sampling point (**Figure 6A**). The figure also indicates the lack of any major conversion of DMAV in the denitrifying culture, noninoculated control, and heat-killed inoculum control. MMAV accumulated to similar extents in the methanogenic and sulfate-reducing assays. MMAV concentrations in the biologically active treatments were significantly greater than an incidental detection in the heat-killed control. A similar experiment was repeated under sulfate-reducing conditions, which showed that the extensive degradation of DMAV could be reproduced; however, AsV was observed as the biotransformation product instead of MMAV (experiment 7-1 in **Table 1**).

Mass Balance of Anaerobic MMAV and DMAV Conversion. A mass balance was conducted for selected experiments in which volatile arsenic in the headspace, residual arsenic in the sludge, and total arsenic in the liquid phase were measured (**Table 2**). The total recovery of arsenic ranged from 55 to 76% for MMAV and from 53 to 68% for DMAV. The mass balance indicates only minor speciation to volatile arsenic compounds, which only accounted for 0.0002−0.0345% of the added arsenic. Identifiable arsenic species extracted from the sludge with NaOH accounted for 0.5−5.5% of added arsenic, of which inorganic arsenicals only accounted for 0.02−0.2% of the added arsenic. The bulk of the recovered arsenic was present in the liquid. The sum of identifiable species in the liquid was generally in agreement with total arsenic of the MMAV experiments; however, in one experiment the identified species were only 75% of the total. This observation was in agreement with the occurrence of unidentified peaks in the HPLC−ICP occurring at retention times (RTs) of 6.5 and 18.4 min. (For comparison, the RTs of MMAIII and MMAV were 1.9 and 3.7 min.). In the DMAV experiments, the sum of identifiable species in the liquid was generally only a small fraction of the total arsenic in the liquid (7−31%). Several unidentified peaks in the HPLC−ICP were observed that might account for the difference. These included peaks at RTs of 4.4 and 6.5 min as well as smaller peaks at 7.8 and 12.8 min. (For comparison, the RT of DMAV was 2.5 min.)

Biotransformation Kinetics. DMAV and MMAV were observed to degrade according to first-order kinetics. Based on the first-order rate expression

$$C_x = C_0 e^{-kt}$$

where C_x = concentration organoarsenical at a given time, C_0 = initial concentration, k = first-order rate constant, and t = time, k was estimated from natural logarithmic plots of the data wherever feasible as shown in **Table 1**. The k of MMAV ranged from 0.011 to 0.041 day^{-1}, whereas the k of DMAV ranged from 0.013 to 0.098 day^{-1}. The higher k values for DMAV removal observed in experiments 4-1 and 7-1 are associated with a newer sample of the methanogenic sludge utilized for the experiments. Only a few direct comparisons of kinetics were feasible. DMAV and MMAV biodegradations were compared in parallel during experiments 4-1 and 4-2 as well as 7-1 and 7-2. In those experiments, the rates of DMAV removal were

Table 2. Balance of Arsenic in Headspace (Volatiles), Sludge (NaOH Extractable), and Liquid from Selected Experiments with MMAV (A) and DMAV (B) as Substrates[a]

				liquid				
		volatiles	sludge	HPLC–ICP	ICP only			
expt no.	duration (days)	ICP only	NaOH extraction (HPLC–ICP)	identified species		sum (vol + slu + lig)[b]	input As	recovery (%)
				(A) MMAV as Substrate				
expt 1-1	235.4	9.27×10^{-6}	0.12	2.1	2.80	2.92	5.34	54.68
expt 1-2	235.4	4.15×10^{-5}	0.34	16.41	14.64	14.98	26.69	56.13
expt 3-3	216.7	5.30×10^{-4}	0.30	3.18	3.16	3.46	5.62	61.51
expt 3-4	216.7	4.04×10^{-4}	0.31	4.59	3.96	4.27	5.62	76.00
				(B) DMAVas Substrate				
expt 2-1	208.3	1.94×10^{-3}	0.24	0.29	3.20	3.44	5.62	61.24
expt 2-2	208.3	5.71×10^{-5}	0.13	18.22	18.98	19.11	28.10	68.01
expt 3-1	216.7	5.24×10^{-4}	0.15	0.13	1.83	1.98	3.75	52.82
expt 3-2	208.3	2.91×10^{-5}	0.13	0.67	2.13	2.26	3.75	60.39

[a] Unless indicated otherwise, values are expressed as milligrams of arsenic per bottle. Volatile and liquid fractions are averages of triplicates, whereas as sludge values are averages of duplicates. [b] Sum of arsenic in the volatile fraction (vol), sludge (slu), and liquid (liq) fractions; the value from the liquid fraction was based on the total As (ICP only) measurement.

from 8.3- to 8.8-fold higher than those of MMAV. On two occasions, *k* values of MMAV removal were obtained in parallel under methanogenic and sulfate-reducing conditions. In experiments 6-4 and 6-5, the *k* value under sulfate-reducing conditions was 2.6-fold higher. However, the *k* values in a second comparison (experiments 4-2 and 7-2) were similar under methanogenic and sulfate-reducing conditions.

Metabolism of Endogenous Substrate. In most assays, MMAV and DMAV were added as sole sources of exogenous carbon and energy. However, the electron-donating content of these compounds was relatively small compared to endogenous substrate available in the sludge inoculum. The theoretical oxygen demand (ThOD) of MMAV and DMAV supplied at 0.7 and 0.5 mM, respectively, is 45 and 64 mg L^{-1}. On the basis of the methane production and sulfate consumption in experiments just incubated with the sludge inoculum, the endogenous substrate level corresponds to 156 and 284 mg of ThOD L^{-1}, respectively, after approximately 140 days of incubation (**Figure 7**). The addition of 0.5 mM DMAV did not greatly impact the methane production or the sulfate reduction. The lack of any noteworthy effect suggests two things. First, DMAV and its biotransformation products were not causing inhibition. Second, the additional 64 mg of ThOD L^{-1} of added DMAV was not mineralized to a large extent. The largest statistically significant differences between the DMAV treatment and the sludge blank was an additional 15.9 mg of ThOD L^{-1} of methane on day 115, which could account for 25% mineralization of DMAV. The addition of 0.7 mM MMAV caused serious inhibition of methane production as well as sulfate reduction (**Figure 7**). Since MMAV itself is not inhibitory to methanogenesis (*16*), the results suggest that one of the biotransformation products is responsible.

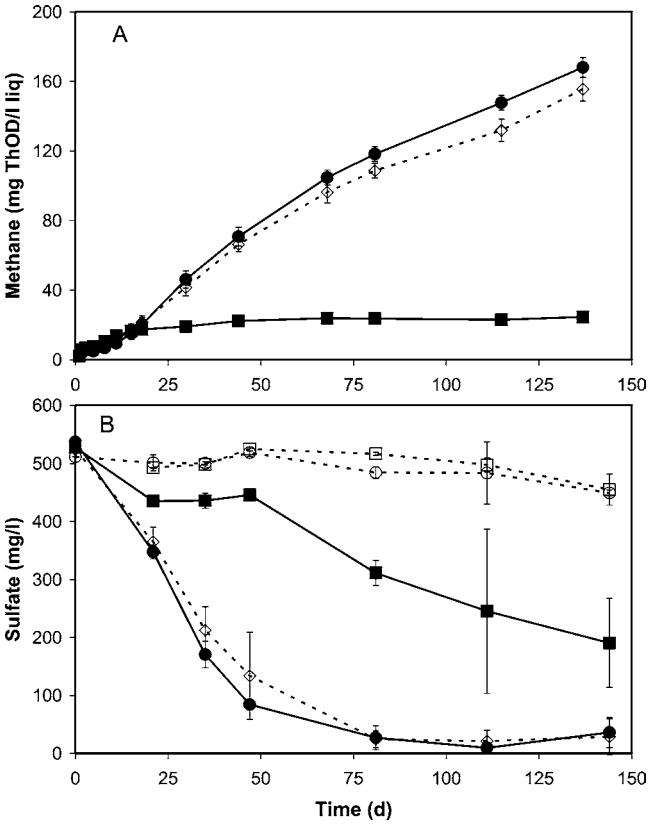

Figure 7. Impact of MMAV and DMAV on endogenous decay of sludge inoculum. (**A**) Production of methane expressed as theoretical oxygen demand (ThOD) produced per liter of medium; (**B**) consumption of sulfate. Open circles, noninoculated control; open squares, heat-killed control; open diamonds, biologically active sludge inoculum without any addition of organoarsenicals; filled circles, biologically active treatment containing 0.5 mM DMAV; filled squares, biologically active treatment containing 0.7 mM MMAV.

DISCUSSION

Biodegradation of MMAV and DMAV. Degradation of MMAV and DMAV was demonstrated in this study under strict anaerobic conditions. These organoarsenicals were largely removed in the presence of biologically active sludge, while they were not significantly removed in noninoculated or heat-killed sludge controls. Degradation percentages of MMAV and DMAV ranged from 24 to 96% and from 33 to 100%, respectively. Degradation occurred under either methanogenic or sulfate-reducing conditions but not in the presence of nitrate. This constitutes the first report of extensive degradation of MMAV and DMAV under methanogenic and sulfate-reducing redox conditions. Previous studies conducted with marine sediments under strict anaerobic conditions have only demonstrated minor conversions of MMAV and DMAV (*17*). Several investigators have studied MMAV and DMAV bioconversion in soils at high moisture contents that presumably correspond to anaerobic or low oxygen conditions. When compared to fully aerobic conditions, the saturated soils provide improved removals of these compounds (*7*, *9*, *18*). Up to 85% removal of DMAV was observed after 70 days of incubation in wet soil (*9*).

Little is known about the bacteria responsible for metabolizing MMAV and DMAV. The few isolates evaluated so far have been tested under aerobic conditions. *Mycobacterium neoaurum* was shown to convert MMAV by 30% to AsV and AsIII during growth on rich microbial medium (*19*). Two DMAV metabolizing strains were isolated from lake water (*20*). Strain 12M16, closely related to *Aeromonas hydrophila*, and strain 10M1, closely related to *Comamonas testosterone*, removed DMAV by 78 and 34%, respectively; resulting in the formation of inorganic arsenicals and MMAV (*20*). *Escherichia coli* A3-6 isolated from the gastrointestinal tract of rats was shown to convert DMAV to two unidentified metabolites in the presence of cysteine (*21*).

Biotransformation Products of MMAV. In this study, MMAIII was consistently observed as an important metabolite of MMAV degradation and it was recovered in molar yields ranging from 5 to 47%. This is the first time that MMAIII has been reported as a microbial biotransformation product of MMAV. The occurrence of MMAIII suggests that reduction of MMAV is an important mechanism of the biotransformation. The only known enzyme responsible for the reduction of MMAV to MMAIII is glutathione-*S*-transferase ω hGSO1-1 from mammals (*22*). Aside from hGSO1-1, the mammalian gene *cyt19* is hypothesized to carry out the reduction of MMAV to MMAIII (*23*). Recombinant *cyt19* proteins are responsible for the methylation of AsIII to MMAV and DMAV via cycles of oxidative methylation and reduction in a process known as the Challenger mechanism. The Challenger mechanism is also implicated in the microbial methylation of inorganic arsenic (*24*). Genes homologous to *cyt19* have recently been shown to occur in the prokaryote *Halobacterium* sp. NRC-1 (*25*).

In addition to MMAIII, DMAV was observed as a metabolite of MMAV biotransformation in one experiment corresponding to a molar yield of 2−2.5%. The Challenger mechanism would account for this metabolite through the reduction of MMAV to MMAIII followed by oxidative methylation to DMAV. The metabolite DMAV was also observed previously as a biotransformation product of MMAV salts applied to golf greens (*8*). DMAV was recovered at a molar yield of 8% in the percolate water collected over 98 days after the application of MMAV.

Biotransformation Products of DMAV. An important biotransformation product from DMAV metabolism identified in this study was MMAV, which was recovered in molar yields ranging from 8 to 65%. The occurrence of MMAV as an important biotransformation product points to demethylation as an important mechanism of the biotransformation. Evidence for demethylation of DMAV under strict anaerobic conditions in marine sediments was previously observed based on the appearance of an unresolved metabolite peak that corresponded to either MMAV, AsV, or ASIII that coelute at the same retention time (*17*). MMAV was detected as a product of the biotransformation of DMAV in wet soil (*6*). The most anaerobic condition tested was wet soil amended with organic matter (50 tons ha^{-1} of hay, manure, or sludge), and this resulted in DMAV being converted to MMAV with a molar yield of 61% in 60 days. Under aerobic conditions, DMAV is largely converted to AsV with temporal accumulation of MMAV as an intermediate in the degradation process (*6*, *9*, *20*). The molar yields of MMAV ranged from 0.8 to 8% of the DMAV consumed during aerobic degradation. The evidence in the literature suggests that DMAV is mineralized to AsV and CO_2 under aerobic conditions (*6*, *9*, *18*, *20*), while under anaerobic conditions DMAV is largely converted to biotransformation products (*6*, *9*). This pattern seems to hold true under the strict anaerobic conditions tested in this study, where little evidence of any inorganic arsenical formation was observed.

Mineralization Not an Important Fate. For both pesticides tested, there was no strong evidence of conversion to inorganic forms of arsenic, AsV or AsIII. In 15 out of 16 experiments conducted, freely soluble inorganic arsenicals were either nondetectable or present in trace amounts. These compounds were also not recovered beyond background levels even after extracting the sludge with hot NaOH, which is known to extract 60−70% of inorganic arsenicals sequestered by anaerobic sludge (Sierra-Alvarez et al., unpublished results). This clearly indicates that mineralization of arsenic was not an important fate during anaerobic biotransformation of MMAV and DMAV in this study. Furthermore, the extra methane production from DMAV-amended cultures could have only accounted for 25% of the electron equivalents in the DMAV in the best scenario, clearly suggesting that carbon in DMAV was not mineralized to a great extent. In contrast to MMAV and DMAV, partial mineralization of N-substituted phenylarsonates to AsIII and AsV was observed under similar experimental conditions (*26*).

Volatile Arsenical Species. AsV, MMAV, and DMAV are known to be microbially converted to volatile arsine and methylarsine compounds (*24*). However, such compounds are usually only formed at extremely low yields. After incubating DMAV with aerobic and flooded soils for 60−70 days, only 0.002−1.5% was converted to alkylarsines (*9*, *27*). Similar results were obtained with MMAV incubated in aerobic soil (*9*). Yields of volatile arsines are also low (0.00015−0.4%) in pure cultures of methanogens or methanogenic consortia administered with either AsV (*28*, *29*) or AsIII (*16*). Consistent with these observations, volatile forms of arsenic volatiles in this study only accounted for 0.0002−0.035% of the added arsenic.

Implications. The results of this study suggest that the biotransformation of organoarsenical pesticides MMAV and DMAV occurs in methanogenic and sulfate-reducing consortia. An important biotransformation product from MMAV was found to be MMAIII, and the methane production from the decay of endogenous substrate in the sludge inoculum ceased after the initial formation of MMAIII. This observation agrees with the high toxicity of MMAIII to methanogenesis. The 50% inhibiting concentration of MMAIII was found to be 9.1 μM, which is greater than 550-fold more toxic than MMAV (*16*). Aside from microbial toxicity, MMAIII is a concern for public health. MMAIII is more toxic to various human cell lines compared to AsV and AsIII. Furthermore, MMAIII has genotoxic activity (*30*). The literature evidence suggests that MMAV is largely mineralized to AsV under aerobic conditions, albeit with a slower removal rate compared to anoxic conditions (*9*, *19*, *31*). Considering the elevated toxicity of the anaerobic biotransformation product compared to the relatively benign fate in aerobic environments, regulators should carefully assess the use of MMAV in areas adjacent to anaerobic environments (shallow aquifers, water bodies, wetlands, etc).

The biotransformation products detected during anaerobic degradation of DMAV were MMAV and several unidentified metabolites. The methanogenesis of the endogenous substrate was not inhibited during the degradation assays with DMAV, indicating a much lower toxicity of its biotransformation products compared to those from degradation assays with MMAV as the parent compound. There was no evidence in this study that the MMAV formed during DMAV degradation was further biotransformed to MMAIII, although the possibility needs to be considered. Anaerobic bioconversion of DMAV was

faster than MMAV as evidenced by an approximately 9-fold higher first-order rate constant.

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