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Methanogenic inhibition by roxarsone (4-hydroxy-3-nitrophenylarsonic acid) and related aromatic arsenic compounds

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ABSTRACT

Roxarsone (4-hydroxy-3-nitro-phenylarsonic acid) and *p*-arsanilic acid (4-aminophenylarsonic acid) are feed additives widely used in the broiler and swine industry. This study evaluated the inhibitory effect of roxarsone, *p*-arsanilic, and other phenylarsonic compounds on the activity of acetate- and H_2-utilizing methanogenic microorganisms. Roxarsone, *p*-arsanilic, and 4-hydroxy-3-aminophenylarsonic acid (HAPA) inhibited acetoclastic and hydrogenotrophic methanogens when supplemented at concentrations of 1 mM, and their inhibitory effect increased sharply with incubation time. Phenylarsonic acid (1 mM) inhibited acetoclastic but not H_2-utilizing methanogens. HAPA, a metabolite from the anaerobic biodegradation of roxarsone, was found to be sensitive to autooxidation by oxygen. The compound (2.6 mM) caused low methanogenic inhibition (only 14.2%) in short-term assays of 12 h when autooxidation was prevented by supplementing HAPA solutions with ascorbate. However, ascorbate-free HAPA solutions underwent spontaneous autooxidation in the presence of oxygen, leading to the formation of highly inhibitory compounds. These results confirm the microbial toxicity of organoarsenic compounds, and they indicate that biotic as well as abiotic transformations can potentially impact the fate and microbial toxicity of these contaminants in the environment.

1. Introduction

Roxarsone (4-hydroxy-3-nitrophenylarsonic acid) and *p*-arsanilic acid (4-aminophenylarsonic acid) are aromatic arsenicals commonly used as feed additives in the poultry industry for improving feed efficiency, increasing the rate of weight gain, and for treatment and prevention of coccidial intestinal parasites [1]. These phenylarsonic derivatives are also used in the swine industry to control enteric diseases or to improve productivity. They are incorporated in the feed at levels of 25–50 mg/kg for roxarsone, and 50–100 mg/kg for *p*-arsanilic acid [2]. Based on broiler production and roxarsone feed dosage, it is estimated that approximately 900 metric tons of roxarsone are released into environment in the U.S. annually by the poultry industry alone, an amount that is equivalent to 250 metric tons of arsenic [3]. Roxarsone and *p*-arsanilic show a low bioaccumulation potential and are largely excreted [4]. Consequently, high concentrations of arsenic ranging from 0.4 to 119 mg/kg have been detected in the litter of animals which diet is supplemented with these organoarsenic compounds [5–9]. Poultry litter has been reported to contain concentrations of roxarsone ranging from 14 to 54 mg/kg (equivalent to 36–93% of the total arsenic) [4,6,7]. Fresh poultry litter was also shown to contain 4-hydroxy-3-aminophenylarsonic acid (HAPA), a reduced biotransformation product of roxarsone, accounting for 25% of the species identified [10]. Animal waste is currently not classified as hazardous waste by the U.S. EPA, and land application of manure tainted with organoarsenic compounds is a common practice in crop growing fields near to poultry and swine houses which can contribute to environmental emissions of arsenic. Elevated levels of arsenic have been detected in pore water from agricultural fields amended with poultry litter (<29 μg/L) and in sediments of several Chesapeake Bay tributaries near areas with intensive poultry and swine farming (12 mg/kg) [11,12].

Organic arsenic compounds and other herbicides when applied in agriculture fields can be subjected to biotic and abiotic transformations that can lead to degradation products of increased toxicity and mobility. As an example, roxarsone can undergo microbial reduction in anaerobic environments resulting in the formation of the amino-aromatic compound, 4-hydroxy-3-aminophenylarsonic acid (HAPA), and inorganic arsenic [13–15]. Arsenate is formed from roxarsone during composting of poultry litter [6] and during incubation of contaminated swine waste [15] and soil [16]. In poultry litter, 4-hydroxyphenylarsonic acid, phenylarsonic acid, and methylated arsenic species have been detected in addition to HAPA and inorganic arsenic [5–7,10,17]. High concentrations of phenylarsonic acid and other phenyl arsenicals have also been detected in soil and groundwater at several former ammunition depots and warfare agent production sites worldwide [18–20].

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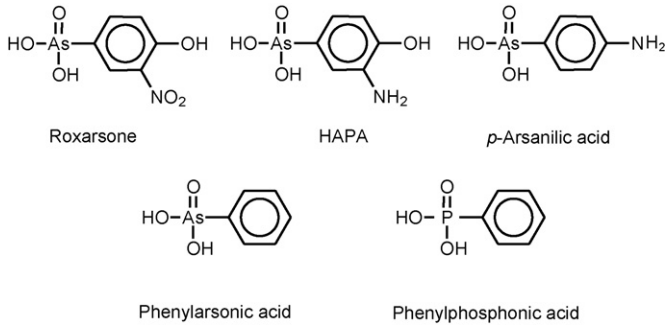

Fig. 1. Chemical structure of aromatic arsenic compounds considered in this study. HAPA = 4-hydroxy-3-aminophenylarsonic acid.

These contaminants are products from the oxidation and hydrolysis of more complex arsenic containing chemical warfare agents such as diphenylchloroarsine (Clark I) or diphenylcyanoarsine (Clark II) and phenyldichloroarsine. Daus et al. [20] reported that 93% of a total of 2.5 mg/L arsenic in German groundwater at an abandoned site contaminated by warfare agents was in the form of phenylarsonic acid and diphenylarsinic acid.

The inhibitory effect of inorganic arsenic species (arsenite (As^{III}), and arsenate (As^{V})) on microorganisms has been reported in numerous studies [21–25], and the mechanisms of bacterial tolerance to inorganic arsenic are well understood [26,27]. In contrast, there is little information on the microbial toxicity of aromatic organoarsenic pesticides. The objective of this study was to investigate the inhibitory effect of the widely used pesticide roxarsone and its anaerobic metabolite, 4-hydroxy-3-aminophenylarsonic acid, to methanogens. Other compounds structurally related to roxarsone were also tested, *i.e.* *p*-arsanilic acid, phenylarsonic acid, and phenylphosphonic acid, to determine the impact of different chemical functional groups on their toxic impact. Information on the inhibitory impact of organoarsenicals on methanogenic archaea is important, because methanogenesis is the final step in the microbial degradation of organic matter in many anaerobic environments, including sediments, anoxic groundwater, and anaerobic wastewater treatment systems. Fig. 1 illustrates the chemical structure of the compounds evaluated.

2. Material and methods

2.1. Anaerobic microorganisms and basal mineral medium

Anaerobic methanogenic sludge in the form of biofilm granules was used as inoculum in the bioassays. The consortium was obtained from an industrial anaerobic treatment plant treating recycled paper wastewater (Industriewater, Eerbeek, The Netherlands). The content of volatile suspended solids (VSS) in the sludge was 12.9%. The sludge was stored at 4 °C under a N_2 atmosphere.

The basal mineral medium (pH 7.20) used in the toxicity tests had the following composition (mg/L): KH_2PO_4 (41); $CaCl_2{\cdot}2H_2O$ (11); $MgSO_4{\cdot}7H_2O$ (11); $MgCl_2{\cdot}6H_2O$ (87); NH_4Cl (742); $NaHCO_3$ (3333); yeast extract (20) and 1 mL/L of a trace element solution containing (in mg/L): $FeC1_3{\cdot}4H_2O$, 2000; $CoCl_2{\cdot}6H_2O$, 2000; $MnCl_2{\cdot}4H_2O$, 50; $AlCl_3{\cdot}6H_2O$, 90; $CuCl_2{\cdot}2H_2O$, 30; $ZnCl_2$, 50; H_3BO_3, 50; $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 90; $Na_2SeO_3{\cdot}5H_2O$, 100; $NiCl_2{\cdot}6H_2O$, 50; EDTA, 1000; HCl 36% (1 mL). The pH of the medium was adjusted with HCl or NaOH, as needed.

2.2. Methanogenic toxicity bioassays

2.2.1. Short-term bioassays

The inhibitory effect of organic arsenical compounds towards acetoclastic methanogens was evaluated in shaken batch bioassays. Serum flasks (160 mL) were supplemented with 25 mL of mineral media and acetate (1.88 g/L as sodium acetate), and inoculated with the anaerobic consortium (1.5 g VSS/L). Treatments were conducted in triplicate. The bottles were sealed with butyl rubber septa and crimp aluminum seals and flushed with a gas mixture of N_2/CO_2 (80:20, v/v) for 2–3 min to create an anaerobic atmosphere. Treatments were incubated for 12–14 h before the addition of the organic arsenic compound. After the adaptation period, the headspace was flushed with N_2/CO_2 for 2–3 min to remove the methane and, subsequently, the desired concentration of the toxicant was supplemented by injecting a known volume of a concentrated stock solution. Control flasks lacking the organoarsenical were run in parallel. Flasks were again incubated for 12 h. Samples of the gas in the headspace were obtained periodically (every 1.5–3.0 h) and analyzed for methane.

2.2.2. Long-term toxicity assays

The long-term inhibitory effect of organic arsenic compounds to acetoclastic and hydrogen-utilizing methanogenic microorganisms was evaluated in shaken batch bioassays incubated for 19 d as described above for the short-term bioassays. The exogenous substrate (acetate or hydrogen) was supplemented in three feedings. The first feeding was provided at the beginning of the experiment, the second at day 7, and the third at day 13. Previous to each feeding, the methane accumulated in the headspace was removed by flushing with a mixture of N_2/CO_2 gas (80/20, v/v). Acetate (1.88 g/L as sodium acetate) in the second and third addition was replenished by injecting a neutralized, concentrated stock solution (0.5 mL) in order to minimize dilution of the culture medium. Hydrogen was supplied as gas mixture of H_2/CO_2 (80:20, v/v) in the headspace (170 MPa). Controls lacking the organic arsenic compounds were run in parallel. The culture medium and inoculum was not replaced during the course of the experiment. Samples of the headspace were analyzed for methane every 1.5–3.0 h for 12–14 h on the first day, then one or twice per day during the next 7 d. The same procedure was followed to monitor methane production in successive feedings.

All bioassays were conducted in an orbital shaker (120 rpm) at a temperature of 30 ± 2 °C. The specific methanogenic activities (mg CH_4/(g VSS d)) were calculated from the slope of the cumulative methane production versus time, and the biomass concentrations at the end of the assay as the means of the values for triplicate culture flasks. In each case, the maximum specific activity at a given concentration of the organic arsenic compound considered was determined during the time period when the organoarsenical-free control displayed maximum specific activity. The inhibition observed was calculated as shown below:

$$\text{Inhibition}(\%) = 100 - \left[100 \times \frac{\text{Maximum Specific Activity at the Tested Concentration}}{\text{Maximum Specific Activity of the Control}}\right]$$

The initial concentrations of a toxicant causing 20, 50 and 80% reduction in activity compared to an uninhibited control were referred to as IC_{20}, IC_{50} and IC_{80}, respectively. These values were calculated by interpolation in the graph plotting the inhibition observed (expressed as percent) as a function of the inhibitor concentration. Unless otherwise indicated, reported inhibitory concentrations are average values of triplicate assays and corresponding standard deviations.

2.2.3. Preparation of HAPA solutions

HAPA solutions were prepared by dissolving the compound in distilled water amended with the reducing agent, ascorbic acid (1.14 mM), unless otherwise indicated. Ascorbic acid was supplement to prevent autooxidation of HAPA. To ensure complete

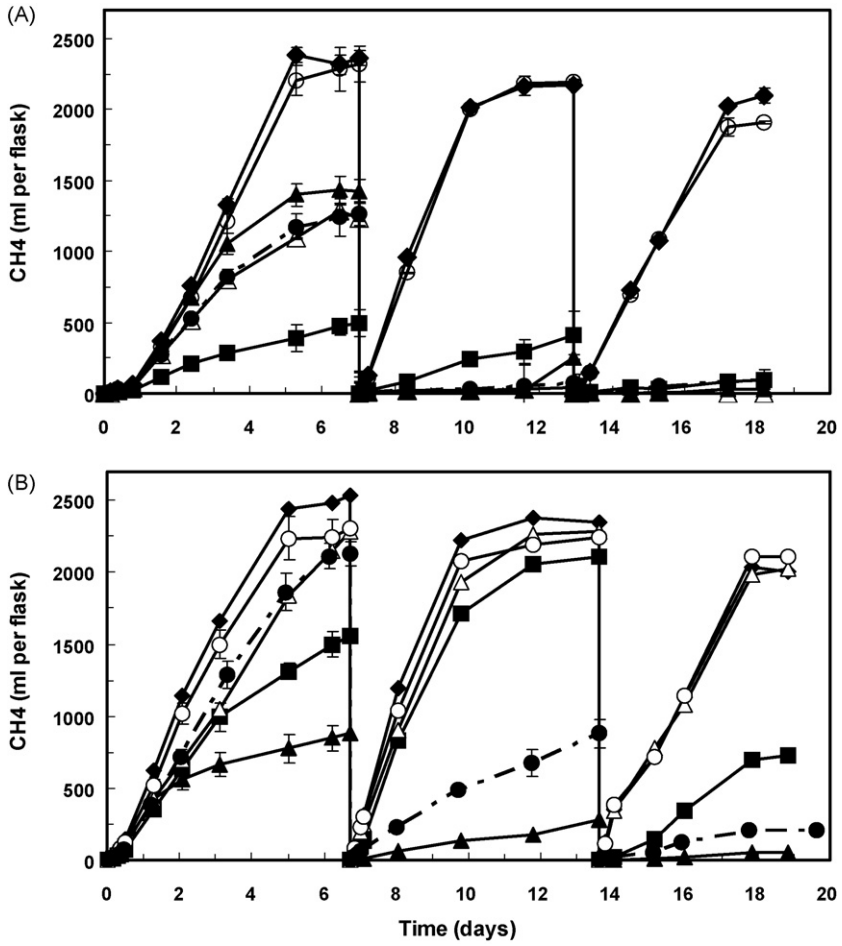

Fig. 2. Cumulative methane production with organic arsenical compounds (1 mM) by acetate-utilizing methanogens (A) and hydrogen-utilizing methanogens (B) as a function of time in long-term incubation bioassays. Control lacking toxicant (♦); roxarsone (■); *p*-arsanilic acid or *p*-Ars (▲); phenylarsonic acid or Ph-A (△); 4-hydroxy-3-aminophenylarsonic or HAPA (●), and phenylphosphonic acid or Ph-P (○). *Note*: Ascorbic acid was supplied to the mineral media to prevent oxidation of HAPA and *p*-arsanilic to a final concentration of 1.14 mM.

dissolution of the compound, the solution was stirred at 60 °C for approximately 10 min.

Oxidized HAPA solutions were prepared by raising the pH of the solution to 9.10 with NaOH and sparging air for the desired period of time. The oxidation was stopped by adjusting the pH to 7.0 with HCl, and immediately adding ascorbic acid to a final concentration of 1.14 mM. Distilled water was added to compensate for evaporation losses.

2.2.4. Analytical methods

The methane content in the headspace of the flasks was determined by gas chromatography using a 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 ID, 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 and a split flow of 32.4 mL/min. Samples from the headspace (100 μL) were collected using a pressure-lock gas syringe.

The total concentration of arsenic in liquid samples was determined using an Agilent 7500a ICP-MS fitted with an ASX500 autosampler (CETAC Technologies, Omaha, NE). The ICP-MS system was operated at a Rf power of 1500 W, a plasma gas flow of 15 l/min and a carrier gas flow of 1.2 L/min. The acquisition parameters used were: arsenic measured at *m*/*z* 75; terbium (IS) measured at *m*/*z* 159; 3 points per peak; 1.5 s dwell time for As, 1.5 s dwell time for Tb; number of repetitions = 7. Inorganic arsenic species (As^{III} and As^{V}) and organic arsenic species (roxarsone, *p*-arsanilic acid and HAPA) in liquid samples were analyzed by HPLC/inductively coupled plasma/mass spectrometry (HPLC–ICP–MS) using a method adapted from Gong et al. [28]. 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 mm × 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. 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 of the detector were as described above for the analysis of total arsenic. The injection volume was 10 μL. The detection limit for the various arsenic species was 0.1 μg/L.

Some roxarsone and HAPA analyses were performed using an HPLC with a diode array detector (Hewlett-Packard 1090, Agilent Technologies, Santa Clara, CA). The chromatograph was equipped with an Ion Pac-AS14 column (4 mm × 250 mm) and an Ion-Pac-AG14 pre-column (4 mm × 50 mm) (Dionex, Sunnyvale, DA), which were maintained at room temperature. Phosphate (10 mM; pH 7.2) was used as the eluent at a flow rate of 2 mL/min. The injection volume was 25 μL. Roxarsone and HAPA were detected at a wavelength of 222 nm.

Other parameters (e.g., pH, VSS) were determined according to Standard Methods [29].

2.2.5. Chemicals

Roxarsone (CAS 121-19-7; 98% purity), and *p*-arsanilic acid (CAS 98-50-0; 98% purity) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Phenylarsonic acid (CAS 98-05-5; 99% purity) and 4-hydroxy-3-aminophenylarsonic acid; CAS 2163-77-1; 97% purity) were purchased from Platz and Bauer, Inc. (Waterbury, CT). Phenylphosphonic acid (CAS 1571-33-1; 98% purity) was acquired from Alfa Aesar (Ward Hill, MA). All compounds were utilized as received.

3. Results

3.1. Methanogenic toxicity of aromatic arsenic compounds

The inhibitory effect of various aromatic arsenicals (1 mM) towards acetate- and H_2-utilizing methanogens in an anaerobic mixed microbial consortium was evaluated in batch bioassays with a total duration of 19 d (Fig. 2). Initially, roxarsone was the most severe inhibitor to acetate-utilizing methanogens and exposure to this compound resulted in 84% decrease of the methanogenic activity in the first substrate feeding, respectively (Fig. 3). HAPA, *p*-arsanilic and phenylarsonic acid displayed little or no inhibition during the initial 4 d of exposure. However, all the organoarsenic compounds caused a marked decrease of the methanogenic activity afterwards (>90% inhibition). In contrast with these results, phenylphosphonic acid, a structural analogue of phenylarsonic acid, only had marginal effect on the acetoclastic methanogenic activity.

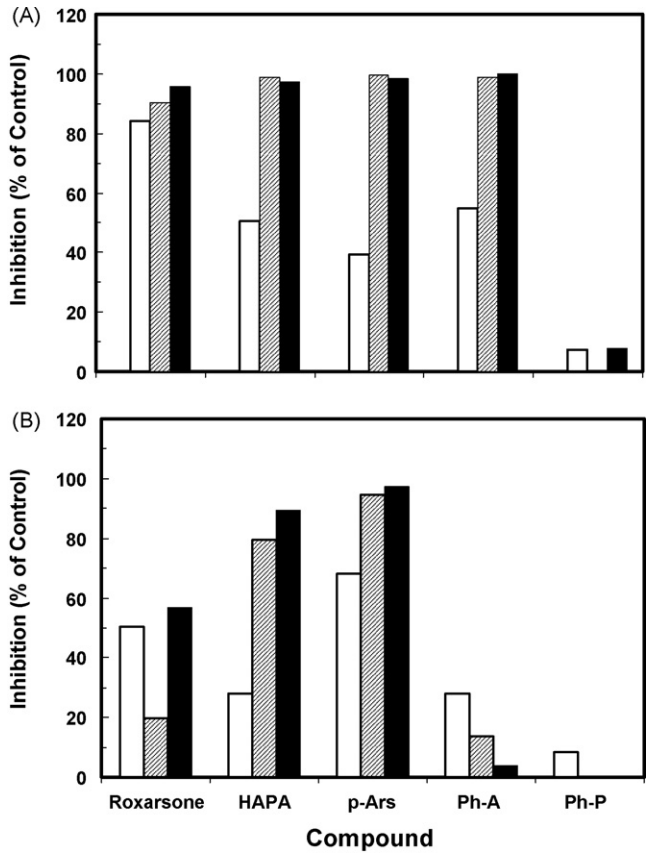

Fig. 3. Impact of exposure time on the inhibitory effect of aromatic arsenic compounds (1 mM) to acetate-utilizing methanogens (panel A) and hydrogen-utilizing methanogens (panel B). Substrate feeding: (□) first; (▨) second, and (■) third. *Note*: Ascorbic acid (1.14 mM) was supplied to the culture medium to prevent compound autooxidation.

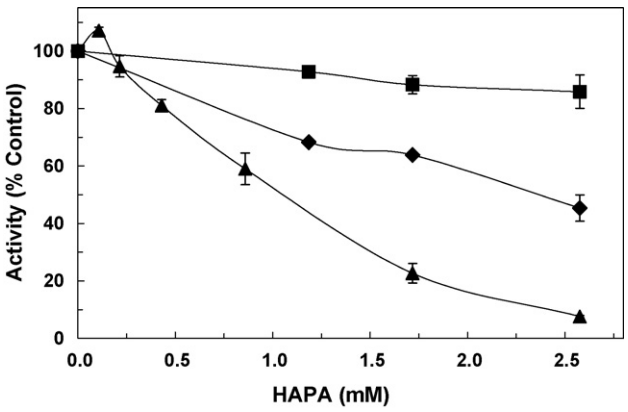

Fig. 4. The effect of the initial concentration of 4-hydroxy-3-aminophenylarsonic acid (HAPA) on the activity of acetoclastic methanogens in the presence and absence of the reducing agent, ascorbic acid (1.14 mM). The methanogenic activity is expressed as percent of the activity determined in uninhibited controls. HAPA + ascorbic acid (■); HAPA oxidized by gentle stirring for: 12 min (♦); and 40 min (▲).

H_2-utilizing methanogens were generally less susceptible to inhibition by the organoarsenicals than acetoclastic methanogens. *p*-Arsanilic acid was the most severe inhibitor to the hydrogenotrophic methanogens (Figs. 2B and 3), and exposure to this compound resulted in 68, 95, and 97% decrease of the methanogenic activity determined in the first, second and third assays feeding, respectively. The inhibitory effect of roxarsone and HAPA was also observed to increase with increasing exposure time (Fig. 2B), and these compounds caused 57 and 89% inhibition, respectively, in the third substrate feeding (Fig. 3). Phenylarsonic acid and phenylphosphonic acid were not or only mildly inhibitory to H_2-utilizing methanogens.

3.2. Methanogenic inhibition by oxidized HAPA

Freshly prepared solutions of HAPA were observed to turn from colorless to light yellow with time, due to spontaneous autooxidation reactions of the compound in the presence of oxygen. The impact of mild aeration on the inhibitory effect of HAPA towards acetoclastic methanogens was evaluated in short-term bioassays with a total duration of 12 h. Aeration was provided by gentle mechanical stirring. The inhibitory effect of HAPA in solutions amended with the reducing agent, ascorbic acid (1.14 mM), was compared to that of ascorbate-free HAPA solutions stirred for 12 and 40 min. In the presence of ascorbic acid, HAPA does not undergo oxidative transformations by reaction with atmospheric oxygen as was confirmed by chromatographic analysis (results not shown).

HAPA was not or only moderately inhibitory to acetoclastic methanogens in assays spiked with ascorbic acid (Fig. 4). In contrast, oxidized HAPA solutions caused severe inhibition. A considerable decrease in the methanogenic activity with increasing oxidation time was noted. As an example, 14.2% methanogenic inhibition was observed in assays supplemented with HAPA (2.6 mM) and ascorbate. In contrast, as much as 54.7 and 92.4% methanogenic inhibition was determined in assays supplemented with the HAPA solutions (2.6 mM) previously exposed to gentle aeration for 12 and 40 min, respectively.

Additional bioassays were performed to evaluate the impact of oxidation duration on the methanogenic toxicity of HAPA. To promote oxidation, alkaline solutions (pH 9.10) of HAPA were subjected to air sparging for different periods of time ranging from 4 min to 16 d. Fig. 5 shows the effect of different oxidation times on

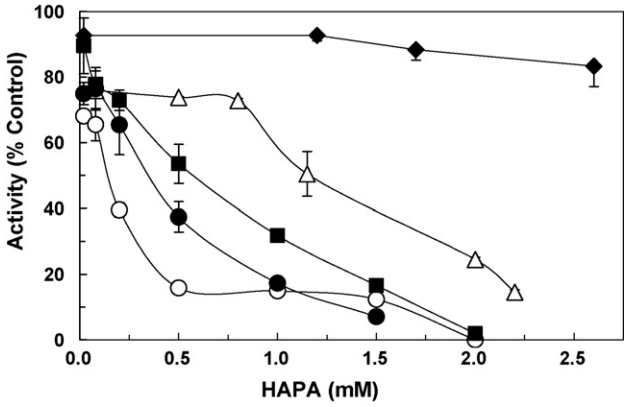

Fig. 5. Impact of the initial concentration of 4-hydroxy-3-aminophenylarsonic acid (HAPA) and oxidation time on the activity of acetoclastic methanogens. Alkaline solutions of HAPA (pH 9.1) were oxidized aggressively by sparging air for 4 min (△), 1 h (■), 16 h (○), and 4 d (●); HAPA supplied with ascorbic acid (1.14 mM), not aerated (♦).

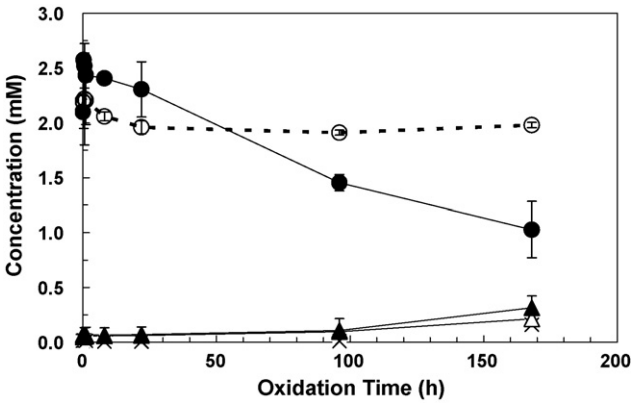

Fig. 7. Change in the concentration of 4-hydroxy-3-aminophenylarsonic acid (HAPA) and other arsenic species with oxidation time. Alkaline solutions of HAPA (pH 9.10) were oxidized by sparging air. HAPA (●), total soluble arsenic (○), As^{V} (△); As^{III} (×), soluble inorganic arsenic (▲).

the residual methanogenic activity determined in assays supplemented with varying concentrations of HAPA. Fig. 6 plots the IC_{50} values determined for autooxidized HAPA solutions as a function of oxidation time. The results show that the methanogenic toxicity of HAPA increased sharply with the time of oxidation up to 16 h, and that thereafter the oxidation resulted in a partial decrease of the inhibitory effect.

HAPA oxidation also caused appreciable changes in the color and composition of the solution. While aqueous solutions of HAPA containing ascorbic acid were colorless, solutions oxidized for short periods of times presented a light yellow color which turned dark yellow-brownish when the solutions were oxidized aggressively by aeration at alkaline pH values (pH 9.10). The concentration of HAPA in the oxidized solution decreased steadily with oxidation time (Fig. 7). A concomitant release of inorganic arsenic species (As^{III} and As^{V}) was observed albeit at sub-stoichiometric concentrations, suggesting that autooxidation of HAPA resulted in the formation of unknown transformation products. The sharp increase in toxicity observed after very short times of oxidation (Figs. 5 and 6), when only a small fraction of HAPA had been transformed, indicates that some of the autooxidation products displayed high antimicrobial activity.

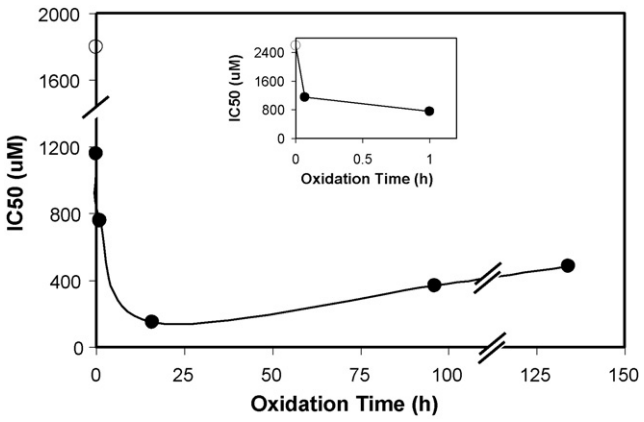

Fig. 6. Impact of aeration time on the 50% inhibitory concentration (IC_{50}) of HAPA. Legend: (●), IC_{50} of oxidized HAPA solutions; (○), highest concentration tested of unoxidized HAPA, causing 14.2% inhibition. Insert shows time course over the first hour of oxidation. HAPA solutions (pH 9.10) were oxidized by air sparging.

4. Discussion

4.1. Methanogenic inhibition by phenylarsonic compounds

Several phenylarsonic acid derivates, including roxarsone, *p*-arsanilic, and HAPA when supplemented at concentrations of 1 mM were found to cause low to intermediate methanogenic inhibition during the first day of exposure. However, the inhibitory effects of these compounds increased sharply with increasing exposure time (Fig. 2). The low methanogenic inhibition observed for *p*-arsanilic and HAPA during the initial phase of these bioassays is in good agreement with those obtained in an earlier study utilizing short-term tests (8–12 h) [21].

In contrast with the severe inhibitory effects displayed by the nitro- and amino-substituted phenylarsonic compounds against acetate- and H_2-utilizing methanogens, phenylarsonic acid (1 mM) was only inhibitory to acetoclastic methanogens, and phenylphosphonic acid (1 mM) did not have any impact on the specific methanogenic activity of the anaerobic consortium utilized in this study. In agreement with our results, amino-aromatic compounds have been reported to be less toxic towards acetoclastic methanogens than their nitro-aromatic counterparts [30]. There is a general lack of information on the microbial toxicity of phenylarsonate compounds, but the methanogenic inhibition of nitro-aromatic compounds is well documented in the literature [30–32]. Donlon et al. [30] demonstrated that the toxicity of nitro-substituted aromatic compounds was related to the chemical reactivity of the compounds, and that hydrophobic compound partitioning on bacterial membranes played a minor role. Nitro-aromatic compounds can interact with proteins causing enzyme inactivation [33], and they can uncouple phosphorylation reactions [34]. Gorontzy et al. [35] suggested that the methanogenic toxicity of nitro-aromatics or their reduced intermediate compounds may be due to interaction with the unique cell membrane of methanogenic archaea.

The microbial toxicity of the compounds assayed generally increased with increasing incubation time, as shown in Figs. 2 and 3. The gradual decrease observed in the specific methanogenic activity with time, particularly during the initial 2–3 d, might reflect the period required for effective interaction of the toxicant with the methanogenic population in the relatively "thick" granular biofilms. Microbial transformation of some organoarsenic compounds with formation of more toxic metabolites might have also contributed to exacerbate microbial inhibition in some treatments.

Roxarsone is rapidly reductively transformed by microorganisms in anaerobic sludge [13] and swine waste [15], and by the

arsenate-respiring bacterium, *Clostridium* sp. strain OhILAs [14]. Previous research has shown complete biotransformation of roxarsone (1 mM) to its corresponding amino derivative; HAPA, by methanogenic granular sludge in 5 d [13]. Conversion of roxarsone to HAPA, a compound which displays low microbial toxicity under strict anaerobic conditions, might explain the significant toxicity decrease observed in the second feed (days 7–14) in assays supplemented with H_2 (Fig. 2). Further biomineralization of HAPA with release of highly toxic arsenite could be responsible for the subsequent increase in methanogenic inhibition observed after extended exposure in the assays spiked with roxarsone (days 14–19), and HAPA (days 7–19). Arsenite and, to a lesser extent, arsenate were the main biotransformation products of HAPA under anaerobic conditions [13,15].

4.2. Impact of oxidative reactions on the methanogenic toxicity of HAPA

The intrinsic methanogenic toxicity of HAPA appears to be very low as confirmed by the results in short-term experiments supplemented with HAPA concentrations up to 2.6 mM and supplemented with ascorbic acid to prevent the autooxidation of the organoarsenic compound (Fig. 4). Although unoxidized HAPA was essentially non-inhibitory to acetoclastic methanogens, even short periods of exposure to passive aeration (4–40 min) resulted in a considerable increase of the microbial toxicity (Fig. 4). Oxidative decomposition of HAPA, an amino-aromatic compound, is expected to result in the formation of polymeric aromatic compounds. Aromatic amines are known to be susceptible to polymerization reactions, leading to further incorporation in complex humic compounds [36,37]. To the best of our knowledge, the identity of products from the autooxidation of HAPA has not been described in the literature. However, abiotic oxidation of HAPA catalyzed by clay minerals has been demonstrated to result in the formation of oligomeric azobenzene arsenical derivatives [38]. Preliminary MS–MS studies conduct in our study also revealed the presence of large unidentified fragmentation products with m/z values of up to 1851.5, suggesting oligomers up to 8–9 units.

Autooxidation of HAPA can be promoted by aeration under alkaline pH conditions. Our results indicated that air sparging of alkaline HAPA solutions led to the formation of highly inhibitory compounds which were slowly transformed to less toxic products after extensive aeration (>16 h) (Figs. 5 and 6). Similarly, the molecular weight of polymers formed by autooxidation of tannins and phenolic compounds with vicinal hydroxyl groups has been related to their inhibitory potency [39,40]. In the latter studies, increased methanogenic inhibition was observed for oligomeric compounds (MW < 3000 Da) when compared to the corresponding monomers, which was attributed to improved interaction between the phenolic compounds and bacterial proteins (*i.e.*, enzymes). However, the microbial inhibition decreased as the compounds were polymerized further, a trend hypothesized to result from poor penetration of large aromatic polymers into the cell.

5. Environmental implications

This study demonstrated that phenylarsonic acid derivates, which include widely used animal-feed additives of broiler chickens and swine, e.g. roxarsone and *p*-arsanilic acid, are inhibitory to acetate- and hydrogen-utilizing methanogenic microorganisms. Our results also indicate that, upon environmental release, some of these organoarsenicals are likely to undergo chemical as well as microbial-mediated transformations which can alter considerably their environmental fate and, in particular, their inhibitory effect on sensitive microbial communities. Roxarsone is rapidly transformed in the absence of oxygen to the corresponding aromatic amine, 4-hydroxy-3-aminophenylarsonic acid. In anaerobic environments, such as water-logged and sediments, HAPA can undergo further decomposition resulting in the formation of arsenite, an arsenic species which is highly toxic not only to microorganisms [21], but also to higher organisms, including mammals. In aerobic environments, on the other hand, HAPA can readily undergo autooxidative reactions leading to the formation of oligomeric and polymeric compounds. Although the oxidative transformation products formed in the initial phase of the oxidation are considerably more toxic than HAPA and roxarsone, further polymerization to high molecular weight compounds is expected to decrease toxicity. Similarly to other amino-aromatic compounds [41,42], HAPA autooxidation in soils and sediments is likely to result in incorporation of the organoarsenic compound into humic structures. Copolymerization of amino-aromatic compounds with humic structures is an irreversible process that often leads to a sharp decrease in the mobility and toxicity of the aromatic compounds. Further experiments are required to confirm the reactivity of HAPA with humus and characterize the impact of this potential transformation in the environmental fate of the compound.

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