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

Reyes Sierra-Alvarez*, Irail Cortinas, Jim A. Field

Department of Chemical and Environmental Engineering, University of Arizona Tucson, AZ 85721-0011, USA

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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].

* Corresponding author. Tel.: +1 520 626 2896; fax: +1 520 621 6048.
E-mail address: rsierra@email.arizona.edu (R. Sierra-Alvarez).

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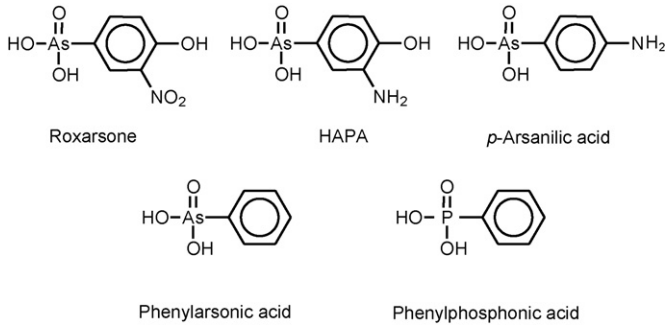

Fig. 1. Chemical structure of aromatic arsenic compounds considered in this study. HAPA = 4-hydroxy-3-aminophenylarsonic acid.

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