APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. **14**, 364–370 (2000)

Interaction of *Scopulariopsis brevicaulis*, and other microorganisms, with 10,10′-oxybisphenoxarsine (OBPA)

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The fungicide 10,10′-oxybisphenoxarsine (OBPA) is widely used in consumer products, such as shower curtains, wall coverings and carpets. A possibility exists that microorganisms might be able to degrade OBPA to produce volatile trimethylarsine. If this did occur, then in certain situations enough trimethylarsine might be produced to be a hazard. In this study, we cultured microorganisms in medium containing OBPA, and examined the medium for possible degradation products. We used *Scopulariopsis brevicaulis* in one experiment, because this microorganism is known for its ability to biomethylate arsenic. OBPA-tolerant microorganisms, isolated from a soil contaminated with arylarsenic compounds, were used in a second series of experiments. We found no evidence of complete microbiological cleavage of aryl–arsenic bonds in any of the cultures, and no significant amount of trimethylarsine was detected in the headspace of *S. brevicaulis* cultures.

Keywords: arsenic; 10,10′-oxybisphenoxarsine; OBPA; *Scopulariopsis brevicaulis*; SIDS; cyanodiphenylarsine; trimethylarsine

Received 24 July 1999; accepted 18 January 2000

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Contract/grant sponsor: US Department of the Army.
Contract/grant sponsor: NATO.
Contract/grant sponsor: NSERC of Canada.

INTRODUCTION

In a submission to the Expert Group to Investigate Cot Death Theories: Toxic Gas Hypothesis,[1] Richardson wrote: 'It was recognized by Richardson in 1988 that microbial deterioration of plasticised polyvinyl chloride (PVC) containing arsenical preservatives might result in the generation of extremely toxic gaseous arsines. It was considered that this process might be a cause of sudden infant death.'[2]

This statement was based on the recognized phenomenon by which volatile toxic arsines, usually trimethylarsine, are produced when some microorganisms, e.g. *Scopulariopsis brevicaulis*, interact with simple arsenic species such as arsenate or arsenite.[3–8] The assumption seems to have been made that microbial action on the arsenical preservatives used in the PVC might also produce toxic arsines. The *only* arsenical used as a fungicide in PVC is 10,10′-oxybisphenoxarsine (OBPA, **1;** Fig. 1). However, OBPA is not commonly used in cot mattress covers, which is probably why Richardson extended his hypothesis to suggest that 'the primary cause of sudden infant death syndrome (SIDS) is poisoning by gaseous phosphines, arsines and stibines, generated by deterioration of cot mattress material by microorganisms, particularly *Scopulariopsis brevicaulis* an otherwise harmless fungus that is normally found in all domestic environments.' The focus of most toxic-gas hypothesis research then shifted to production of stibines from antimony trioxide (present in bedding material as a flame retardant) by *S. brevicaulis*.[1]

Although OBPA is not commonly used in cot mattress covers, it is marketed as Vinyzene® for use in many consumer products, such as shower curtains, wall coverings and carpets, which often become damp and dirty and so could be exposed to

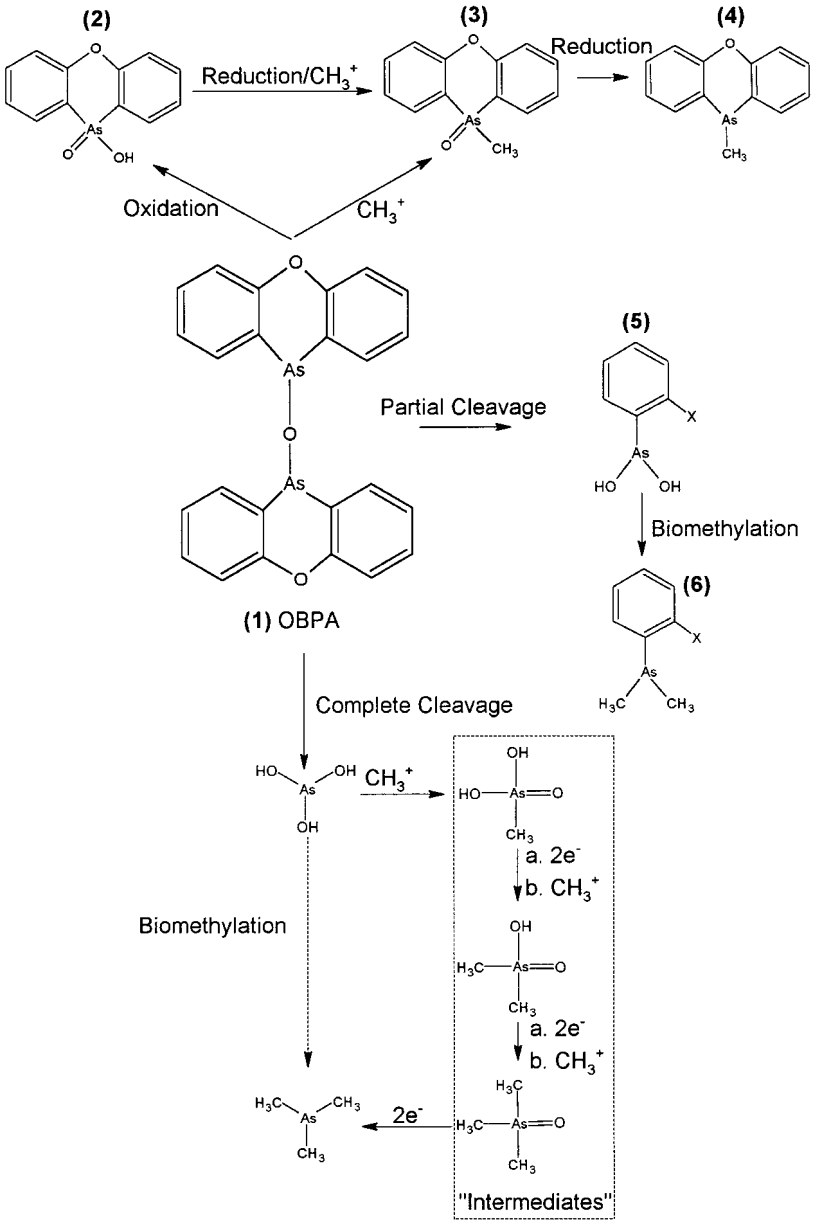

Figure 1 Structure of 10,10′-oxybisphenoxarsine (OBPA), and some possible biotransformation pathways.

microbial action. If OBPA is readily degraded by microorganisms to trimethylarsine, as originally implied by Richardson, this could be cause for concern.

Microorganisms could act on OBPA in a variety of ways (Fig. 1). For instance, the biomethylation of OBPA might produce species **3** or **4;** such a process for arylarsenicals is known.[9] However, these species would not be volatile at room temperature. Partial cleavage of the aryl–arsenic bond of OBPA and subsequent biomethylation could produce various species **(6)**, but again these species would not be volatile at room temperature. The production of volatile species, such as trimethylarsine, would first require the biologically catalyzed cleavage of all aryl–arsenic bonds, a process that was unsubstantiated in 1988. Thus, there was no experimental foundation, at the time it was first proposed, for

Richardson's toxic-gas hypothesis based on arsine production from OBPA.

The currently accepted mechanism of trimethylarsine production by *S. brevicaulis* begins with oxidative methylation of arsenite, followed by a series of reduction and oxidative methylation steps (the Challenger mechanism[3]). Thus, for OBPA, trimethylarsine production can only occur after cleavage of all aryl–arsenic bonds. Methylarsenic(V) intermediates (Fig. 1) in Challenger's mechanism have been detected.[10] Indeed, at low concentrations of inorganic arsenic species, volatile trimethylarsine is not produced in significant yields, although trimethylarsine oxide is produced.[10] Therefore, in most experiments there is no need to determine trimethylarsine production directly, because the determination of the major intermediates on the pathway will indicate if trimethylarsine production is likely.

The microorganisms most likely to degrade OBPA, by aryl–arsenic bond cleavage, are ones that have been exposed to OBPA or similar compounds in the environment. With this in mind, microorganisms were isolated from a soil contaminated with cyanodiphenylarsine (Fig. 2), which is chemically very similar to OBPA and was used in World War I as a chemical warfare agent (Clark II). If microorganisms from such an environment are unable to cleave aryl–arsenic bonds, then it is unlikely that such cleavage could be accomplished by other common microorganisms.

We describe here results from two studies. In one of these, we incubated *S. brevicaulis* with OBPA, and headspace gases and medium from these cultures were examined by using GC–ICP MS and HG–GC–AAS, respectively, to determine whether OBPA was degraded by this microorganism to volatile arsenic species or their precursors. In the other study, OBPA-tolerant microorganisms were isolated from a soil contaminated with cyanodiphenylarsine, then incubated with OBPA, and the medium was examined for OBPA-degradation products and biomethylation intermediates, to assess whether arsenic volatilization from OBPA was likely.

MATERIALS AND METHODS

Materials

OBPA was prepared in our laboratory by using a standard literature method.[11] Nutrient agar (NA) and nutrient broth (NB) were acquired from Difco Laboratories. A glucose/minimal-salts medium[4] was prepared from reagent-grade chemicals. The media were autoclaved at 121 °C and 19 psi (131 kpa) for 20 min. The OBPA was added to the NA and NB before autoclaving. Reagent-grade chemicals were used for the hydride generation analysis.

Soil sampling

A small quantity of cyanodiphenylarsine was spilt onto soil during a disposal operation in 1991 at the Defense Research Establishment Suffield (DRES), Alberta, Canada. The site (known as 14A) is easily located because it is completely devoid of vegetation. Two soil samples (0–10 cm depth) were collected on 17 February 1998 from randomly selected points within the area of the spill.

Cultures isolated from soils

A small amount of each soil sample was streaked onto Petri plates containing nutrient agar (1/10 strength) saturated with OBPA (5 mg l^{-1}). One set of Petri plates was incubated at 17 °C and the second set at 30 °C. All plates were examined daily for the appearance of colonies; those that grew are described in Table 1. The isolated microorganisms were then inoculated into nutrient broth (5 ml full-strength NB in 16 mm × 100 mm test tubes) saturated with OBPA (1 mg of OBPA per test tube), and placed on a rotary shaker [25 °C, ~135 rpm, 1.75-inch (4.45-cm) displacement] for a month. A control that consisted of nutrient medium and OBPA was also incubated on the rotary shaker. The medium from each culture was filtered (0.45 μm) after one month of incubation and analyzed by hydride generation–gas chromatography–atomic absorption (HG–GC–AAS).

HG–GC–AAS analysis

Analysis was performed by using semi-continuous HG–GC–AAS as described in detail elsewhere.[10] Appropriate experimental conditions (1 M hydrochloric acid/2% $NaBH_4$ in water) were used so that standards of sodium arsenite, sodium arsenate, monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide all readily formed volatile hydrides. The sample volume was 5 ml, which corresponds to concentration detection limits of 1 ng As ml^{-1} and 2 ng As ml^{-1} for inorganic arsenic species and methylarsenic species respec-

Table 1 Microorganisms isolated from cyanodiphenylarsine-contaminated soil[a]

Microorganism	Type	Macroscopic	Microscopic
OPBA-1	Fungus	Dark green center, white rhizoid	
OBPA-2	Bacterium	Shiny white, convex, 2 mm diameter	Gm(−) coccoid, 0.5 μm, clusters
OBPA-3	Bacterium	Shiny white, convex, 1 mm diameter	Gm(−) coccoid, clusters
OBPA-4	Bacterium	Pale pink, shiny, convex, 2 mm diameter	Gm(−) rods, terminal or central spores, 1.2 μm, singlets and clusters
OBPA-5	Bacterium	White, shiny, blob-like, various sizes	Gm(−) coccoid, 0.7 μm, singlets and clusters
OBPA-6	Bacterium	Yellow, shiny, convex, 2 mm diameter	Gm(−) coccoid, 0.5 μm, clusters
OBPA-7	Bacterium	Bright yellow, shiny, irregular borders, 2 mm diameter	Gm(−) rods in chains, 1 μm
OBPA-8	Bacterium	Dark pink, shiny, 2 mm diameter	Gm(−) rods, 1.1 μm, very big central or terminal spores
OBPA-9	Bacterium	Salmon pink, 1 mm diameter, dull	Gm(−) rods, 1.2 μm, clusters, very big terminal spores
OBPA-10	Bacterium	White, dull, central peak, 2 mm diameter	Gm(−) rods in chains, 1 μm
OBPA-11	Bacterium	Clear, flat, various sizes	Gm(−) coccoids, in pairs and clusters, 0.5 μm

[a] Microorganisms were isolated on nutrient agar (1/10 strength) containing OBPA (5 mg l^{-1}).

tively. OBPA does not form a volatile hydride under these conditions.

Scopulariopsis brevicaulis cultures

Medium analysis

A minimal-salts/glucose medium[4] (400 ml) was seeded with 40 ml of *S. brevicaulis* (ATCC no. 7903) mycelial balls (20–30 balls, *Ca* 1 mm diameter). Two replicate cultures were prepared. By adding an appropriate volume of 1000 mg OBPA l^{-1} in methanol, the cultures were made up to 1 mg OBPA l^{-1} (in preliminary experiments we had observed that $\geq$2 mg OBPA l^{-1} significantly inhibited the growth of *S. brevicaulis*). A control was prepared consisting of minimal-salts/glucose medium and OBPA. The cultures and the control were incubated on a rotary shaker [25 °C, *ca* 135 rpm, 1.75-inch (4.45-cm) displacement] for one month, after which the medium from each culture and the control was filtered (0.45 μm) and analyzed by HG–GC–AAS.

Headspace analysis

Four cultures were prepared that each contained 400 ml of minimal-salts/glucose medium and *S. brevicaulis*. Two of these cultures contained 1 mg OBPA l^{-1} (Cultures A and B), made by adding 0.4 ml of 1000 mg OBPA l^{-1} in methanol to each; the other two were controls (Cultures C and D), and had 0.4 ml of methanol added to them. The cultures were placed in sterile 1-liter Erlenmeyer flasks topped with ground-glass male joints and capped with female ground-glass joints, which were fitted with inlet and outlet glass tubing. The cultures were continuously purged with sterile (0.2 μm filtered) compressed air and the headspace gases were sampled by trapping them in U-shaped glass traps (packed with 10% Supelcoport SP-2100 on Chromosorb) immersed in dry-ice/acetone. The contents of the traps were determined by using GC–ICP MS. Full details of the gas sampling and analysis procedure are described elsewhere.[12] Gas samples were taken 4, 9 and 12 days after preparing the cultures, over approximately 4 h.

RESULTS AND DISCUSSION

Contents of media from cultures of *S. brevicaulis* and microorganisms isolated from contaminated soil

We did not observe inhibition of growth when we cultured *S. brevicaulis* in medium containing 1 mg OBPA l^{-1}. At $\geq$ 2 mg OBPA l^{-1}, growth was inhibited. After one month of incubation of *S. brevicaulis* in medium containing 1 mg OBPA l^{-1}, the medium was analyzed by using HG–GC–AAS. No methylarsenic species were detected (Table 2). Inorganic arsenic species were detected in the

Table 2 Concentration of hydride-forming arsenicals in cultures and controls after one month of incubation with OBPA

Microorganism	Inorganic arsenic concentration (ng As ml^{-1})	Organoarsenic concentration (ng As ml^{-1})
S. brevicaulis	2.9	<2
S. brevicaulis	3.1	<2
OPBA-1	8.3	<2
OBPA-2	3.3	<2
OBPA-3	6.4	<2
OBPA-4	10.6	<2
OBPA-5	6.8	<2
OBPA-6	8.3	<2
OBPA-7	8.1	<2
OBPA-8	8.5	<2
OBPA-9	7.5	<2
OBPA-10	6.6	<2
OBPA-11	8.5	<2
Controls:		
Minimal-salts/glucose medium	4.5	<2
Nutrient broth medium	8.3	<2

medium but similar levels were detected in the controls and active cultures, i.e. the inorganic arsenic species detected arose from impurities in the medium and OBPA. Therefore, *S. brevicaulis* is unable to cleave all the aryl–arsenic bonds of OBPA.

About 80 microorganisms were isolated by standard microbiological methods from the two. cyanodiphenylarsine-contaminated soil samples. The number may be slightly lower because of the possibility of duplicate isolation from more than one soil sample. This is not as many as would be expected from a healthy soil but it is certainly an indication that the soil is not sterile. By using nutrient agar saturated with OBPA, we were able to isolate 11 macroscopically different microorganisms, 10 bacteria and 1 fungus as pure cultures, described in Table 1. These microorganisms were subsequently grown for one month in a liquid medium saturated with OBPA. The medium was examined by using HG–GC–AAS and the results are reported in Table 2. Controls, containing medium and OBPA, contained *ca* 5–10 ng As ml^{-1} as inorganic arsenic (either arsenite or arsenate). Similarly all cultures contained approx. 5–10 ng As ml^{-1}. No methylarsenic species were detected. Therefore, microbially assisted cleavage of all aryl–arsenic bonds of OBPA did not take place. The 11 OPBA-tolerant microorganisms either had some mechanism to exclude OBPA, or they detoxified it by some other means.

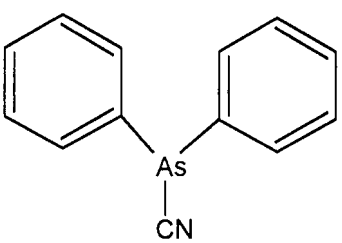

Figure 2 Structure of cyanodiphenylarsine (Clark II).

The solubility of OBPA in water (pH 7, 20 °C) is 5 mg OBPA l^{-1}. Thus, for the cultures of OBPA-tolerant microorganisms isolated from soils, *complete* degradation of only 5% of the dissolved OBPA to inorganic arsenic species would yield 75 ng As ml^{-1} and would be readily detected in our system. For the *S. brevicaulis* cultures containing 1 mg OBPA l^{-1}, degradation of more than 10% OBPA would be readily detected. However, if only partial aryl–arsenic bond cleavage occurred, the resulting arylarsenic species (Fig. 1) would not be detected by our methodology. Reverse-phase HPLC with ICP MS as a detector would enable the detection of such partial-breakdown products; however, only complete-breakdown products are relevant to any hypothesis linking OBPA with poisoning by volatile arsines.

It has been shown that the concentration of methylarsenicals in the medium would have to

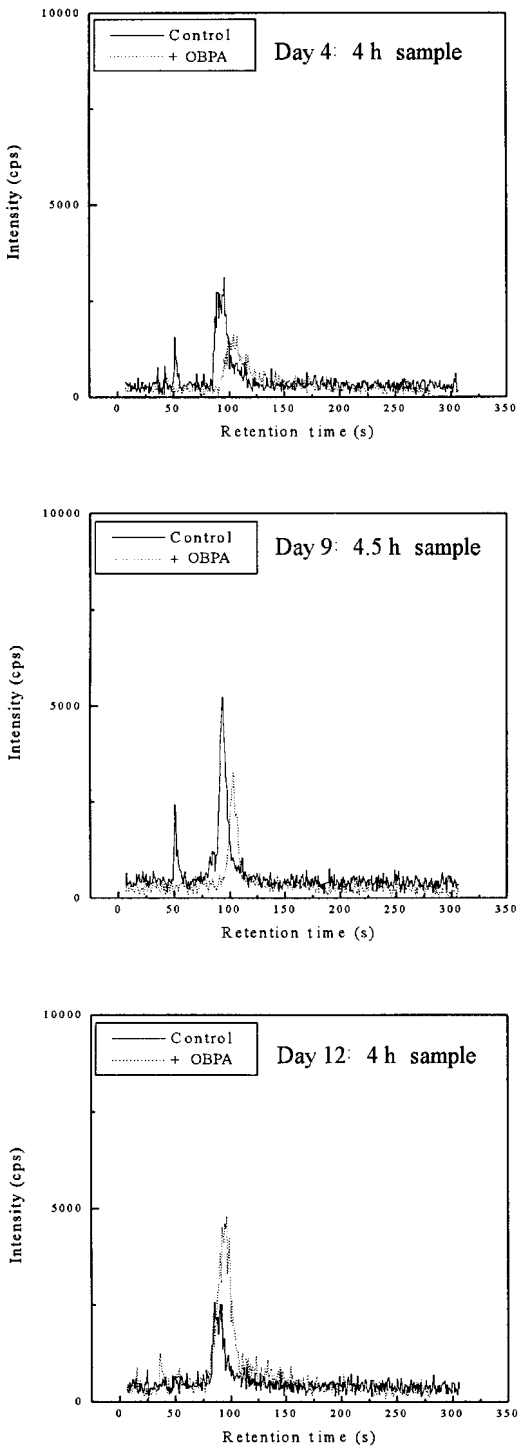

Figure 3 GC–ICP MS chromatograms ($m/z = 75$) obtained by analyzing headspace gas samples taken on days 4, 9 and 12 from Cultures A (*S. brevicaulis* + OBPA) and C (control: *S. brevicaulis*).

exceed at least 40 ng As ml^{-1} before trimethylarsine production occurs.[10] The present results provide no evidence that microorganisms can break down OBPA to the extent that arsenic could be volatilized as trimethylarsine.

Contents of *S. brevicaulis* headspace

We believe that trimethylarsine would not be produced from OBPA without prior cleavage of all aryl–arsenic bonds of OBPA. If this is the case, these degradation products (inorganic arsenic species and methylated intermediates on the pathway to trimethylarsine) should be readily detected if trimethylarsine is indeed an end product. However, because of the remote chance that direct transformation of OBPA to trimethylarsine might be possible, it was deemed necessary to examine *S. brevicaulis* headspace gases for trimethylarsine. Such a pathway would presumably require sequential cleavage/methylation reactions starting with the formation of **5** (Fig. 1).

Two cultures were prepared that contained 1 mg OBPA l^{-1} (Cultures A and B) and two control cultures were prepared that did not contain OBPA (Cultures C and D). The cultures were continuously purged with compressed air over a 12-day incubation period. On days 4, 9 and 12 headspace gases were sampled by trapping them in U-shaped traps. At the end of the experiment, the contents of the traps were determined by using GC–ICP MS. The chromatograms ($m/z = 75$) obtained on each sampling day, for one of the control cultures (Culture C) and one of the cultures that contained OBPA (Culture A), are shown in Fig. 3. The only compound produced in significant quantities is trimethylarsine and all cultures produced similar quantities (*ca* 1 pg As h^{-1}).

The content (pg As) of each trap was calculated by using a one-point calibration/internal standard method described by Feldmann.[13] We also calculated the amount of trimethylarsine produced over the sample time (pg As h^{-1}). By assuming that the rate of trimethylarsine production between sampling is the same as that over the sample period, we estimated the total amount of trimethylarsine produced over 12 days of incubation to be 0.6 (Cultures A and B) and 0.4 ng As (Cultures C and D).

The amounts of trimethylarsine detected were extremely small: over the 12-day period, less than 0.0005% of the arsenic in OBPA was volatilized, which might be attributed to inorganic arsenic impurities in the OBPA. In contrast, 1%, of the

arsenic was biomethylated to trimethylarsine oxide over five days when *S. brevicaulis* was cultured in 1 mg As l^{-1} as sodium arsenite or arsenate.[10] Thus, there is no evidence that *S. brevicaulis* can transform OBPA to significant amounts of trimethylarsine.

Acknowledgements The authors are indebted to John McAndless and his colleagues at DRES for assistance in obtaining the soil samples. Financial support for this work was provided by the US Department of the Army, NATO and NSERC of Canada.

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