Phosphine and methane generation by the addition of organic compounds containing carbon–phosphorus bonds into incubated soil

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Abstract

Formation of phosphine and methane in anaerobic incubation systems was investigated under stirred and unstirred conditions. The PH_3 and CH_4 levels in the headspace, as well as the matrix-bound PH_3 content in the stirred soil, significantly increased upon the addition of phosphonoacetic acid $\text{P(O)(OH)}_2\text{CH}_2\text{COOH}$. Both the levels of matrix-bound PH_3 and CH_4 are positively correlated to the buffered dithionite fraction of reactive phosphorus in the soil samples, while a negative correlation was observed between matrix-bound PH_3/CH_4 levels and the reactive phosphorus fraction.

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1. Introduction

The hypothesis that microbial phosphate reduction is the predominant mechanism responsible for phosphine formation is subject to controversial discussion (Barrenscheen and Beckh-Widmanstetter, 1923; Tsubota, 1959; Iverson, 1968). Because of their insufficient analytical techniques for phosphine detection, the above-mentioned authors could not provide convincing evidence on phosphine formation. The occurrence of phosphine in the natural environment has remained in dispute for nearly half a century. Since the enrichment and analytical techniques for ambient phosphine were improved, phosphine has been detected from numerous anaerobic sources (Gassmann and Schorn, 1993; Gassmann, 1994; Devai and Delaune, 1995; Glindemann and Bergmann,

1995; Glindemann et al., 1996a,b; Devai et al., 1988; Liu et al., 1999; Han et al., 2000). Han et al. (2000) reported the seasonal variation of phosphine flux from paddy fields.

However, the mechanism of phosphine formation remained unclear. Some tentative experimental results indicated that addition of chicken manure, bone powder, or lecithin can lead to an increment of phosphine emission (Eismann et al., 1997a; Cao et al., 2000). Moreover, Eismann et al. (1997b) found a correlation between the evolution of phosphine and the production of methane/hydrogen sulfide during anaerobic fermentation of fresh swine manure. Bayer et al. (1972) identified phosphinothricin, a microbial secondary metabolite with a (+1)-phosphorus atom in its molecule. In all the cited reports, phosphine and matrix-bound phosphine have been reported to be a minor fraction of the organic phosphorus in soil and sediment. Not all the organic phosphorus can be converted to phosphine. This implies that the major constituents of organic phosphorus, which are esters of phosphoric acid, cannot be

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reduced directly to phosphine. Phosphine should be derived from organic compounds other than phosphates.

In this study, we turned to organic phosphorus compounds with C–P bonds. These organic phosphorus compounds are available in the environment, but in minor quantities. The first organic phosphorus compound with a C–P bond identified in the environment was 2-aminoethylphosphonic acid, which was isolated from rumen protozoa by Horiguchi and Kandatsu (1959). Alkylphosphonates were then reported to occur widely in nature, combined either in lipids (Rosenberg, 1973), or in polysaccharides (Korn et al., 1973), and in proteins as well (Hilderbrand et al., 1973; Hilderbrand and Henderson, 1983). Phosphonates have also been found in bacteria, fungi, and higher organisms, such as the snail schistosome vector *Biomphalaria* (Mastalerz, 1984; Thompson and Lee, 1985). Although alkylphosphonates are regarded as having great chemical stability as a result of their C–P bonds, it has been found that bacteria are capable of using different alkylphosphonates as substrates, which are isolated from sewage and soil (Cook et al., 1978). Daughton et al. (1979) reported that bacteria caused C–P bond cleavage to yield alcohols, alkanes and inorganic phosphates. Nevertheless, these authors have not tried to detect the presence of phosphine during C–P bond cleavage.

It is, therefore, the purpose of this study to investigate phosphine and methane generation upon the addition of organic compounds containing carbon–phosphorus bonds into incubated soil. The compound we used in our study (phosphonoacetic acid, PA) is a biogenic phosphonate found by Hilderbrand and Henderson (1983).

2. Materials and methods

2.1. Anaerobic fermentation experiments

For anaerobic fermentation experiments, we used a paddy soil sample taken from Houbajia, Beijing as the culture inoculum. Aliquots of 3-kg thoroughly mixed inoculum were put separately into two 20-l glass thermostated fermentors together with 5 l of water. In one of these fermentors, 1.03 g of phosphonoacetic acid ($\text{P}(\text{O})(\text{OH})_2\text{CH}_2\text{COOH}$, 98%, Aldrich Chem. Co.) was added too. It is equivalent to an increment of 0.076 mg P per gram of dry soil. In the other fermentor, which served as a control, no phosphonoacetic acid was introduced. The two fermentors were at first evacuated to remove air, and were then purged with high-purity nitrogen. This manipulation was repeated twice. To prevent air intrusion into the anaerobic fermentors, the outlets of the fermentors were isolated from ambient air by liquid seals. Then the fermentors were incubated in

darkness at $30 \pm 1^\circ\text{C}$ with magnetic stirring. Incubation time was about one month. Four to six parallel gas samples from the headspace were taken with 50-ml plastic syringes every day or every other day. At the end of experiments, the matrix-bound phosphine levels and reactive phosphorus levels in the incubated soil samples were determined.

A second batch of anaerobic incubation experiments was run under unstirred conditions. About 40-g aliquots of paddy soil were weighed and added to 120-ml glass vials together with 50-ml water. To one group of vials, 0, 100, 200, and 300 mg of phosphonoacetic acid were added, respectively. To the other group of vials, NaOH and H_2SO_4 solutions were added to adjust the pH value to 4.85, 6.74, and 8.2, respectively. All the vials were then purged with high-purity nitrogen for 5 min, and were stoppered with septa. For each substrate studied, six vials and two controls were run in parallel. The sealed vials were then incubated without agitation at $30 \pm 1^\circ\text{C}$ under a dark cover. Headspace gas samples were then taken with the aid of two 50-ml plastic syringes. High-purity nitrogen in one syringe with a long needle (10 cm) was injected through the sediment into the vial, while the other empty syringe with a short needle (2 cm) was used to take headspace gas from the same vial simultaneously. The headspace gas samples, as well as the matrix-bound phosphine samples liberated from soil samples, were subjected to GC analysis as described elsewhere (Glinemann and Bergmann, 1995; Liu et al., 1999; Cao et al., 2000; Han et al., 2000).

2.2. Matrix-bound phosphine collection and the determination of phosphine and methane

The procedures for matrix-bound phosphine collection and phosphine determination described by Glinemann and Bergmann (1995) and by Han et al. (2000) were adopted in this study. Methane was measured by GC-FID.

2.3. Total phosphorus and reactive phosphorus detection

Total phosphorus detection follows the procedures described in the Handbook of Agro-Chemistry Analysis (Li et al., 1984).

Sequential fractionation of reactive phosphorus (RP) was performed according to the procedure described by Fu et al. (2000). Incubated soil samples in acid-washed polypropylene tubes were at first centrifuged for 20 min at 3000 rev min^{-1} to remove pore water. About 250 mg of the “de-watered” sediment was placed in a 50-ml acid-washed centrifuge tube, and subjected to sequential extraction according to the procedures listed in Table 1 (Fu et al., 2000). The supernatants of each extraction after centrifugation for 10 min at 3000 rev min^{-1} were analyzed for molybdate reactive phosphorus (MRP)

Table 1

Extraction scheme and fractional composition of reactive phosphorus in soil samples

Extraction medium	Fraction acronym	Main species
H ₂ O (for 10 min)	H ₂ O-RP	Orthophosphate, <i>o</i> -P
BD (0.11 mol l ⁻¹ buffered sodium dithionite for 30 min at 40 °C)	BD-RP	Reductant soluble P, RSP (iron-bound P)
NaOH (1.0 mol l ⁻¹ sodium hydroxide for 16 h at room temperature)	NaOH-RP	Iron-bound P (Fe-P)
HCl (0.5 mol l ⁻¹ hydrochloric acid for 24 h at room temperature)	HCl-RP	Calcium- and magnesium-bound P (Ca, Mg-P)
NaOH (1.0 mol l ⁻¹ sodium hydroxide for 24 h at 85 °C)	NaOH-RP (85 °C)	Refractory P, inorganic or organic poly-phosphates

(Murphy and Riley, 1962). All analyses were run in triplicate, and the MRP results were given on a dry-weight basis (dw).

3. Results and discussion

3.1. Phosphine and methane levels in the headspace of fermentors

A soaring increase of PH₃ and CH₄ levels was observed in the headspace of the fermentor with phosphonoacetic acid (Fig. 1). The PH₃ level in this fermentor was 100-folds as high as that in the control. The CH₄ level was about 3–5-fold more than that in the control. Furthermore, the duration of PH₃ and CH₄ release with phosphonoacetic acid added was longer than that in the control. Further investigation is needed to elucidate the causes of this phenomenon.

In our fermentation experiments with a small amount of phosphonoacetic acid added, we did not observe the inhibition effect of phosphine to methane formation. This is in agreement with Eismann's observation that anaerobic fermentation of acetate and yeast extracts was not affected by the presence of PH₃ up to 1000 ppm (Eismann et al., 1997c).

We found that the first CH₄ peak appeared about nine days after the first phosphine peak occurred under anaerobic incubation. Statistical treatments of the results show that for the sample with phosphonoacetic acid added the PEARSON or CORREL correlation coefficient between methane and phosphine is 0.983, while the correlation coefficient for the control sample is 0.500. The PH₃ mean emission rate is about 2.2 ng PH₃ kg⁻¹ h⁻¹.

The presence of organic compounds containing C–P bonds may be crucial for the occurrences of high phosphine levels. When microorganisms under anaerobic

Fig. 1. Gas levels in the headspace of the stirred fermentors with PA added and without PA (control). (a) Phosphine (PH₃) level, (b) methane (CH₄) level.

conditions degrade these C–P compounds, both PH_3 and CH_4 might be formed. This point will be discussed again in the next section.

3.2. Matrix-bound phosphine and methane in the cultivating soil samples

The total phosphorus content in our paddy soil is $0.96 \text{ mg P g}^{-1} \text{ dw}$. The addition of 1.03-g phosphonoacetic acid to 3 kg of paddy soil in the fermentor is equivalent to an increment of $0.076 \text{ mg P g}^{-1} \text{ dw}$. In other words, the total phosphorus in the sample was increased by 8%, as compared to the control sample. After 1-month anaerobic incubation, the matrix-bound phosphine content in the soil sample with phosphonoacetic acid addition became $8371.9 \text{ mg P g}^{-1} \text{ dw}$. It means there was a 90-fold increment in matrix-bound phosphine as compared with the control, or an 800-fold increment as compared with the dry paddy soil without incubation (see Table 2).

Jenkins et al. (2000) reported that bacteria (such as *Escherichia coli*, *Salmonella gallinarum* etc.) and solvent fermentors (*Colstridium sporogenes*, etc.) could generate phosphine, and these fermentative bacteria participate in a multi-stage process of methanogenesis in nature. Probably, phosphine and methane are produced at different stages of fermentation.

From Fig. 2, we can see that phosphonoacetic acid has a double-edge effect. Addition of small amounts of phosphonoacetic acid (100 mg phosphonoacetic acid in 40-g soil, dry weight) promoted PH_3 and CH_4 formation in the soil sample, while larger amounts of phosphonoacetic acid (200 and 300 mg phosphonoacetic acid in 40 g soil, dry weight) inhibited PH_3 and CH_4 generation. Inhibition might be caused either by the toxicity of phosphonoacetic acid itself, or by the acidity of this fairly strong acid. Under different pH values of incubated soil, the amount of matrix-bound PH_3 generated was different.

Fig. 3 shows the effect of pH on matrix-bound phosphine formation. The highest matrix-bound PH_3 content occurred under a neutral condition that is suitable for microbial reproduction. Under an alkaline condition, the matrix-bound PH_3 level is lower. The matrix-bound phosphine level in acidic soil was found to be the lowest.

3.3. Sequential fractionation of reactive phosphorus

Phosphorus release is a function of the quantities and distribution of various phosphorus fractions in the soil. It is also a function of the degree of saturation of exchangeable phosphorus. It is linked closely to the intensity of biological processes in soil and water, as well as to the hydrological conditions (Jansson et al., 1988). It was

Table 2
Matrix-bound PH_3 and CH_4 levels in stirred samples

Sample	Matrix-bound PH_3 ($\text{pg g}^{-1} \text{ dw}$) $\pm$ s.d.	Matrix-bound CH_4 ($\text{mg g}^{-1} \text{ dw}$) $\pm$ s.d.	Reference
Soil sample with PA	8371.9 ± 923.9 ($n = 4$)	10.1 ± 2.0 ($n = 6$)	This work
Soil sample without PA (control)	91.2 ± 11.3 ($n = 5$)	43.2 ± 5.1 ($n = 3$)	This work
Dry soil sample from the same site without incubation	10.4	–	Han et al. (2000)

Fig. 2. Matrix-bound PH_3 and CH_4 in unstirred samples with different amounts of PA added. (a) Matrix-bound PH_3 level, (b) matrix-bound CH_4 level.

Fig. 3. Matrix-bound PH₃ level in unstirred samples under different pH values.

reported, that the ‘reductant’ soluble phosphorus species, which were extracted mainly from the iron hydroxide surface and the buffered dithionite reactive phosphorus (BD-RP) fraction of the soil sample, may play a key role in phosphorus release (Zinder and Stumm, 1985; Psenner and Pucsko, 1988; Fu et al., 2000).

It was shown in Fig. 4 and Table 3 that H₂O-RP and BD-RP levels under anaerobic conditions were obviously higher than those in dry soils without incubation.

Fig. 4. Reactive phosphorus extracted from stirred samples with PA and without PA (control).

When organic phosphorus compounds containing C–P bonds like phosphonoacetic acid were added, the amount of BD-RP fraction extracted by buffered sodium dithionite solution increased dramatically. The share of the BD-RP fraction in Tot-RP for stirred samples was as high as 62.5%. On the contrary, the NaOH-RP fraction decreased.

For unstirred incubation samples, the results on reactive phosphorus levels are shown in Fig. 5 and Table 4. They are similar to those stirred samples with the only difference that the BD-RP contents were not as high as in the stirred samples upon phosphonoacetic acid addition. Sondergaard et al. (1992) also reported that agitation or disturbance of sediments promoted phosphorus release. They found that wind-induced resuspension of sediments occurred frequently in shallow and eutrophic lakes, and that phosphorus release from disturbed sediments could be 20–30 times higher than the release from undisturbed sediments.

Sridharan and Lee (1974) pointed out that mineralization of organic phosphorus is one of the mechanisms of phosphorus release. Daughton et al. (1979) first reported that bacteria caused C–P bond cleavage to yield alcohols, alkanes and phosphate. In our case, we found an increment in phosphine and methane levels both in the headspace and in the soil samples upon incorporation of a compound with a C–P bond. The correlation between matrix-bound PH₃ levels and various fractions of reactive phosphorus, as well as that between matrix-bound CH₄ levels and fractions of reactive phosphorus, are presented in Table 5.

Fig. 5. Reactive phosphorus extracted from unstirred samples with different amounts of PA added.

Table 3

Shares of fractionated reactive phosphorus in total reactive phosphorus (Tot-RP) of stirred samples (%)

Sample	H ₂ O-RP	BD-RP	NaOH-RP	HCl-RP	NaOH-RP (85 °C)
Sample with PA	2.7	62.5	9.1	19.2	6.5
Sample without PA (control)	2.3	16.9	25.6	38.5	16.6
Dry soil sample from the same site without incubation	1.9	12.0	25.7	43.9	16.5

Table 4

Shares of fractionated reactive phosphorus in total reactive phosphorus (Tot-RP) of unstirred soil samples (%)

Amount of PA added	H ₂ O-RP	BD-RP	NaOH-RP	HCl-RP	NaOH-RP (85 °C)
0 mg	2.0	27.8	18.6	39.1	12.6
100 mg	4.6	42.2	9.2	27.7	16.4
200 mg	5.6	48.9	8.7	28.8	8.0
300 mg	6.0	31.5	8.8	26.3	27.4
Dry soil sample from the same site without incubation	1.9	12.0	25.7	43.9	16.5

Table 5

Correlation between matrix-bound PH₃/CH₄ concentrations and reactive phosphorus (RP) in unstirred samples

Fractions of RP	Equation for PH ₃ ^a : $y_1 = a \cdot e^{bx}$			Equation for CH ₄ ^a : $y_2 = c \cdot e^{dx}$		
	<i>a</i>	<i>b</i>	<i>R</i> ₁ ²	<i>c</i>	<i>d</i>	<i>R</i> ₂ ²
H ₂ O-RP	20.502	92.736	0.1588	0.7511	51.251	0.3190
BD-RP	9.0134	30.346	0.6357	0.2996	24.346	0.9948
NaOH-RP	92.332	−51.97	0.5394	1.8682	−38.025	0.9029
HCl-RP	293.68	−48.08	0.4334	4.2439	−33.894	0.9635
NaOH-RP (85 °C)	35.659	−1.8691	0.0011	1.1217	−3.4639	0.0368

^a*y*₁ is the matrix-bound PH₃ concentration in pg g^{−1} dw; *y*₂ is the matrix-bound CH₄ concentration in mg g^{−1} dw; *x* is the share of reactive phosphorus in soil (% dw); *R*₁ and *R*₂ are the regression coefficients.

Judging from Table 5, we found that matrix-bound PH₃ and CH₄ levels have a positive exponential correlation with the BD-RP fraction, and a negative exponential correlation with the NaOH-RP and HCl-RP fractions. However, the H₂O-RP and NaOH-RP (85 °C) fractions have no correlation with the matrix-bound PH₃ and CH₄ levels.

This implies hitherto unknown but important phosphorus cycling processes, which might have both positive and negative effects on the environment. In view that phosphine is an intermediate product of anaerobic processes in soil, its subsequent oxidation to phosphoric acid is actually an activation process of soil phosphorus originally unavailable to plants. This is an overlooked positive aspect of phosphine on plant growth. Phosphine is also a reactive atmospheric trace gas, which competes with methane and other greenhouse gases for hydroxyl radicals, because PH₃ reacts quickly with hydroxyl radicals in the atmosphere (Fritz et al., 1982), and thus enhances an indirect greenhouse effect, or the so-called coupling effect (Prinn, 1994). On the other hand, phosphine itself is a toxic substance, which inhibits the growth of soil microorganisms and damages crop roots. These toxic effects would not only influence the emission fluxes of other greenhouse gases from soil, but also could cause disturbance in the rhizosphere. Matrix-bound phosphine in the sediment of water bodies might cause a sudden release of reactive phosphorus and hence eutrophication, if the sediment is brought to the surface layers by upward water current or anthropogenic agitation.

4. Conclusions

PH₃ and CH₄ emissions in paddy soil increased with the addition of phosphonoacetic acid, a biogenic compound containing a carbon–phosphorus bond. Mixing and agitation promoted the formation of phosphine. Upon the introduction of phosphonoacetic acid into incubated soil, both BD-RP and NaOH-RP fractions underwent obvious changes. The matrix-bound phosphine level in the BD-RP fraction increased abruptly upon stirring. A positive correlation was observed between matrix-bound PH₃/CH₄ levels and the BD-RP content. The NaOH-RP fraction, which contains the iron-bound phosphorus, had a negative correlation with matrix-bound phosphine and methane.

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