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Preliminary investigation on the role of microorganisms in the production of phosphine

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Abstract

The relationships between the phosphine content and various microbial populations, activities of different enzymes were investigated firstly. The results indicated that the phosphine content of samples from various environments was positively related to total anaerobic microorganisms, organic phosphate compound-dissolving bacteria, denitrifying bacteria, and the activities of alkaline phosphatase and dehydrogenase, with correlation coefficients (R^2) up to 0.93, 0.90, 0.69, 0.79, and 0.82, respectively. Results also showed that the phosphine content was not related to total aerobic microorganisms, inorganic phosphate compound-dissolving bacteria, sulfate-reducing bacteria, and the acidic phosphatase activity. Nutrients such as yeast extract and glucose were added, at a time and individually, to normal or autoclaved soil samples. The soil samples were inoculated with sulfate-reducing bacterial (SRB) enrichments and/or denitrifying bacterial (DNB) enrichment. After incubation for one month at 30°C, the phosphane content of these samples was analyzed. The results indicated that the addition of glucose or yeast extract could greatly increase the phosphane content. Moreover, it was revealed that inoculation with SRB or DNB could also promote the formation of phosphine. The DNB, however, was more efficient in this regard. The highest phosphine content, about 5 times that of the control, was detected in the sample that was added with both glucose and yeast extract and inoculated with SRB and DNB simultaneously. SRB and DNB were enriched for several generations and the phosphane content of different generations was analyzed. Furthermore, SRB and DNB enrichments were inoculated into different media, in the beginning of enriching, the phosphane content was about the same for different enrichments, and differed more significantly as the enrichment process was carried further. In fourth generation, the phosphane content of DNB enrichment was about 3 times of that of SRB enrichment, indicating that the inoculation of different enrichments into different media caused the variations of the phosphine content. The highest phosphine content was detected in the sample which was inoculated with DNB enrichment into the denitrifying medium without nitrate. It is inferred from these results that microorganisms play an important role in the production of phosphine in different environments.

Key words: phosphane content; microbial populations; enzymatic activities; sulfate reducing bacteria; denitrifying bacteria

Introduction

Phosphine (PH_3) was first detected in nature in 1988 (Dévai *et al*., 1988). From then, this phosphorous compound has been found in various environments, such as soil (Eismann *et al*., 1997), marshy soil (Dévai and Delaune, 1995), paddy field (Han *et al*., 2000), air (Liu *et al*., 1999), marine sediment (Gassmann and Schorn, 1993; Yu and Song, 2002; Zhu *et al*., 2005), fresh lake sediment (Liu *et al*., 2004), and rocks (Glindemann *et al*., 2005), and so on. These results showed that phosphine is widely distributed in the environments, and such variability of the chemical valency of phosphorus may have an important role in its global biogeochemical cycle (Dévai and Delaune, 1995; Han *et al*., 2000; Glindemann and Bergmann, 1996). Phosphine concentration in samples from different environments varied significantly, ranging from 3 ng/kg soil (Liu *et al*., 1999) to 21,307 ng/kg soil (Liu *et al*., 2004). Yu and Song (2002) reported that phosphine content was closely related to the presence of organic phosphate compounds. Ding *et al*. (2005) reported that matrix bound phosphine was promoted at low pH, and this might be due to the acidic bio-corrosion of metal particles in the sludge or of metal phosphides. Moreover, in our previous work (Liu *et al*., 2004), we found that phosphine content was closely related to the occurrence of total organic carbons, reducing sugars, organic phosphate compounds, and anaerobic heterotrophic bacteria. The aerobic heterotrophic bacteria, on the other hand, were found to be unrelated to the phosphine production. The mechanism for the production of phosphine in the environments is still unknown. In their review, Roels and Verstraete (2001) proposed that phosphine in the environment might be due to biological formation, but the biotic reduction of phosphate to phosphine is controversial (Glindemann *et al*., 1998). Up to date, the attempts to isolate bacterial strains capable of reducing phosphate to phosphine have met failure. In this study, firstly the relationships between phosphine

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content and microbial populations and the activities of phosphatase and dehydrogenase of the samples were analyzed. Next, the phosphine content of different soil samples such as, with glucose or yeast extract added; inoculated with SRB enrichment or DNB enrichment; and role of the two enrichments, regarding the phosphine production, was also evaluated in different media. This was done to preliminarily investigate the role of microorganisms in phosphine production, and to provide us with clues for the isolation of phosphine producing strains.

1 Materials and methods

1.1 Samples

Twelve samples were collected from anaerobic environments. Six of them from the sediments of Taihu Lake at sites T1 (120°13′20.8″E, 31°32′30.1″N), T2 (120°12′45.9″E, 31°30′20.3″N), T3 (120°02′16.8″E, 31°27′10.7″N), T4 (120°13′14″E, 31°13′50″N), T5 (119°57′25″E, 31°12′35″N), and T6 (120°26′11″E, 31°01′57″N), 10–15 cm beneath the sediment surface. Four samples were from the paddy field soil of the suburbs of Beijing, at 0–5 cm, 5–10 cm, 10–15 cm, and 15–20 cm beneath the surface; and two samples were from the activated sludge of a wastewater treatment plant in Beijing. One-half of each sample was packed into polytetraflorinethylene bottles, which was sealed tightly and covered with three layers of black plastic membrane, and stored at –70°C for the analysis of phosphine. The other half of the samples was packed in sample bags and used for chemical and microbial analysis. pH value, moisture content, and other parameters of the samples have been shown previously (Liu *et al*., 2004).

Garden soil sample was collected 5 cm beneath the surface from the suburb of Beijing. After the plant roots and gravel were removed, the soil sample was divided into parts, each with 600 g (wet weight). Half of these samples were autoclaved thoroughly (twice at 121°C for 30 min). Later they were packed into brown bottles which were sealed tightly and covered with 3 layers of black plastic membrane. The bottles were incubated in dark at 30°C, and the phosphine contents were analyzed after a month.

Activated sludge sample was used for the enrichment of sulfate-reducing bacteria and denitrifying bacteria.

1.2 Media

LB broth was used for counting of aerobic heterotrophic and anaerobic heterotrophic bacteria. Ingredients of the medium used for the counting of organic phosphate-dissolving bacteria were as follows (g/L): glucose 10.0; $(NH_4)_2SO_4$ 0.5; $MgSO_4{\cdot}7H_2O$ 0.3; NaCl 0.3; KCl 0.3; $FeSO_4{\cdot}7H_2O$ 0.03; $MnSO_4{\cdot}H_2O$ 0.03; $CaCO_3$ 10; Glycerophosphate 2.0; agar 15. And medium used for the counting of inorganic phosphate-dissolving bacteria contained (g/L): glucose 10.0; $(NH_4)_2SO_4$ 0.5; $MgSO_4{\cdot}7H_2O$ 0.3; NaCl 0.3; KCl 0.3; $FeSO_4{\cdot}7H_2O$ 0.03; $MnSO_4{\cdot}H_2O$ 0.03; $Ca_3(PO_4)_2$ 10; agar 15.

Other media, with detail of ingredients, used in this study are listed in Table 1. The media were boiled and gassed with nitrogen to remove oxygen.

1.3 Enrichment of sulfate-reducing bacteria (SRB) and denitrifying bacteria (DNB)

Ten milliliters of activated sludge sample was suspended in 90 ml of sterile saline solution. After the suspension settled down, a 10-ml of the supernatant was inoculated into 90 ml of medium A, B, modified A, and modified B, respectively, and incubated at 30°C for 10 d as one generation. Then 10 ml of the culture was transferred into 90 ml of the corresponding media and incubated again at 30°C for another 10 d. The transfer process was repeated for another 3 or 4 times to obtain the SRB and DNB enrichments.

1.4 Numerical counting of microbial populations

Aerobic heterotrophic bacteria, anaerobic heterotrophic bacteria, nitrifying bacteria, denitrifying bacteria, and sulfate-reducing bacteria were counted by most probable number (MPN) method (Levin, 1992). Inorganic phosphate-dissolving bacteria and organic phosphate-dissolving bacteria were counted by colony forming units (CFU) method (Levin, 1992).

1.5 Enzyme assays

1.5.1 Dehydrogenase (DH) assay

Assay of dehydrogenase was completed according to the method of Alef (1994). In specific, the DH assay was performed by incubating 2 g sample with 2 ml of triphenyltetrazolium chloride (TTC) solution (0.1 g TTC/100 ml, 100 mmol/L Tris buffer, pH 7.6) in sterile glass test tubes at 30°C for 24 h. Acetone (20 ml) was added to each sample followed by further incubation for 2 h at room temperature in dark. Each sample suspension was filtered and measured for absorbance at 546 nm. Absorbance results were compared against a standard series of triphenyl formazan solutions, the end product of TTC reduction. The DH activity was calculated for each sample and expressed

Table 1 Media and the ingredients (unit: g/L)

Ingredient	Medium			
	A	B	Modified A	Modified B
Glucose	–	10.0	–	10.0
K_2HPO_4	0.5	0.5	2.0	2.0
NH_4Cl	1.0	–	1.0	0.5
Na_2SO_4	0.5	–	–	–
KNO_3	–	1.0	–	–
$CaCl_2{\cdot}2H_2O$	0.1	0.5	0.1	0.5
$MgSO_4{\cdot}7H_2O$	2.0	–	0.7	–
Sodium lactate	3.5	–	3.5	–
Yeast extract	1.0	–	1.0	–
$FeSO_4{\cdot}(NH_4)_2SO_4{\cdot}6H_2O$[a]	0.1	–	0.1	–
$Na_2S_2O_4$[a]	0.1	–	0.1	–
Vitamin C[a]	0.1	–	0.1	–
pH	7.0–7.2	7.2	7.0–7.2	7.2

* Media A and modified A were used for enriching sulfate-reducing bacteria (SRB); medium B and modified B were used for enriching denitrifying bacteria (DNB). [a] These ingredients were sterilized by filtration and added after the media were autoclaved.

Table 2 Phosphine (PH_3) content and some microbial populations of samples

Sample	PH_3 (ng/kg soil)	IPB (CFU/g)	Nitrifier (MPN/g)	SRB (MPN/g)
Sediment				
T1	37.73 ± 4.32	5.6×10^5	2.8×10^4	1.7×10^6
T2	49.37 ± 5.57	3.8×10^5	2.9×10^4	3.6×10^5
T3	62.12 ± 5.46	1.8×10^5	8.2×10^4	3.5×10^5
T4	124.12 ± 6.78	1.5×10^3	1.5×10^4	1.1×10^3
T5	14.85 ± 2.25	1.6×10^2	1.0×10^2	1.5×10^4
T6	66.70 ± 5.13	2.2×10^2	3.7×10^1	7.4×10^4
Paddy field soil				
0–5 cm	2,394.49 ± 15.66	8.2×10^5	8.2×10^5	1.2×10^6
5–10 cm	950.44 ± 8.94	1.2×10^5	3.7×10^5	1.1×10^6
10–15 cm	672.60 ± 7.55	0	1.1×10^6	2.3×10^5
15–20 cm	856.38 ± 7.36	0	3.7×10^4	1.8×10^5
Sludge				
Precipitated sludge	21,307.4 ± 57.81	0	1.2×10^6	2.6×10^8
Concentrated sludge	14,055.5 ± 43.76	0	1.5×10^7	3.2×10^9
R^2	–	0.055 (P = 0.46)	0.30 (P = 0.066)	0.31 (P = 0.060)

IPB: inorganic phosphate-dissolving bacteria.

as μg TTC reduced/g dry sample.

1.5.2 Phosphatase assay

Phosphatase activity was determined as described by Xu and Zheng (1986). For the assay of acidic phosphatase and alkaline phosphatase, acetate buffer (pH 5.0, 0.1 mol/L) and Tris-HCl buffer (pH 8.5, 0.1 mol/L) were used, respectively. One unit (U) of phosphatase activity was defined as the amount needed for the release of 1 μmol PO_4^{3-}/min.

1.6 Analysis of phosphine

Samples were treated and analyzed according to Gassmann (1994), and the gas chromatography conditions were the same as Niu *et al*. (2003). Each sample was analyzed triply.

2 Results

2.1 Phosphine content of the samples and its relationship with microbial populations

Phosphine contents and some microbial populations of samples were analyzed (Table 2 and Fig.1). The results showed that there were not relationships between phospine content and inorganic phosphate-dissolving bacteria, nitrifying bacteria, and SRB, since R^2 were all less than 0.4. However, the R^2 for the relationship between phosphine content and organic phosphate-dissolving bacteria was 0.90 (Fig.1a). This strong correlation clearly establishes the important role of phosphate-dissolving bacteria in the production of phosphine. This result was in agreement with that of Yu and Song (2002) and Liu *et al*. (2004) regarding the positive correlation of phosphine to the content of organic phosphate compounds, and the organic phosphate-dissolving bacteria, one of the main microbial populations involved in the metabolism of organic phosphate compounds. The R^2 value for the relationship between phosphine content and denitrifying bacteria was 0.69 (Fig.1b), showing a moderately positive correlation between the two. This result inferred that denitrifying bacteria might be involved in the production of phosphine since they are important reducing bacteria in an ecosystem.

2.2 Relationships between phosphine content and the enzymatic activities

The activities of dehydrogenase and phosphatase of the samples were analyzed. The results (Figs.2 and 3) indicated that phosphine content was closely related to the activities of dehydrogenase and alkaline phosphatase, but not to the activity of acid phosphatase, since its correlative index (R^2) was only 0.40 (Fig.3). R^2 for the relationship between phosphine content and dehydrogenase was 0.82 (Fig.2), implying that the higher the activity of dehydro-

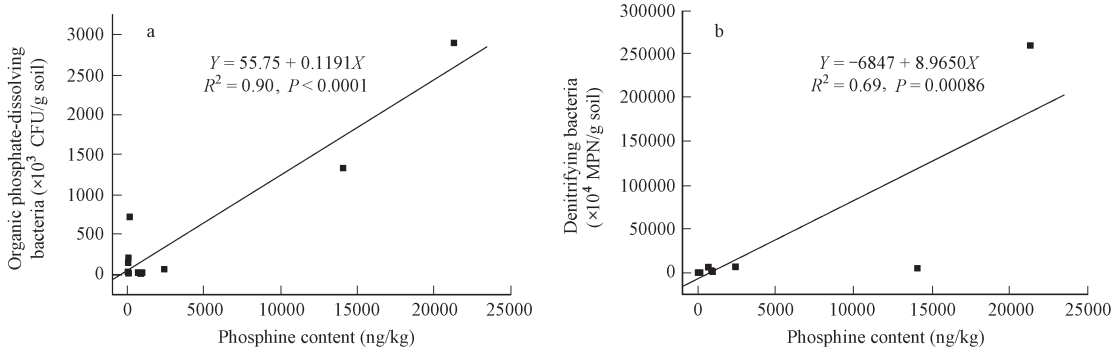

Fig. 1 Relationships between phosphine content and organic phosphate-dissolving bacteria, and denitrifying bacteria. (a) organic phosphate-dissolving bacteria; (b) denitrifying bacteria. CFU: colony forming units; MPN: most probable number

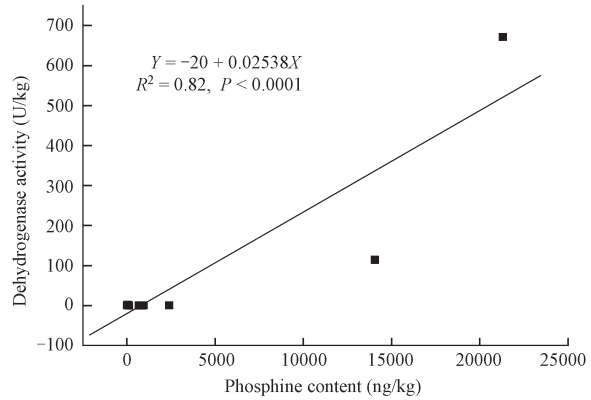

Fig. 2 Relationship between phosphine content and the activity of dehydrogenase.

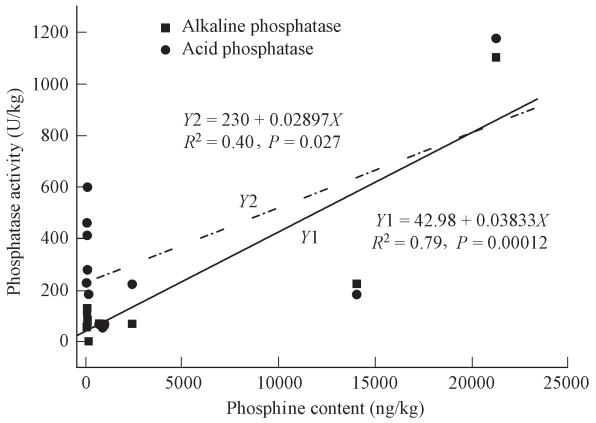

Fig. 3 Relationships between phosphine content and the activity of phosphatases. *Y*1: relationship with alkaline phosphatase; *Y*2: relationship with acid phosphatase.

genase the greater the phosphine content. Dehydrogenase includes a group of the enzymes that catalyze the dehydrogenation reaction of a large number of organic compounds, such as carbohydrates (especially reducing sugars), organic acids, amino acids, alcohols, humic acids, and so on. Activity of dehydrogenase results in the production of reducing compounds such as NADH or NADPH and so on, which provide an ecosystem with enough reducing power for various biochemical processes. The extremely positive relationship between phosphine content and dehydrogenase shows that the production of phosphine is a reducing biochemical process. R^2 for the relationship between phosphine content and alkaline phosphatase was 0.79 (Fig.3), signifying a direct proportionality.

2.3 Advantage of the inoculation of some microbial populations to soil samples in phosphine production

The phosphine contents of garden soil samples with different treatments are listed in Table 3. The results indicated phosphine detection in normal garden soil, though it's concentration was quite low (22.22 ng/kg soil). It was also revealed that the addition of glucose and yeast extract to soil could greatly promote the production of phosphine; its content was increased to 52.79 and 65.91 ng/kg soil, respectively. In addition, the results in Table 3 also indicate that the inoculation of SRB or DNB to both non-autoclaved soil and autoclaved soil could also promote the production of phosphine; the phosphine contents of the non-autoclaved samples were increased to 39.53 and 51.60 ng/kg soil, respectively, and for autoclaved samples, this promotion was even greater, the phosphine contents were increased to 46.36 and 66.90 ng/kg soil, respectively. Conclusively, regarding the phosphine production, the inoculation of SRB or DNB to the autoclaved soil was more advantageous than to the non-autoclaved soil, and inoculation with DNB furnished better yield than that of SRB. Furthermore, the phosphine content of the autoclaved soil inoculated with a blend of SRB and DNB was higher than the one inoculated with SRB or DNB solely. The autoclaved soil with the addition of glucose and yeast extract, and inoculation of SRB and DNB together, possessed the highest phosphine content (112.25 ng/kg soil), which was about 5 times of the control.

2.4 Phosphine content of the enrichments

2.4.1 Phosphine content of various generations of the different enrichments

Phosphine content of various generations of the different enrichments were also analyzed (Table 4). The results showed that the phosphine contents increased with the progress of enrichment from generation to generation for both SRB and DNB. At the first generation, the phosphine contents for both enrichments were about the same, but

Table 3 Garden soil samples and their phosphine (PH_3) content

Sample No.	Soil weight (g)	Sterile water (ml)	Sterile glucose 30% (ml)	Sterile YE 15% (ml)	Inoculation[a]	PH_3 (ng/kg soil)
Non-autoclaved						
1	600	80	–	–	–	22.22
2	600	40	40	–	–	52.79
3	600	40	–	40	–	65.91
4	600	60	–	–	20 ml SRB	39.53
5	600	60	–	–	20 ml DNB	51.60
Autoclaved						
6	600	80	–	–	–	24.72
7	600	60	–	–	20 ml SRB	46.36
8	600	60	–	–	20 ml DNB	66.90
9	600	60	–	–	10 ml SRB +10 ml DNB	80.29
10	600	0	40	40	10 ml SRB +10 ml DNB	112.25

YE: yeast extract; [a] SRB or DNB contain 10^9 cells/ml.

Table 4 Enrichments and their phosphine contents

Enrichments and their generations	Phosphine content (ng/m^3)
SRB enrichment	
First generation	0.0178
Second generation	0.0216
Third generation	0.0264
Fourth generation	0.0321
DNB enrichment	
First generation	0.0186
Second generation	0.0346
Third generation	0.0579
Fourth generation	0.0867

the difference became obvious with the maturation of enrichment. The phosphine content of DNB enrichment was higher than that of SRB enrichment. For SRB the phosphine content increased from 0.0178 to 0.321 ng/m^3, about a two-fold increment, but for the DNB, the phosphine content increased from 0.0186 to 0.0867 ng/m^3, the increment was about four folds during the enrichment.

2.4.2 Phosphine contents of different cultivations

Samples of 20 ml from the fourth generation of SRB and DNB enrichments were separately inoculated into different media and incubated in dark at 30°C for one month, and then the phosphine contents of different incubations were analyzed (Table 5). The phosphine contents of the samples 1 to 3 were similar. However, the phosphine content for sample 4 was comparatively higher. The medium used for sample 4 was modified A, indicating greater suitability of this medium for phosphine production. The difference of the phosphine contents of samples 5 to 8 was more pronounced. The phosphine contents of media or modified B inoculated with DNB enrichment were much higher (about 3 to 4 times) than those inoculated with SRB enrichment. This inferred that the denitrifying bacteria have an important role in the production of phosphine in the environments. In addition, media B and modified B were both suitable for the production of phosphine despite of the decrease of nitrate and increase of phosphate in modified B.

Table 5 Phosphine contents of different cultivations

Sample No.	Medium (500 ml)	Inoculation (20 ml)	Phosphine content (ng/m^3)
1	A	SRB	0.0420
2	A	DNB	0.0453
3	Modified A	SRB	0.0347
4	Modified A	DNB	0.0577
5	B	SRB	0.0190
6	B	DNB	0.0858
7	Modified B	SRB	0.0386
8	Modified B	DNB	0.0913

3 Discussion

Microorganisms are the most important components of an ecosystem and carry out the main role in all the biogeochemical cycles. Microbial flora structure and the number of the populations as well as their metabolic and enzymatic activities in an ecosystem could stand the function and cycling rate of the elements of the ecosystem. Different microbial populations possess various functions and collectively maintain the balance of an ecosystem. Nevertheless, the conditions of the environment significantly affect the structure and function of the microbial florae. In the oxygen deficient environments the anaerobic microorganisms such as sulfate-reducing bacteria and denitrifying bacteria flourish. These bacteria use SO_4^{2-} or NO_3^- as final electron acceptors and reduce them to H_2S or N_2. Similarly, the phosphate-reducing bacteria also need anaerobic conditions to reduce phosphate to phosphine. Many reports demonstrated that phosphine is mainly released in the anaerobic environments (Devai and Delaune, 1995; Glindemann and Bergmann, 1996; Eismann *et al*., 1997a). Since fermentation is an anaerobic metabolic process of microbes, the release of phosphine from manure fermentation (Eismann *et al*., 1997b) strongly suggests the importance of microbes in the production of phosphine. In our previous work, the results showed that phosphine content was closely related to anaerobic microbes (R^2=0.92, Liu *et al*. 2004), and not to the aerobic ones. Thus it is eminent that anaerobic microorganisms act an important role in the production of phosphine in the environments. Further analysis showed that phosphine content was also closely related to organic phosphate-dissolving bacteria (R^2=0.90, Fig.1a), indicating their predictable involvement in the formation of phosphine. This result was in agreement with those reported by Yu and Song (2002) and Liu *et al*., (2004) illustrating that phosphine content was closely related to the concentration of organic phosphate compounds. Our results also indicated that phosphine content was closely related to the activities of dehydrogenase and alkaline phosphatase (R^2=0.82 and 0.79, respectively, Figs.2 and 3). Dehydrogenase enzymes are involved in catalyzing the dehydrogenation reaction and acting as middle transporters of hydrogen in the ecosystem, they can deduce the hydrogen from carbohydrates (such as reducing sugars), organic acids, amino acids, and so on. The released hydrogen was transported to oxygen or other hydrogen acceptors such NAD(P) and forming NAD(P)H, providing the ecosystem with enough reducing power for the reduction-metabolisms, such as reduction of sulfate, nitrate, and possibly the phosphate. Glucose and yeast extract are good reducing substrates for microorganisms in the ecosystem, and their addition to soil samples not only elate the activity of microorganisms, but also provide a lot of reducing power for many biochemical processes including the reduction of phosphate to phosphine (Table 3). Phosphine content was closely related to denitrifying bacteria (R^2 = 0.69, Fig.1b), but not to other anaerobic microbial populations. Denitrifying bacteria are the microorganisms possessing diverse metabolic abilities; they can grow both aerobically and anaerobically–nitrate respiration. The results in Tables 3, 4, and 5 further demonstrated the role of denitrifying bacteria in the production of phosphine. It shows higher phosphine contents for the soil samples inoculated with denitrifying bacteria and the samples of denitrifying bacterial enrichments compared

to those of sulfate-reducing bacterial samples, though the phosphine contents of sulfate-reducing bacterial samples were also a bit higher than that of control. All of these results inferred that microorganisms, especial denitrifying bacteria, take an important role in the production of phosphine in the environments. The investigation of the role of pure culture of denitrifying bacteria in the production of phosphine is currently undertaken.

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