Biol Fertil Soils (2002) 35:136–146
DOI 10.1007/s00374-002-0454-8

ORIGINAL PAPER

M.I. Makarov · L. Haumaier · W. Zech

The nature and origins of diester phosphates in soils: a ^{31}P-NMR study

Received: 10 August 2001 / Published online: 20 March 2002

Abstract Soils of two climosequences in Russia were investigated by ^{31}P-NMR spectroscopy. They comprised Dystric Podzoluvisols, Haplic Greyzems, Calcic Chernozems, and Gypsic Kastanozems, which are located along temperature and precipitation gradients of the Russian Plain. Another sequence of soils included forest Humic Cambisols and Umbric Leptosols of subalpine and alpine meadows, which are formed in different climatic conditions along a climosequence of the Mt. Malaya Khatipara (northern Caucasus). The results showed that accumulation of DNA was high in the cold, wet, and acid soils (Dystric Podzoluvisol, alpine Umbric Leptosol), while phospholipids and teichoic acids mainly accumulated in the more microbially active soils. We performed a laboratory incubation experiment to test the relationship between microbial biomass P and P species identified in soil extracts. The proportions of P compounds resonating at 0.5–3.0 ppm in the $NaHCO_3$ and H_2SO_4 extracts from the incubated Humic Cambisol increased. The amounts of phosphate diesters resonating at 0 ppm in the same extracts and in the subsequent NaOH extracts decreased after incubation. Based on the results of ^{31}P-NMR spectroscopy of native soils and of the laboratory incubation experiment we concluded that signals at 0 ppm in spectra of soil alkaline extracts belong to DNA P which is mainly stabilised in soil organic matter outside microbial cells (at least in soils with relatively low microbial activity). Phospholipids-teichoic acids P extracted with 0.5 M $NaHCO_3$ seems to be derived from soil microbial biomass, and its proportion can reflect the microbial activity in the soil.

M.I. Makarov (✉)
Department of Soil Science, Moscow State University,
119899 Moscow, Russia
e-mail: mmakarov@soil.msu.ru

L. Haumaier · W. Zech
Institute of Soil Science and Soil Geography,
University of Bayreuth, 95440 Bayreuth, Germany

Keywords Soil organic phosphorus · Microbial biomass phosphorus · Diester phosphates · ^{31}P-NMR spectroscopy

Introduction

A large proportion of the organic phosphorus (P_{org}) in soils is associated with the polymeric structure of humic compounds. Phosphorus (P) in these humic fractions may be moderately resistant (if associated with fulvic acids, FA) or highly resistant (if associated with humic acids, HA). However, ^{31}P nuclear magnetic resonance (NMR) spectroscopy has shown that HA and FA fractions of various soils contain diester P compounds which are most frequently attributed to the labile part of the soil P_{org} pool. Recently, we established new assignments of the diester P resonances at 0 ppm (DNA P) and 0.6–1.9 ppm (phospholipids-teichoic acids P) in ^{31}P-NMR spectra of alkaline soil extracts (Makarov et al., submitted for publication). This new assignment will be used in the present paper to interpret diester P accumulation in soils.

Knowledge of the factors regulating the accumulation of labile P_{org} compounds in soil is necessary to understand their role in the phosphorus cycle. According to Tate and Newman (1982) the amount of potentially readily mineralisable phosphonates and phosphate diesters in soils increased when conditions for microbial decomposition became limited by climate. These authors showed that the largest proportions of labile P_{org} species in a climosequence of soils in New Zealand tussock grassland accumulated in the highly acid alpine meadow soils receiving high precipitation. Similar conditions favourable for the accumulation of labile P_{org} compounds were revealed in acid soils of the Bavarian Alps (Germany; Zech et al. 1987), in a pseudo-alpine Ranker of Galicia (Spain; Gil-Sotres et al. 1990), and in a climosequence of Caucasian mountain soils (Russia; Makarov et al. 1996). Within a toposequence of alpine soils in the northern Caucasus the contribution of labile P species to total P in HA of surface soil horizons also increased at the lower eleva-

tions, where the snow cover increased during winter and soil was most acidic due to leaching (Makarov et al. 1997). Therefore, according to these results, labile P_{org} compounds (phosphonates and phosphate diesters) seem to accumulate in cold, wet and acidic soils as the result of reduced microbial decomposition.

However, in a recent ^{31}P-NMR study Sumann et al. (1998) demonstrated that the concentration of phosphate diesters resonating at 0 ppm increased in bulk soils and clay fractions of 18 uncultivated soils of North American grasslands when conditions were more favourable for microbial activity. These authors believed that considerable amounts of these compounds were represented by products of microbial metabolism, which accumulated in microbially active soils due to stabilisation in the clay fraction. At the same time, there was no accumulation of other P species resonating in the diester region which are considered also to be of microbial origin (teichoic acids and unknowns). Phosphonates also did not reflect any influence of climate.

Studies using sequential extraction and ^{31}P-NMR spectroscopy indicate that diester P is more labile than monoester P. However, little is known about the relationship between labile soil P_{org} and diester P. Rubæk et al. (1999) found that the concentration of labile resin-P_{org} was linearly related to the concentration of diester P in dialysed NaOH extracts from soil-particle-size fractions. Robinson et al. (1998) and Zhang et al. (1999) demonstrated that soil phosphate diesters had different extractability. The fraction of diester P extractable by 0.5 M $NaHCO_3$ was represented by easily mineralisable P_{org}. However, the major phosphate diester fraction was only dissolved in more strongly alkaline extracts (0.1 M NaOH or 0.25 M NaOH/0.05 M EDTA). Furthermore, Taranto et al. (2000) showed that most (>90%) of the labile P compounds extracted by the Chelex 20 cation-exchange resin were monoesters.

At least four separate signals were found in the diester P region (from –1.5 to 3.0 ppm) but no attempts have been made to estimate the relations between different diester compounds that accumulate in soils. The data of Sumann et al. (1998) gives only indirect evidence of relatively independent accumulation of DNA P (0 ppm) and other compounds resonating between –1.5 and 3.0 ppm. This data and the results of Robinson et al. (1998) and Zhang et al. (1999) demonstrated that soil diester P comprises a heterogeneous group of P compounds, which may accumulate differently in soil and have different roles in the soil phosphorus cycle.

Total amounts of diester P in soils, as determined by ^{31}P-NMR spectroscopy, were of the same order of magnitude as soil microbial biomass P measured in numerous investigations (most frequently 5–20% of total or organic P; Brookes et al. 1984; Srivastava and Singh 1988; Joergensen et al. 1995). However, no experimental data exist about the relation of microbial biomass P and diester-P compounds identified in alkaline soil extracts. It is unknown if phosphate diesters mainly derive from lysed microbial cells or if they mainly are components of humic substances.

Therefore the aim of the present study was to investigate the mechanism of labile P_{org}-species accumulation in soils using ^{31}P-NMR spectroscopy. We investigated the distribution of P species in soils of climosequences in the Russian Plain and in the northern Caucasus. The dynamics of P_{org} compounds during short-term incubation of the soil and the relationship of microbial biomass P to soil diester P were investigated in a laboratory incubation experiment.

Materials and methods

Soil samples

The soils investigated were a Dystric Podzoluvisol, a Haplic Greyzem, a Calcic Chernozem, and a Gypsic Kastanozem, which were located along temperature and precipitation gradients of the Russian Plain. Soils were collected under native vegetation: spruce forest (*Picea abies*), lime forest (*Tilia cordata*), meadow steppe and half-shrub bunch-grass steppe for the Podzoluvisol, Greyzem, Chernozem, and Kastanozem, respectively. Another sequence of soils included a Humic Cambisol of *Abies nordmanniana* forest and Umbric Leptosols of subalpine and alpine meadows, which were located along temperature and precipitation gradients of the Mt. Malaya Khatipara (northern Caucasus).

Mean annual temperature along that part of the Russian Plain where soils were sampled ranges from +3.4°C in the forest zone of the Podzoluvisols to +9.2°C in the dry-steppe zone of the Kastanozems. Corresponding mean annual precipitation ranges from 590 to 410 mm. Temperature and precipitation parameters for the Caucasus are +6.3°C and 700 mm for the lowest investigated position (forest Cambisol) and –1.2°C and 1,400 mm for the highest position (alpine Leptosol).

From two to four samples of humic horizons of each soil type were collected for ^{31}P-NMR spectroscopy. Some properties of the soils, and sampling depths, are presented in Table 1.

Incubation experiment

The experiment was carried out with a mixed soil sample (0–15 cm soil depth, five profiles) of a Humic Cambisol from the northern Caucasus with the following properties: pH 5.9, organic C 6.9%, total N 0.6%, total P 780 mg kg^{-1}, and organic P 580 mg kg^{-1}. The soil was incubated, and then P species were extracted with 0.5 M $NaHCO_3$, 0.05 M H_2SO_4, and 0.1 M NaOH from initial, incubated, and $CHCl_3$-treated incubated samples, and characterised by ^{31}P-NMR spectroscopy. In brief, six series of triplicate 10-g samples were placed in 100-ml plastic bottles. Distilled water with dissolved glucose, NH_4Cl, and KH_2PO_4 was added to series 1–4 to create 60% of water holding capacity and to supply the soil with C, N, and P in concentrations of 6.0, 0.6, and 0.06 mg g^{-1} soil, respectively. Series 5 and 6 were treated in the same way but excluding P supply. The samples were incubated at 28°C for 72 h. Then, samples of series 1 and 5 were extracted with 0.5 M $NaHCO_3$ (pH 8.5) followed by extraction with 0.1 M NaOH, and samples of the second series were extracted with 0.05 M H_2SO_4 followed by extraction with 0.1 M NaOH. Samples of series 3, 4, and 6 were treated with 4.0 ml alcohol-free $CHCl_3$ over 16 h. After treatment chloroform was evaporated over 8 h, and samples of series 3 and 6 were extracted in the same way as those of series 1 and 5. Samples of the fourth series were extracted as for series 2. The initial soil sample was also extracted in triplicate in the same way as the incubated samples (e.g. with 0.5 M $NaHCO_3$ followed by 0.1 M NaOH and with 0.05 M H_2SO_4 followed by 0.1 M NaOH).

The soil-to-solution ratio of each extraction procedure was 1:5. Shaking time on a rotary shaker was 15 min for extraction with H_2SO_4 and 16 h for $NaHCO_3$ and NaOH. All extractions were repeated twice and corresponding extracts were combined for analysis.

Table 1 Properties of the A horizons of typical soil types

Soil type (FAO)	Depth (cm)	pH		C (g kg^{-1})	P_t (mg kg^{-1})	P_{org}	C:P
		(H_2O)	(KCl)				
Dystric Podzoluvisol	3–12	4.8	3.8	25.0	400	198	126
Haplic Greyzem	1–15	5.4	4.9	20.9	759	401	52
Calcic Chernozem	0–35	6.4	5.9	54.6	843	664	82
Gypsic Kastanozem	0–23	7.6	7.2	16.7	609	295	57
Humic Cambisol	5–17	5.9	5.3	69.0	780	580	119
Umbric Leptosol (subalpine)	0–15	5.3	4.5	87.6	1,296	847	103
Umbric Leptosol (alpine)	0–12	4.7	3.9	72.1	1,454	1,019	71

Table 2 Average proportions of P species in dialysed NaOH extracts from different soils (% of total P in extract)

Soil	Phosphonates[a]	Inorganic ortho-phosphate[a]	Monoesters[a]	Phospholipids and teichoic acids[a]	DNA	Unknown	Pyro-phosphates
Dystric Podzoluvisol	1.3a	6.2a	58.7a	6.8a	22.3a	3.6a	1.1a
Haplic Greyzem	2.2a	6.1a	71.8b	9.2a	5.2b	3.8a	1.7a
Calcic Chernozem	2.1a	1.5b	72.0b	12.5b	6.8b	5.1b	–
Gypsic Kastanozem	3.8b	–	76.9c	3.9c	10.7c	4.7ab	–
Humic Cambisol	2.3a	5.7a	60.9a	13.2b	9.7c	6.4b	1.8a
Umbric Leptosol (subalpine)	1.8a	5.2a	70.8b	12.4b	5.5b	4.3a	0.6b
Umbric Leptosol (alpine)	2.8a	8.0a	65.2b	6.7a	13.7d	3.2a	0.4b

[a] Within each column, values followed by the same letter do not differ significantly at $P < 0.05$

Microbial biomass C and P measurement

Microbial biomass P was calculated from the difference between the amount of inorganic P extracted by 0.5 M $NaHCO_3$ from samples treated with $CHCl_3$ and the amount extracted from untreated samples of the incubation experiment. A K_p value of 0.4 was used, assuming that 40% of P in the microbial biomass is released as inorganic P by $CHCl_3$ (Brookes et al. 1982).

Microbial biomass C was determined by the fumigation-extraction method in separate subsamples, after incubation in the same manner as described above. Chloroform vapour was used for fumigation and 0.5 M K_2SO_4 for extraction of organic C from fumigated and unfumigated soils. The organic C extracted was analysed using the $K_2Cr_2O_7$ / H_2SO_4 oxidation method. The unused dichromate was titrated with $(NH_4)_2SO_4$ $FeSO_4$ $6H_2O$ in the presence of N-phenylanthranilic acid as indicator, and the C content was calculated from the dichromate consumed. Microbial biomass C was estimated using a K_c value of 0.45 (Wu et al. 1990). Microbial biomass C in the incubated soil was also estimated by the SIR technique of Anderson and Domsch (1978) as modified by West and Sparling (1986).

Analyses and ^{31}P-NMR spectroscopy

Total P in aliquots of all extracts of the incubation experiment was determined by acid digestion with concentrated H_2SO_4 and $HClO_4$ (20:1). Inorganic P in H_2SO_4 extracts was determined directly, and in alkaline extracts after HA precipitation with 10% H_2SO_4. P_{org} in all extracts was calculated by differences between total and inorganic P. All P determinations were made by the ammonium molybdate-ascorbic acid method (John 1970).

The corresponding extracts of triplicate samples were mixed, dialysed, and freeze-dried to prepare samples for ^{31}P-NMR spectroscopy. Freeze-dried $NaHCO_3$ and H_2SO_4 extracts were dissolved in 2 ml 0.5 M NaOH, and NaOH extracts were dissolved in 8 ml.

For ^{31}P-NMR spectroscopy of native soils, P compounds were extracted with 0.5 M NaOH at a soil-to-solution ratio of 1:9 from humic horizons pretreated with 0.1 M HCl. The samples were shaken for 1 h and left to stand overnight. Then they were centrifuged for 30 min at 2,560 *g*. The supernatants were dialysed for removal of inorganic P and freeze dried. Freeze-dried material was dissolved in 2 ml 0.5 M NaOH. To 2 ml each 0.5 M NaOH solution, 1 ml D_2O was added and the samples were transferred to 10-mm NMR tubes for NMR spectroscopy.

^{31}P-NMR spectra were obtained on a Bruker Avance DRX 500 NMR spectrometer (11.7 T; 202.5 MHz for ^{31}P). Spectra were acquired without proton decoupling using an acquisition time of 0.1 s, a 90° pulse, and a relaxation delay of 0.2 s. Chemical shifts were measured relative to external 85% H_3PO_4. Spectra were recorded with a line-broadening of 20 Hz. Intensities of signals were determined by electronic integration.

The mean proportions of P species in NaOH extracts from soils and the mean concentrations of P in extracts of the incubation experiment were tested for significant differences using a *t*-test at 5% probability. Spearman rank order correlation coefficients between proportions of P species in NaOH soil extracts were calculated.

Results and discussion

Phosphorus compounds in soils

Phosphate monoesters (4.3–5.6 ppm) were the dominant P species in all investigated soils. They accounted for 58–77% of P in the dialysed NaOH extracts. The lowest proportions of monoesters were found in two forest soils (Podzoluvisol and Cambisol), and the highest in the Kastanozem of the dry steppe (Table 2). Most samples also contained phosphonates (19.0 ppm), DNA (about 0 ppm), phospholipids-teichoic acids (0.5–3.0 ppm), and unknown compounds resonating at –1.5 ppm. Inorganic P was represented by orthophosphate (5.8 ppm) and by pyrophosphates (–4.7 ppm). Average proportions of pho-

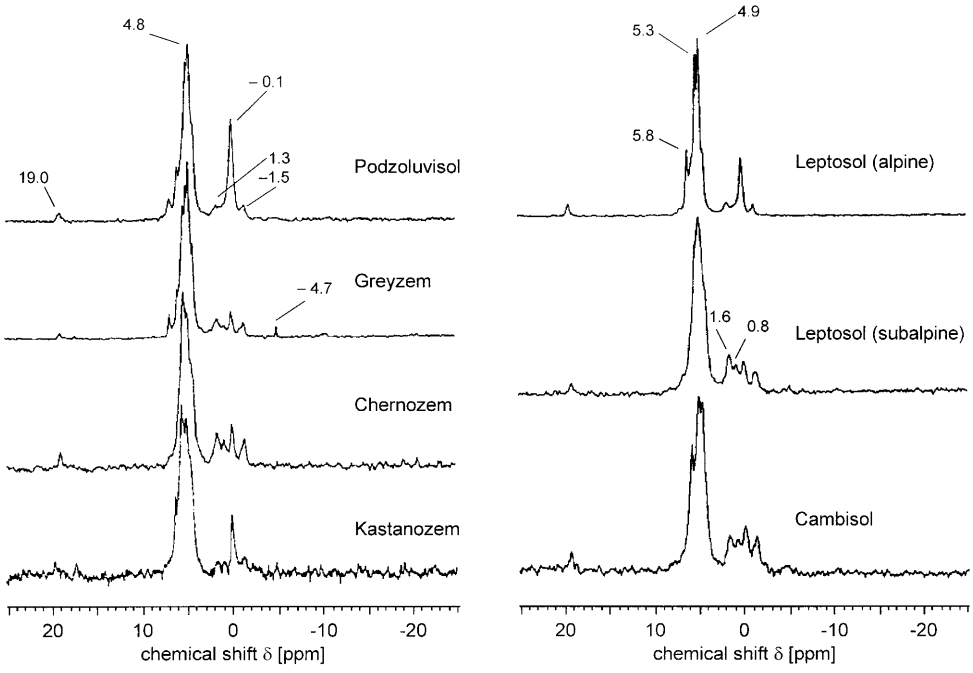

Fig. 1 ^{31}P-NMR spectra of NaOH extracts from soils of the Russian Plain and of the northern Caucasus

sphonates ranged from 1.3% to 3.8%, with a maximum in the Gypsic Kastanozem. DNA P accounted for 5.2–22.3% and phospholipids-teichoic acids P for 3.9–13.2% of the NaOH-extractable P. Unknown compounds comprised 3.2–6.4% of extractable P.

The NMR-spectroscopic results showed that there was no connection between the proportions of phospholipids-teichoic acids P and DNA P. High proportions of DNA P were found in the cold, wet, and acidic soils (Dystric Podzoluvisol and alpine Umbric Leptosol; Table 2, Fig. 1), and also in the Kastanozem. This corresponded with findings presented by Amelung et al. (2001) who found high proportions of diester P in Haplic and Gypsic Kastanozems of the Russian Plain. Thus, our results support the hypothesis of Tate and Newman (1982) that the amount of potentially easily mineralisable phosphate diesters (DNA) in soils increases as conditions for microbial activity become limited by climate. One group of soils with pronounced DNA P accumulation is represented by wet acidic soils, and another group by dry calcareous soil. In contrast, phospholipids and teichoic acids mainly accumulated in the more microbially active soils (Calcic Chernozem, Humic Cambisol, and subalpine Umbric Leptosol).

Though plants produce phosphate diesters (nucleic acids, phospholipids), P_{org} of plant origin may be influenced by microbial resynthesis, and therefore the bulk of diester P compounds in soils could be compised of microbial metabolites (Halstead and McKercher 1975; Cosgrove 1977; Anderson 1980). We therefore investigated, whether microbes regulate the accumulation of different P_{org} compounds in soils.

For all soils under study phospholipids-teichoic acids P was negatively correlated with DNA P (r =–0.53, P <0.05). The correlation of phospholipids-teichoic acids P with unknown P compounds was positive (Fig. 2A, B). However, in the diagram of the relationship between phospholipids-teichoic acids P and DNA P two groups of soils where either phospholipids-teichoic acids P or DNA P predominated can clearly be recognised. Within these separate groups, the relationships between phospholipids-teichoic acids P and DNA P were positive (Fig. 2C, D). Moreover, in soils with a predominance of phospholipids-teichoic acids P, positive correlations were found between all compounds resonating between –1.5 and 3.0 ppm (phospholipids-teichoic acids/DNA – r =0.62, P <0.05; phospholipids-teichoic acids/unknown – r =0.66, P <0.05; and DNA/unknown – r =0.93, P <0.01; Fig. 2C, E, G). In soils with a predominance of DNA P these correlations were not observed (the highest r =0.50, P >0.05 was found for the relationship between phospholipids-teichoic acids P and DNA P).

We assume that in soils with relatively low microbial activity conditions are more favourable for the accumulation of DNA P of plant or microbial origin. A high proportion of DNA P in extracts from plant leaves was recently reported (Makarov et al., submitted for publication). However, microbial metabolites could also be stabilised in soil organic matter. Since different P_{org} species have different stabilities and mineralise at different rates, the proportions of individual microbially derived P compounds in soils can differ from proportions in microbial cells. As P compounds in the microbial biomass of such soils comprise relatively low proportions of labile P_{org} species accumulated in soil, the relationships between individual compounds are weak. In contrast, in soils with high microbial activity a greater proportion of soil diester P could be represented by compounds of microbial biomass, and correlations between different P species of microbial origin are more pronounced.

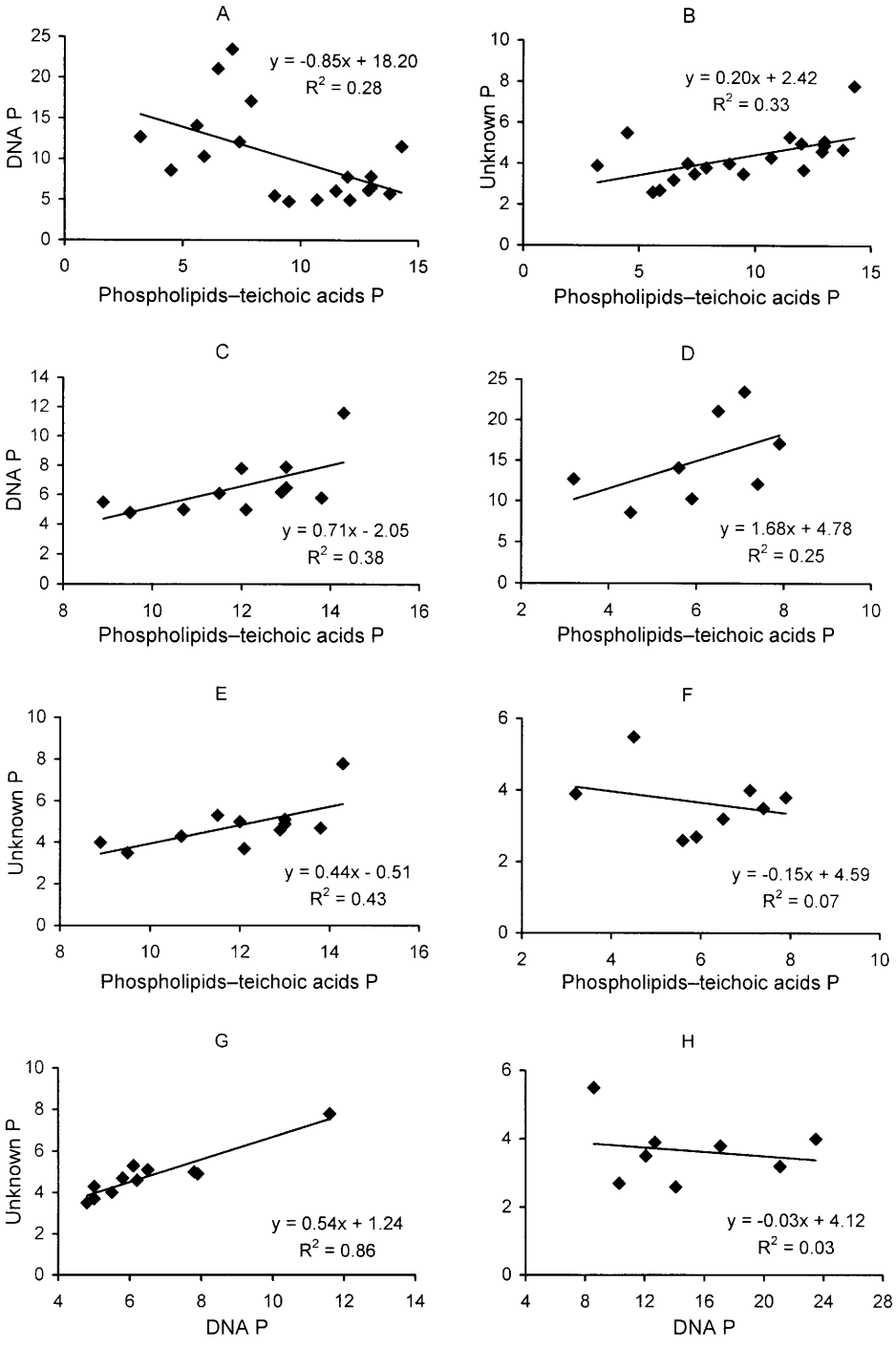

Fig. 2 Relationships between various P compounds resonating between 3.0 and –1.5 ppm expressed as a percentage of total extractable P in all soils studied (**A**, **B**), in soils with phospholipids-teichoic acids P greater than DNA P (**C**, **E**, **G**), and in soils with phospholipids-teichoic acids P less than DNA P (**D**, **F**, **H**)

Incubation experiment

Microbial biomass C and P

Microbial biomass C determined by the fumigation-extraction method did not differ significantly from the value determined by the SIR method. Therefore we used the average concentrations obtained from these two techniques. The results of the microbial C determination showed that biomass growth during incubation was not limited by lack of P. After 72 h of incubation in the soils with and without P addition, respectively, 1,230 µg g^{-1} and 1,280 µg g^{-1} microbial biomass C were accumulated (difference not significant at 0.05 probability level). However, the concentration of microbial biomass P in the soil supplied with P was higher: 34 µg g^{-1} versus 26 µg g^{-1} in the sample not supplied with P (difference significant at 0.01 probability level). Inorganic P was more efficiently extracted by 0.05 M H_2SO_4 after treatment of the soil with $CHCl_3$. Extraction of inorganic P from the $CHCl_3$-treated sample increased by 22 µg g^{-1} compared with the untreated sample (K_p value un-

Table 3 Phosphorus concentrations (mg kg^{-1}) in a Humic Cambisol subjected to different treatments

Extract	Sample	Inorganic[a]	Organic[a]	Remaining after dialysis
0.5 M $NaHCO_3$	Initial soil	9.9a	27.5a	21.4
	Incubated, no P	5.0b	23.9b	20.1
	$CHCl_3$-treated, no P	15.4c	26.7a	21.1
	Incubated, with P	23.7d	25.0b	19.9
	$CHCl_3$-treated, with P	37.2e	26.6a	20.8
0.1 M NaOH after 0.5 M $NaHCO_3$	Initial soil	15.4a	140.2a	129.9
	Incubated, no P	16.5a	143.9b	131.2
	$CHCl_3$-treated, no P	20.8b	142.0a	134.5
	Incubated, with P	25.7c	142.2a	137.3
	$CHCl_3$-treated, with P	29.6d	139.9a	132.2
0.05 M H_2SO_4	Initial soil	11.3a	12.9a	6.3
	Incubated, with P	23.3b	10.1b	6.9
	$CHCl_3$-treated, with P	45.3c	11.5a	7.2
0.1 M NaOH after 0.05 M H_2SO_4	Initial soil	27.5a	272.1a	220.9
	Incubated, with P	34.7b	278.7b	230.8
	$CHCl_3$-treated, with P	39.6c	271.7a	213.5

[a] For each extractant, values within each column followed by the same letter do not differ significantly at $P < 0.05$

known). This result contrasts with the results of McLaughlin et al. (1986) who showed that 0.05 M H_2SO_4 was an unsatisfactory extractant for detecting microbial biomass P released by the biocidal treatment. However, van Veen et al. (1987) used 0.1 M H_2SO_4 to extract $CHCl_3$-released P and showed that P extraction using acid was 91% of the value obtained using 0.5 M $NaHCO_3$ (pH 8.5).

The concentrations of P in microbial biomass were 1.4% and 1.0% in soils with and without P addition, respectively, assuming that dry biomass contains 50% C (Brookes et al. 1984). Corresponding C/P ratios were 36 and 49. Chauhan et al. (1981) and Srivastava and Lal (1994) reported that additions of inorganic P to soil can result in low biomass C/P ratios.

In our study the concentrations of P in the soil biomass were lower than reported by Brookes et al. (1984) for 15 soils of the UnitedKingdom (C/P ratio 11–36, mean 14), by Srivastava and Singh (1988) for tropical soils of India (C/P ratio 9–23), by Joergensen et al. (1995) for 38 beech forest soils of central Germany (C/P ratio 6–26, mean 14), or by Kouno et al. (1999) for a granitic regosol of Japan (C/P ratio 7–16). One reason for our result may be the high availability of C due to glucose supply. The strong influence of C availability on microbial biomass C/P ratios was demonstrated in a laboratory experiment (Anderson and Domsch 1980) and in a field experiment (Sparling and Williams 1986). The latter authors found that the microbial C/P ratio in the mineral horizon of an acidic forest soil increased from 8.3 to 35 after the addition of glucose. Another reason may be the prevalence of *Saccharomyces* sp. in microbial biomass of previously dried and glucose-supplied incubated soil (Babieva and Zenova 1989). Myers et al. (1999) demonstrated that *S. cerevisiae* contained less P than bacteria (0.8–1.5% and 2.2–4.8%, respectively). Brookes et al. (1982) also reported that fungi contained less P than bacteria (0.4–0.6% and 1.4–2.7%, respectively). On the other hand, Anderson and Domsch (1980) cultured 14 species of fungi and 10 species of bacteria in a medium having 1–10 g glucose l^{-1}. The mean concentration of P for fungi ranged from 4.8% at 1.0 g glucose l^{-1} to 3.1% at 10 g glucose l^{-1}. Bacteria grown at 10 g glucose l^{-1} contained 2.8% P on average.

P in soil extracts

The initial soil contained 9.9 mg kg^{-1} and 27.5 mg kg^{-1} of $NaHCO_3$-extractable inorganic and organic P, respectively. The corresponding values for NaOH-extractable P forms were 15.4 and 140.2 mg kg^{-1} (Table 3); 0.05 M H_2SO_4 extracted 11.3 mg kg^{-1} of inorganic P and 12.9 mg kg^{-1} of P_{org}. Acidic pretreatment of the soil increased the P extractability by NaOH (27.5 and 272.1 mg kg^{-1} of inorganic and organic P, respectively). About half of P_{org} in the H_2SO_4 extract of the initial soil was lost during dialysis when the sample was prepared for ^{31}P-NMR spectroscopy. The loss of P_{org} from the $NaHCO_3$ extract was 22%, and only 7% of P_{org} was lost from the NaOH extract of soil pretreated with $NaHCO_3$, while the NaOH extract after pretreatment with H_2SO_4 lost 19% of P_{org}.

Concentrations of inorganic P in all extracts from the incubated soil supplied with P increased. However, only 40% and 32% of P added before incubation was extracted with $NaHCO_3$-NaOH and H_2SO_4-NaOH, respectively. After $CHCl_3$-treatment the extractability of inorganic P increased, but 20–30% of added P was not extracted. Incubation of the soil without P supply decreased inorganic P concentration in the $NaHCO_3$ extract, indicating that part of the labile inorganic soil P was consumed by microorganisms.

Concentrations of P_{org} in $NaHCO_3$ and H_2SO_4 extracts of incubated soil decreased by 2.8–3.6 mg kg^{-1} as

Table 4 Phosphorus-species distribution in soil extracts (*a*, %) and concentrations in a Humic Cambisol (*b*, mg kg^{-1}) subjected to incubation with added inorganic phosphorus

Extract	Sample[a]		Inorganic ortho-phosphate	Phospho-nates	Monoesters	Phospholipids and teichoic acids	DNA	Unknown	Pyro-phosphates
$NaHCO_3$	1	a	14.6	–	34.3 (42.1)[b]	17.4 (21.4)	18.3 (22.5)	11.4 (14.0)	4.0
		b	3.1	–	7.3	3.7	3.9	2.4	0.9
	2	a	12.5	–	29.4 (35.9)	27.4 (33.4)	14.7 (17.9)	10.4 (12.7)	5.5
		b	2.5	–	5.9	5.5	2.9	2.1	1.1
	3	a	23.6	–	29.6 (38.7)	23.4 (30.6)	12.4 (16.2)	10.9 (14.3)	–
		b	4.9	–	6.2	4.9	2.6	2.3	–
NaOH[c]	1	a	10.7	2.3 (2.6)	50.9 (58.6)	14.3 (16.6)	11.6 (13.3)	7.8 (9.0)	2.4
		b	13.9	3.0	66.1	18.6	15.1	10.1	3.1
	2	a	13.2	2.5 (3.0)	51.7 (61.2)	14.6 (17.3)	8.5 (10.1)	7.3 (8.6)	2.3
		b	17.3	3.3	67.9	19.2	11.2	9.6	3.0
	3	a	13.1	2.5 (3.0)	51.7 (61.7)	14.3 (17.1)	8.4 (10.0)	6.8 (8.1)	3.1
		b	17.3	3.3	68.3	18.9	11.1	9.0	4.1
H_2SO_4	1	a	14.3	–	56.2 (65.6)	19.6 (22.9)	5.3 (6.2)	4.6 (5.4)	–
		b	0.9	–	3.5	1.2	0.3	0.3	–
	2	a	24.3	–	32.7 (43.2)	33.7 (44.5)	5.3 (7.0)	3.9 (5.2)	–
		b	1.7	–	2.3	2.3	0.4	0.3	–
	3	a	49.3	–	23.2 (45.8)	21.9 (43.2)	5.6 (11.0)	–	–
		b	3.5	–	1.7	1.6	0.4	–	–

[a] *1* initial soil, *2* incubated soil, *3* incubated $CHCl_3$-treated soil
[b] Values in parentheses are percentage of P_{org} in dialysed extracts
[c] NaOH after $NaHCO_3$ (NaOH after H_2SO_4 not shown because of bad signal resolution in diester region)

compared with the control soil. The differences between P_{org} concentrations in $NaHCO_3$ extracts from soils incubated with and without P addition were not significant. Recently we demonstrated that 0.5 M $NaHCO_3$ and 0.05 M H_2SO_4 extracted only a minor part of microbial P (Makarov et al., submitted for publication). The decrease of labile P_{org} during incubation thus can result from its immobilisation in microbial biomass in spite of inorganic P supply.

In the NaOH extracts of incubated soil P_{org} increased by 3–4 mg kg^{-1}. This also indicated the possibility of immobilisation of labile soil P_{org} in microbial cells during short-term soil incubation. However, all changes in the concentrations of $NaHCO_3$- and NaOH-extractable P_{org} were only about 10% of microbial biomass P, indicating the overlap of microbial biomass P and soil P_{org} pools extractable with alkaline solutions. Changes of P_{org} extractability in incubation experiments can probably be ascribed to individual soil characteristics because these changes can result from the relation between mineralised soil P_{org} and immobilised P.

Most of the P released by $CHCl_3$ treatment was in inorganic form. P_{org} comprised only 11% and 21% of released P in $NaHCO_3$ extracts from the soil incubated with and without P supply, respectively. In the H_2SO_4 extract P_{org} comprised only 6% of released total P. In NaOH extracts there was no additional release of P_{org} after $CHCl_3$ treatment. The mean proportion of inorganic P in the $CHCl_3$-released total P was 90% in 15 United Kingdom soils, and the percentage increased with duration of fumigation (Brookes et al. 1984). However, Hedley and Stewart (1982) showed that P_{org} can also be released by $CHCl_3$ fumigation. A much higher proportion of released P_{org} was typical of bacterial rather than fungal P.

^{31}P-NMR spectroscopy

The ^{31}P-NMR spectra of the various extracts differed from each other (Figs. 3, 4, and 5). The spectrum of the 0.05 M H_2SO_4 extract from the initial soil had a low signal-to-noise ratio because of the low P concentration. The spectra of all three extracts showed similar proportions of inorganic orthophosphate, accounting for 11–15% of extractable P (Table 4). Proportions of monoester and diester P (DNA + phospholipids-teichoic acids) in the $NaHCO_3$ extract were 34% and 36%, respectively, while in the NaOH extract monoester and diester P accounted for 51% and 26% of total extractable P. In the H_2SO_4 extract the corresponding values were 56% and 25% (Table 4). In the $NaHCO_3$ extract proportions of DNA P and phospholipids-teichoic acids P were similar, while in the NaOH extract and especially in the H_2SO_4 extract phospholipids-teichoic acids P prevailed. Phosphonates were present only in the NaOH extracts.

In spite of the high proportion of diesters in the $NaHCO_3$ extract, they accounted for only one sixth of diester P extracted by NaOH. This was not surprising because $NaHCO_3$ extracted only a minor fraction of microbal P. Zhang et al. (1999) investigated the distribution of P forms in $NaHCO_3$ and NaOH extracts from soils under continuous corn and also found that there was more diester P in the NaOH extracts. Robinson et al. (1998) found that, following $NaHCO_3$ pretreatment, the proportion of diester P in an NaOH-EDTA extract increased.

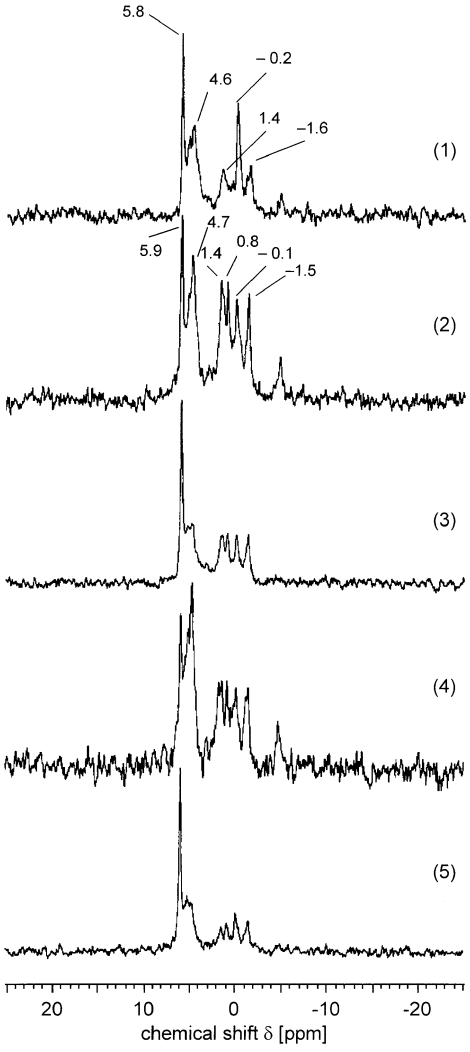

Fig. 3 ^{31}P-NMR spectra of 0.5 M $NaHCO_3$ extracts from a Humic Cambisol: initial sample (*1*), incubated with added P (*2*), incubated with added P and treated with $CHCl_3$ (*3*), incubated without added P (*4*), and incubated without added P and treated with $CHCl_3$ (*5*)

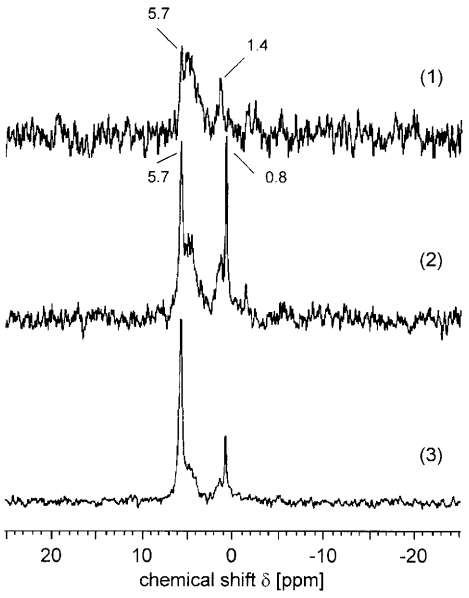

Fig. 4 ^{31}P-NMR spectra of 0.05 M H_2SO_4 extracts from a Humic Cambisol: initial sample (*1*), incubated with added P (*2*), and incubated and treated with $CHCl_3$ (*3*)

Consequently, the size of the labile P_{org} pool may be underestimated when attributed to $NaHCO_3$ extraction only.

Incubation of the soil essentially changed the character of the ^{31}P-NMR spectra of the $NaHCO_3$ and H_2SO_4 extracts (Figs. 3, 4). The proportions of monoester P decreased, and the extracts were enriched with P compounds resonating at 0.5–3.0 ppm (33% of the total extractable P_{org} in $NaHCO_3$ and 45% in H_2SO_4 extracts) compared with the extract of the initial sample (21% and 23% in $NaHCO_3$ and H_2SO_4 extracts, respectively). Two signals at 0.8 and at 1.4 ppm could be clearly distinguished in the spectrum of the $NaHCO_3$ extract from incubated soil. Both signals were attributed to a microbial origin in previous ^{31}P-NMR studies, and were assigned to phospholipids and unknown acid-soluble diesters in our recent investigation (Makarov et al., submitted for publication). The proportions of compounds resonating at 0.4–1.0 ppm (named as diester structures not yet identified), and at 1.0–3.0 ppm (teichoic acids) also increased in the course of the incubation of beech leaf litter over 498 days due to the accumulation of microbial P (Miltner et al. 1998).

Our results clearly show that at least two different P compounds of microbial origin resonated in the region of 0.4–3.0 ppm. Both compounds were partially soluble in acid, which corresponded well with their prevalence in FA fractions of soils (Guggenberger et al. 1996; Makarov et al. 1997). In the spectrum of the H_2SO_4 extract from initial soil, a clear resonance at 1.4 ppm was present. After incubation, the resonance at 1.4 ppm was not changed but an additional clear resonance at 0.8 ppm appeared. These compounds could either belong to microbial cells or to associated metabolites. For instance, McLaughlin et al. (1986) showed that $NaHCO_3$ extraction without $CHCl_3$ treatment may release P from microbial cells. Hedley and Stewart (1982) also showed that $NaHCO_3$ alone can extract up to 48% of total P from fungal cells. However, the data of Brookes et al. (1982) provided evidence that soil extraction with $NaHCO_3$ caused little or no release of biomass P unless the soil was fumigated. We nonetheless believe that the compounds in question are derived from live microbial cells as they increased in $NaHCO_3$ and H_2SO_4 extracts during the short-term soil incubation. This assumption is confirmed by the results of our experiments on P_{org} extraction from microbial cells which demonstrated that the main diester P compounds extractable from microbial cells with $NaHCO_3$ and H_2SO_4 resonated in the low field of the diester region (Makarov et al., submitted for publication).

The proportion of DNA P in the $NaHCO_3$ extracts of incubated soil decreased, especially in the case of

Table 5 Phosphorus-species distribution in soil extracts (*a*, %) and concentrations in a Humic Cambisol (*b*, mg kg^{-1}) subjected to incubation without added inorganic phosphorus

Extract	Sample[a]		Inorganic ortho-phosphate	Phospho-nates[b]	Monoesters[b]	Phospholipids and teichoic acids[b]	DNA	Unknown	Pyro-phosphates
$NaHCO_3$	1	a	13.0	–	36.4 (44.4)	25.1 (30.6)	10.0 (12.2)	10.5 (12.8)	5.1
		b	2.6	–	7.3	5.1	2.0	2.1	1.0
	2	a	28.7	–	33.0 (46.3)	17.9 (25.1)	10.2 (14.3)	10.2 (14.3)	–
		b	6.0	–	6.9	3.7	2.1	2.1	–
NaOH	1	a	15.5	2.6 (3.1)	53.3 (63.1)	13.6 (16.1)	7.7 (9.1)	7.4 (8.8)	–
		b	20.3	3.4	69.9	17.8	10.1	9.7	–
	2	a	13.6	2.8 (3.2)	55.5 (64.2)	12.9 (14.9)	7.4 (8.6)	7.7 (8.9)	–
		b	18.0	3.7	73.4	17.1	9.8	10.2	–

[a] *1* incubated soil; *2* incubated $CHCl_3$-treated soil

[b] Values in parentheses are percentage of P_{org} in dialysed extracts

incubation without P supply (Table 5). This result corresponded with the observation of low DNA-P extractability from microbial cells with $NaHCO_3$ solution (Makarov et al., submitted for publication). The spectra of the subsequent NaOH extracts from the incubated soil were similar to the spectrum of the initial soil (Fig. 5). Only the proportion of monoester P was slightly higher, and DNA P decreased from 13% to 10% of extractable P_{org}. Probably some soil diester P was mineralised by microorganisms during incubation not only from relatively easily available ($NaHCO_3$-soluble) but also from NaOH-extractable organic matter. Though P_{org}-turnover studies have shown that NaOH-extractable P_{org} is moderately stable and turns over slowly in the field, the decrease of diester P under conditions of high soil organic matter mineralisation, for example under cultivation, was shown by Hawkes et al. (1984) and by Condron et al. (1990). Chauhan et al. (1981) also reported that NaOH-extractable P_{org} can serve as a source for microbial P uptake during short-term incubations if inorganic P is limited. In our study, the decrease of DNA P in the $NaHCO_3$ extract and in the subsequent NaOH extract was higher during incubation without P supply, but also took place when inorganic P was readily available during P-supplied incubation. This agreed with the data of McLaughlin et al. (1988) who showed that soil microorganisms consumed the soil P_{org} in an experiment with isotope-labelled inorganic fertiliser application.

The spectra of the $NaHCO_3$ and H_2SO_4 extracts from the $CHCl_3$-treated soil showed an increase in inorganic P, indicating that some orthophosphate P was not removed during dialysis. The proportions of all P_{org} species in the extracts from incubated, untreated and treated soils were similar. This indicated that there was no additional extraction of microbial biomass P_{org} after $CHCl_3$ treatment.

High and similar concentrations of phospholipids-teichoic acids P and DNA P in the NaOH extracts of initial, incubated, and incubated and $CHCl_3$-treated soil indicated that these species were probably relatively stable compounds of soil humus and were not derived from microbial biomass.

There is some evidence for the predominance of non-microbial diester P in soil. First, the concentrations of diester P extractable from incubated samples were higher than for total microbial biomass P (33–39 µg g^{-1} and 26–34 µg g^{-1}, respectively). Considering that alkali-stable microbial P-diesters comprised 5% to 26% of total

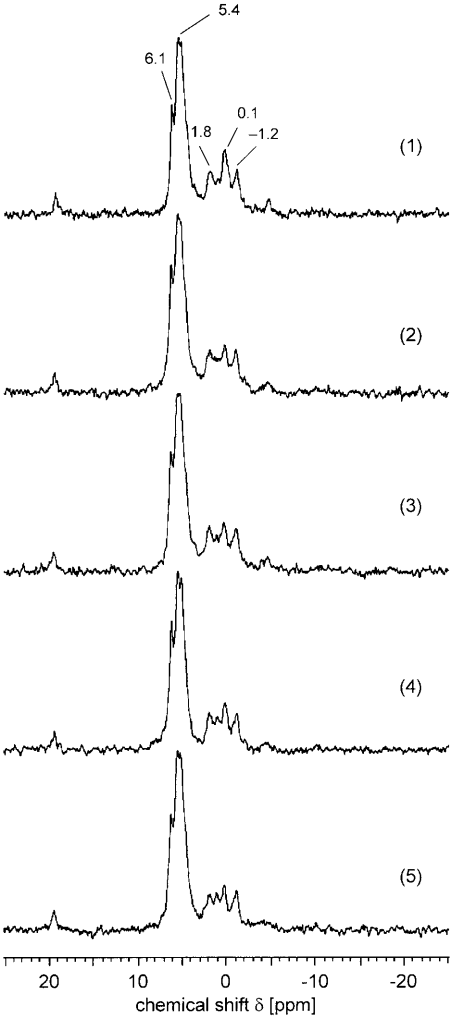

Fig. 5 ^{31}P-NMR spectra of 0.1 M NaOH extracts from a Humic Cambisol: initial sample (*1*), incubated with added P (*2*), incubated with added P and treated with $CHCl_3$ (*3*), incubated without added P (*4*), and incubated without added P and treated with $CHCl_3$ (*5*)

NaOH-extractable P in 3 out of 4 species investigated (only in *Bacillus subtilis* the proportion was 48%; Makarov et al., submitted for publication), we can assume that microbial P-diesters comprised not more than 15–20% of diester P extractable from soil with 0.1 M NaOH. Other indirect evidence of the relatively low proportion of diester P from microbial biomass in the total soil diester pool is the absence in soil extracts of the resonance of α-phosphate groups in NTP and NDP at about –10 ppm. In 0.1 M NaOH extracts from microbial cells this resonance can be readily observed (Makarov et al., submitted for publication).

Conclusion

There was no close connection between phosphate diesters with resonances at 0.5–3.0 ppm (phospholipids and teichoic acids P) and with the resonance at about 0 ppm (DNA P). Accumulation of DNA P in the most cold, wet, and acid soils indicated reduced organic P mineralisation under conditions unfavourable for microbial activity. Phospholipids and teichoic acids P by contrast accumulated in the microbially active soils. Incubation experiments confirmed that microbial growth produced labile ($NaHCO_3$ and H_2SO_4-soluble) P compounds resonating between 0.5 and 3.0 ppm. Simultaneously the proportion of DNA P resonating at 0 ppm decreased. Phosphate diesters identified in alkaline soil extracts are derived from both microbial biomass and humic compounds. Probably, in soils with low microbial activity diesters are stabilised in soil humus, while in microbially active soils the proportion of phosphate diesters derived from microbial biomass increases.

Acknowledgements This work was supported by a Research Fellowship awarded to M.I. Makarov by the Alexander von Humboldt Foundation and the Russian Foundation for Scientific Research. We thank Dr. A.L. Stepanov for microbial biomass C measurement using the SIR method.

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