Effects of Highly Volatile Organochlorine Solvents on Soil Nitrogen Metabolism and Microbial Counts

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Abstract: The effects of highly volatile organochlorine solvents (1,1,1-trichloroethane, TCET; trichloroethylene, TCE; and tetrachloroethylene, PCE) on soil nitrogen cycle and microbial counts were investigated using volcanic ash soil with different fertilizations. All the solvents significantly inhibited the activity of the cycle under the sealed conditions with 10 to 50 mg/g (dry soil) solvents added. No significant difference between the solvents, and between fertilization plots, was observed. Nitrate ion was not accumulated, and instead, ammonium ion was highly accumulated in the presence of the solvents. Nitrite ion was partially detected, while L-glutaminase activity was inhibited. The growths of ammonification, nitritation, nitratation and denitrification bacteria, and filamentous fungi were significantly inhibited in the presence of 10 mg/g (dry soil) of the solvents.

Keywords: microbial counts; soil nitrogen cycle; tetrachloroethylene; 1,1,1-trichloroethane; trichloroethylene

Volatile organic compounds, especially chlorinated aliphatic hydrocarbons such as 1,1,1-trichloroethane (TCET), 1,1,2-trichloroethylene (triclene, TCE) and tetrachloroethylene (perclene, PCE), are of major environmental concern (Cheremisinoff 2001; Mirsal 2008; Filip & Demnerova 2009), and many efforts have been made for the bioremediation (Borden 2007; Guimarães *et al.* 2010) and phytoremediation (Anderson & Walton 1995; James & Strand 2009) of polluted soils. In the previous paper (Kiyota *et al.* 2006), the effects of these solvents on soil respiration and soil biomass were reported using volcanic ash soil and grey lowland soil. All the solvents significantly inhibited the soil microbial activity. On the continuation of our research, the effects of the solvents on soil nitrogen cycle and microbial counts of volcanic ash soil under upland conditions are described.

MATERIAL AND METHODS

Soils and incubation. The plough layer of an upland field consisting of a volcanic ash soil of the Kanto loam type (FAO/UNESCO: Humic Andosols, US Soil Taxonomy: Typic (Lithic) Dystrandepts) located at the Tama farm of the University of Tokyo in Tanashi-City (Tokyo metropolitan area) was used. Properties of the soils were described in the previous paper (Kiyota *et al.* 2006). Soil samples were taken from three plots, one plot without fertilizer (abbreviated to NF), one plot with the application of chemical fertilizer (nitrogen, 12 kg/ha; magnesium phosphate, 300 kg/ha, annually; abbreviated to CF), and one plot with organic manure and chemical fertilizer (farmyard manure, 6 t/ha; nitrogen, 12 kg/ha; fused magnesium phosphate, phosphine, 26 kg/ha and magnesium, 22 kg/ha,

annually; abbreviated to OM). The pre-incubated soil sample (5 g dry weight) was placed in a 125 ml vial (total volume of the gas phase was 125 ml). To this was added each organochlorine solvent, 1,1,1-trichloroethane (TCET), 1,1,2-trichloro-ethylene (triclene, TCE) and tetrachloroethylene (perclene, PCE) at a concentration of 0, 3, 10 or 50 mg/g (dry soil), and the vial was capped tightly. Each vial was kept in the dark at 25°C and opened for analysis after 0, 1, 2, 4, or 8 weeks (Kiyota *et al.* 2006). The experiment was run in duplicate for each sample. For the microbial counts, a series of solvent concentrations of 0, 1, 10, or 50 mg/g (dry soil) was used.

Extraction of soil sample. A suspension of wet soil sample (3 g) in water (20 ml) (or 10% aqueous KCl solution (20 ml)) was shaken in a 50 ml plastic bottle for 2 h (or 30 min for aqueous KCl solution) and the supernatant was collected after centrifugation (5000 rpm, 10 min). The water extracts were filtered through a short ODS column to remove organic compounds for analysis.

Analysis. (1) Ammonium (NH_4^+) ion was quantified by the indophenol method (Sheiner 1975) using KCl extracts, detected at 635 nm (200-20 spectrophotometer, Hitachi, Tokyo, Japan). Each soil was assayed three times to obtain average value.

(2) Nitrate (NO_3^-) and nitrite (NO_2^-) ions were quantified by ion chromatography of water extracts (each 20 μl); t_R (NO_2^-) 3.3 min and 4.8 min (NO_3^-) (HIC-6A system, Shimadzu, Kyoto, Japan; column: Shimpak IC-AI (40°C), eluent: aqueous 2.5mM phthalic acid + 2.4mM tris(hydroxymethylamino)methane solution (pH 4.0), 1.5 ml/min. Each soil was assayed three times to obtain average value.

(3) Total inorganic nitrogen was calculated by adding ammonium, nitrite, and nitrate nitrogen.

(4) L-glutaminase activity was estimated by quantifying the liberated ammonium ion (indophenol method) according to our procedure (Kanazawa & Kiyota 1995). Each soil was assayed three times to obtain average value.

(5) Microbial counts were enumerated by the most probable number (MPN) technique (Rowe *et al.* 1977) as follows: for the complete dispersion of the soil samples, 10 g of moist soil was dispersed in 90 ml of 0.01M tris(hydroxymethylamino)methane buffer (pH 7.2) using a Waring blender at a speed of 16 000–18 000 rpm for 3 min (Kanazawa & Filip 1987). Microbial numbers with 10-fold dilutions and 5 tubes per dilution (*n* = 5) were derived from the literature (Alexander 1965,

1982). (a) Ammonification bacteria: diluted sample solution was inoculated and cultured in a casein-glucose medium at 25°C for 2 weeks at a stationary culture. The presence of ammonification bacteria was estimated by the detection of ammonium ion by Nessler's method (Gedroits 1955). (b) Nitritation bacteria: Each diluted sample solution was inoculated and cultured in an autotrophic medium for nitritation bacteria (Bankworth & Bateson 1964) at 25°C for 6 weeks at a stationary culture. The presence of nitritation bacteria was estimated by the detection of nitrite ion with Griess-Ilosvay reagent (Schmidt & Belser 1994). (c) Nitratation bacteria: the presence of nitratation bacteria was estimated by the method similar to that for nitritation bacteria using another autotrophic media (Alexander 1965). (d) Denitrification bacteria: diluted sample solution was inoculated and cultured in a yeast medium (Valera & Alexander 1961) with an upended Durham tube at 25°C for 2 weeks. The presence of denitrification bacteria was estimated by evolution of gas in the Durham tube. (e) Filamentous fungi: colony counts for filamentous fungi were determined using a spread plate technique with Rose-Bengal streptomycin agar (Martin 1950) after incubation at 25°C for 4 days.

All analytical data are average values of duplicated experiments.

RESULTS

Ammonium ion. The analysis of ammonium ion extracted with aqueous KCl solution is shown in Figure 1. In each plot without solvents, the amount of ammonium ion was almost eliminated under aerobic upland conditions in four weeks. However, the addition of each solvent in any amount resulted in significant accumulation of ammonium ion (10 > 3 > 50 mg/g (dry soil)). The amount of ammonium ion in the OM (organic manure and chemical fertilizer) plots was 1.5 to 2 times as much as in the NF (without fertilizer) and CF (chemical fertilizer) plots. There was no difference in the tendencies between solvents. Amounts of ammonium ion extracted with water showed a similar change to those with aqueous KCl solution, and each total amount was aq. KCl/water = 1:3 to 1:4.

Nitrite and nitrate ions. Nitrate ion gradually accumulated during the incubation time in control plots (NF > CF > OM) (Figure 2). The addition of
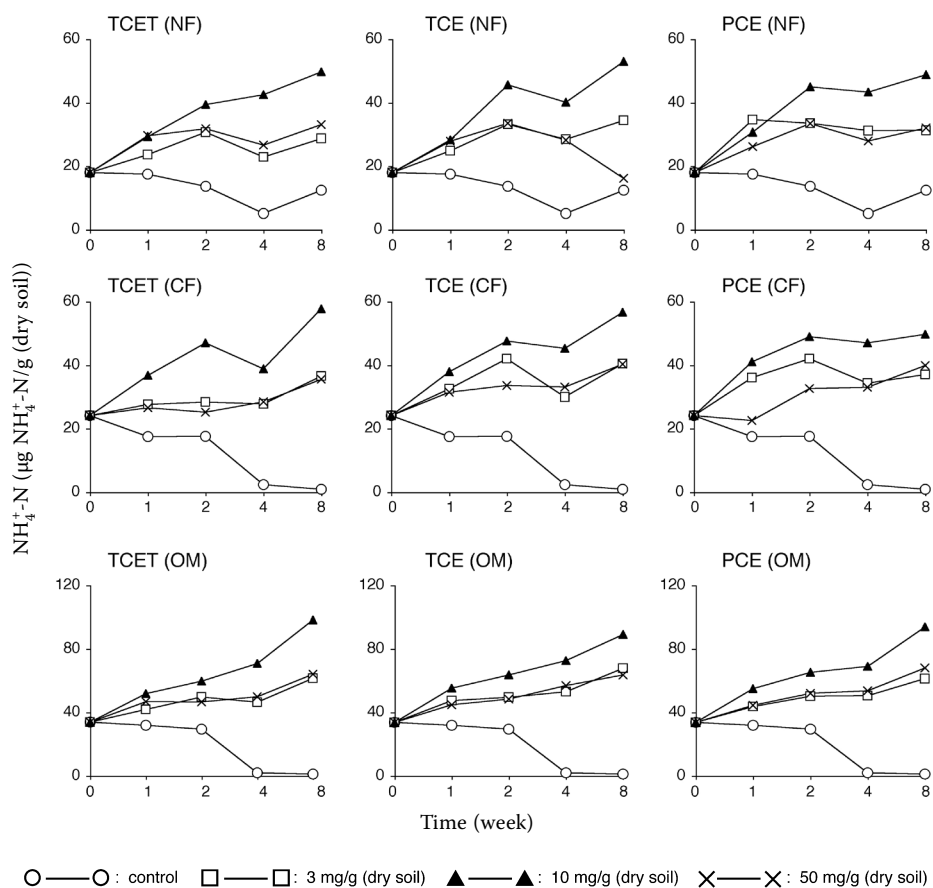
Figure 1. Effects of organochlorine solvents on ammonium ion concentration under upland conditions of volcanic ash soil; soil with 0, 3, 10, and 50 mg/g (dry soil) of solvent was kept in dark at 25°C; TCET – 1,1,1-trichloroethane; TCE – tetrachloroethylene; PCE – tetrachloroethylene; NF – without fertilizer; CF – chemical fertilizer; OM – organic manure and chemical fertilizer
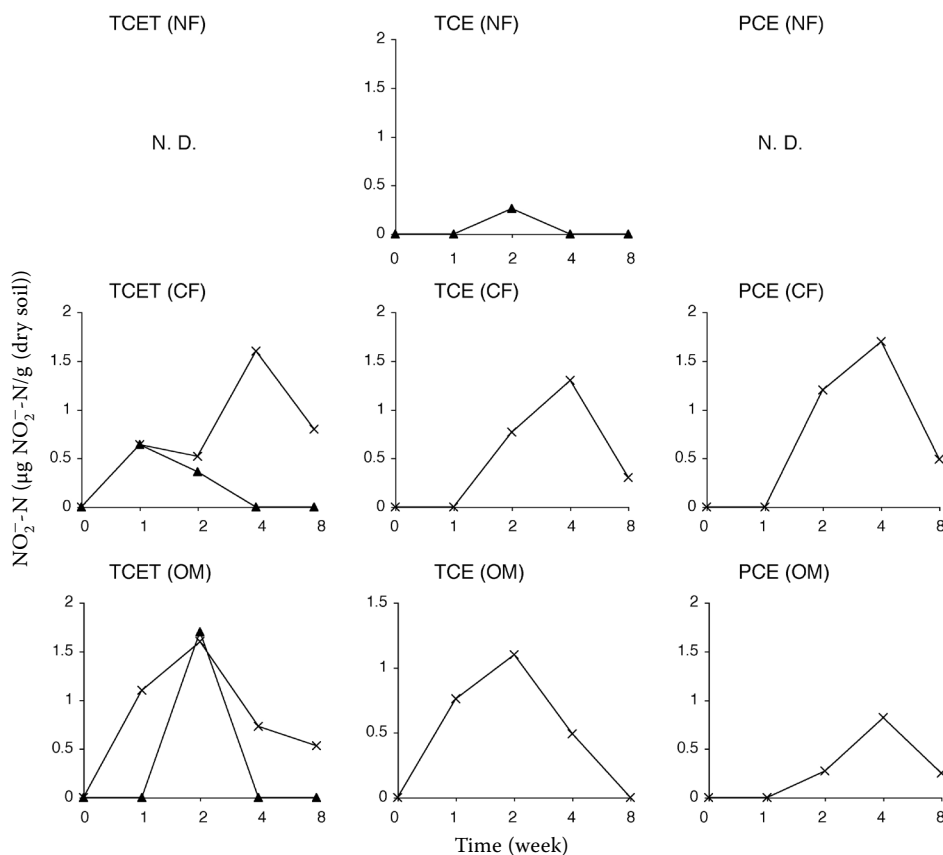

Figure 2. Effects of organochlorine solvents on nitrite ion concentration under upland conditions of volcanic ash soil; for explanations see Figure 1
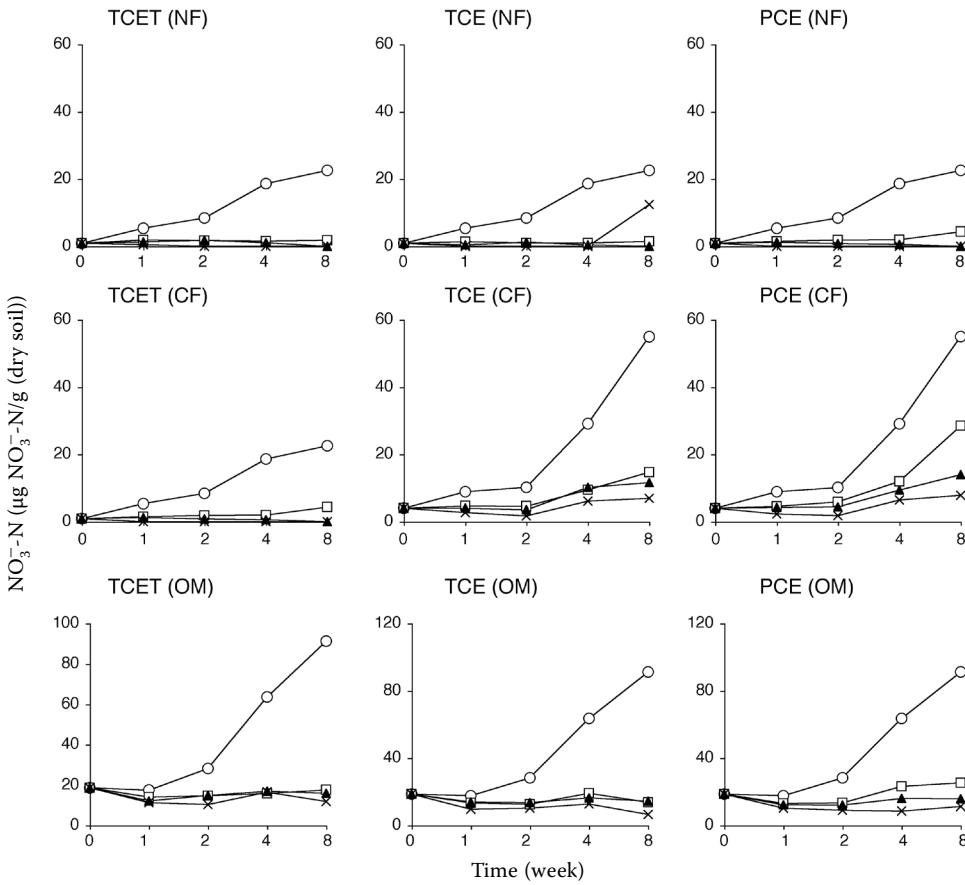
Figure 3. Effects of organochlorine solvents on nitrate ion concentration under upland conditions of volcanic ash soil; for explanations see Figure 1
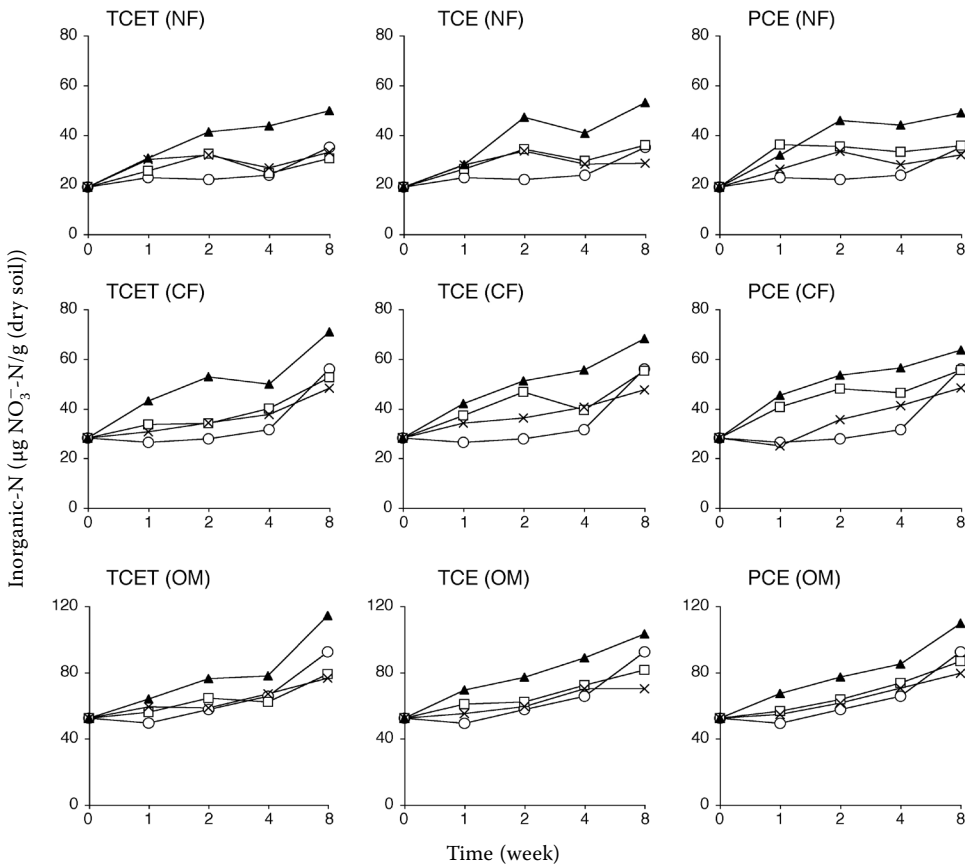

Figure 4. Effects of organochlorine solvents on total inorganic nitrogen (ammonium, nitrite and nitrate ions) concentration under upland conditions of volcanic ash soil; for explanations see Figure 1
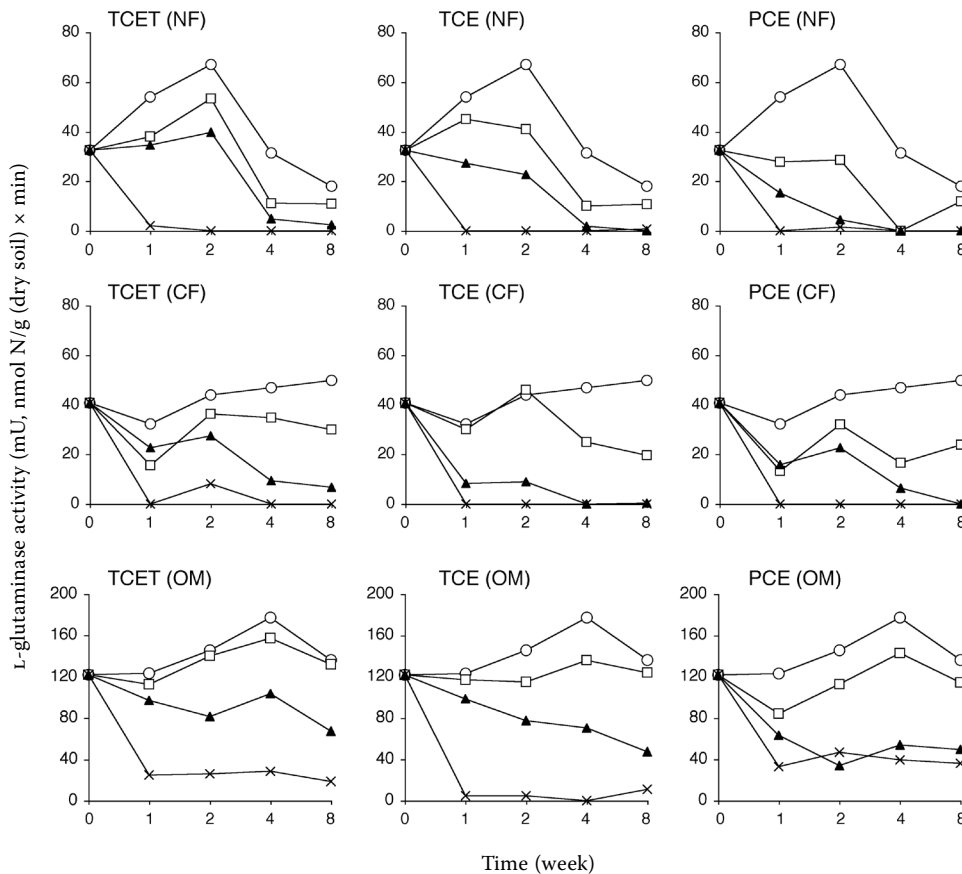
Figure 5. Effects of organochlorine solvents on ʟ-glutaminase activity under upland conditions of volcanic ash soil; for explanations see Figure 1

3 mg/g (dry soil) solvent eliminated significantly the formation of nitrate ion in NF plots. Although the formation of nitrate ion was slower in the TCET (1,1,1-trichloroethane)-CF plot, there was no difference in the tendencies between solvents. Nitrite ion was not detected in each control plot and 3 mg/g (dry soil) plot, but it was observed when a larger amount of solvents was applied to CF and OM plots (Figure 3).

Total inorganic nitrogen. Amounts of total inorganic nitrogen gradually increased during the incubation in each plot, and their tendencies were similar: higher at 10 mg/g (dry soil) plots and hardly any difference between the other three concentration plots (Figure 4).

ʟ-Glutaminase activity. ʟ-Glutaminase activity decreased according to the amount of solvent in each plot (Figure 5). A significant loss of the activity began at the early stage of incubation at 50 mg/g (dry soil) plots with NF and CF application.

Microbial counts. (a) Ammonification bacteria (Figure 6): the number of the bacteria gradually decreased to one-tenth. Initially (1~2 weeks), the presence of 1 mg/g (dry soil) solvent promoted the growth.

(b + c) Nitritation and nitratation bacteria (Figure 6): the presence of 1–10 mg/g (dry soil) of solvents did not affect the bacterial counts of both nitratation and nitritation bacteria so much as compared with the control plots. In addition, 1 mg/g (dry soil) of solvents slightly promoted the growth. (d) Denitrification bacteria (Figure 7): bacterial counts decreased in dependence on the solvent concentration. The presence of 1 mg/g (dry soil) of solvents gradually affected their growth. (e) Filamentous fungi (Figure 8): the addition of 1 mg/g (dry soil) of solvents did not affect the counts of filamentous fungi, but they decreased ten times at 10 and 50 mg/g (dry soil) of each solvent plot since week 1 and they did not recover even in 8 weeks.

DISCUSSION

Nitrogen cycle. Generally, under aerobic upland conditions, NH_4^+-N is gradually oxidized to NO_3^--N, and the intermediary NO_2^--N is not usually detected. Each control plot (without solvents) under NF, CF and OM conditions showed this tendency.
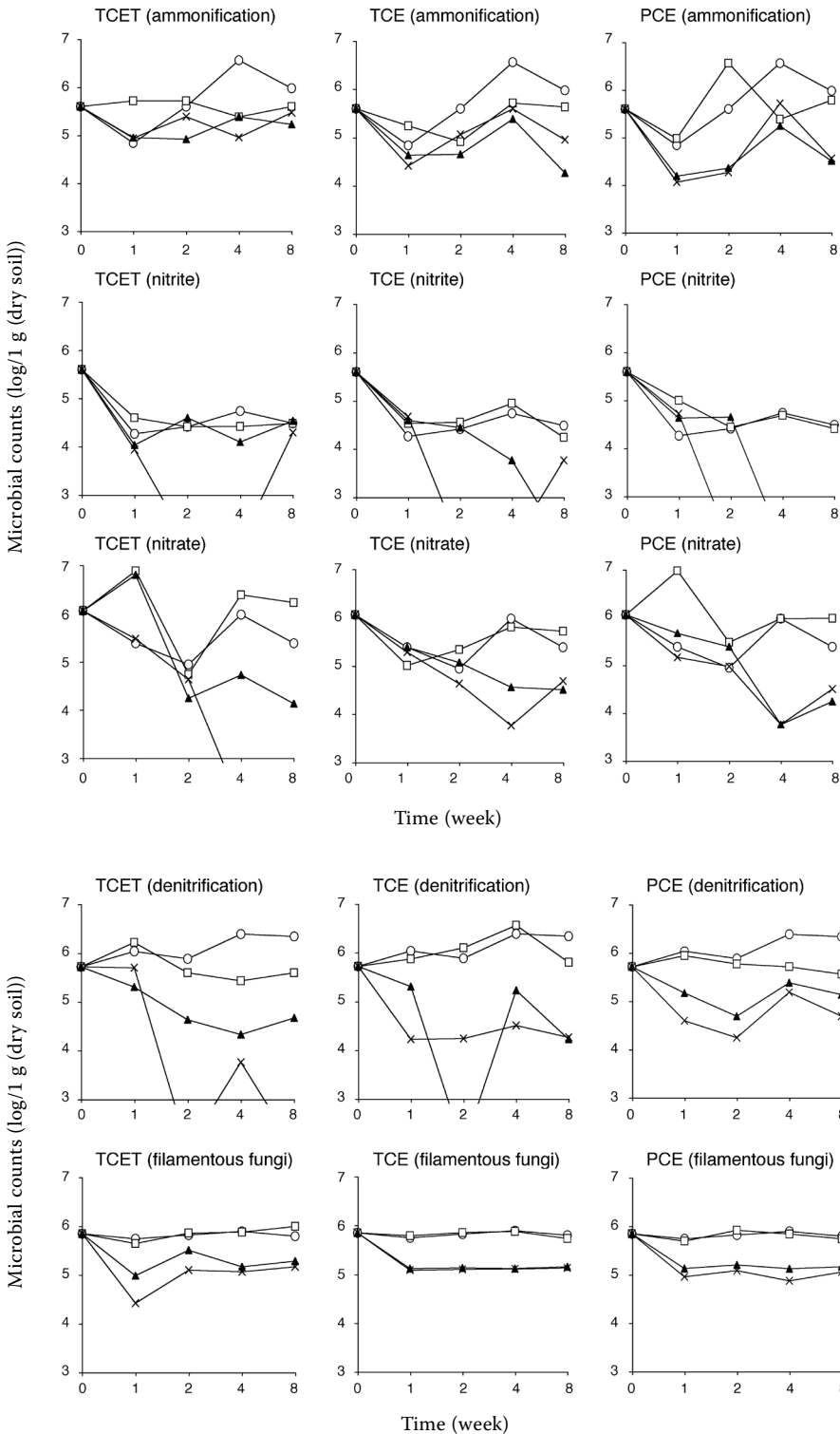
Figure 6. Effects of organochlorine solvents on microbial counts of ammonification, nitritation and nitratation bacteria under upland conditions of volcanic ash soil without fertilizer (NF); soil with 0, 1, 10, and 50 mg/g (dry soil) of solvent was kept in dark at 25°C; TCET – 1,1,1-trichloroethane; TCE – tetrachloroethylene; PCE – tetrachloroethylene; NF – without fertilizer; CF – chemical fertilizer; OM – organic manure and chemical fertilizer

Figure 7. Effects of organochlorine solvents on microbial counts of denitrification bacteria and filamentous fungi under upland conditions of volcanic ash soil without fertilizer (NF); for explanations see Figure 6

However, in the presence of each solvent, NH_4^+-N increased rather gradually and only a small amount of NO_3^--N was formed. In addition, NO_2^--N was abnormally detected in a large amount at CF and OM plots. These results indicate that oxidative enzymes lost the activities in the presence of solvents. As expected according to Kanazawa and

Filip (1986), l-glutaminases were also deactivated in each solvent plot. Buffer ability of the OM plot (Kanazawa & Kiyota 2000) did not function efficiently. On the other hand, total inorganic nitrogen of solvent plots gradually increased in the same manner as in the control plots. This may be due to the degradation of organic nitrogen caused

by the death of microorganisms, especially, the combination of partial death of microorganisms and the activity of the living ones. There seems to be no significant difference in inhibitory activity between three solvents. Even in the CF and OM plots, having high buffer ability for soil respiration (Kiyota *et al.* 2006), nitrogen metabolism was severely damaged.

Microbial counts. Counts of ammonification bacteria had a similar tendency between the solvents, i.e. they were more susceptible to the lower concentration of solvents but less susceptible to the higher concentration than the other bacteria and fungi. This indicates that there are two types of ammonification bacteria: one type is susceptible and the other is tolerant to organochlorine solvents. In addition, it is a general feature of bacteria capable of releasing the ammonium ion from organic nitrogen, thus consists a wide diversity. Its lower susceptibility at a higher concentration agrees with the accumulation of NH_4^+-N (Figure 1) but opposes to the deactivation of L-glutaminase (Figure 5). As extracellular enzymes adsorbed onto the soil surface could be saved from chemical and biological degradation (Ladd & Butler 1975), NH_4^+-N accumulated in the chloroform-fumigated soil (Stevenson 1982). Accordingly, even if most of the susceptible bacteria died at the lower concentration of solvent, extracellular enzymes released from them would degrade organic nitrogen to accumulate NH_4^+-N. As for other nitrogen bacteria regarding NO_2^--N, NO_3^--N and N_2 whose related enzymes exist on the cell membrane, mortal effects of solvents correlate with the formation of inorganic nitrogen.

The growth of nitritation bacteria was inhibited in the presence of 10 mg/g (dry soil) of the solvents. The number of nitratation bacteria decreased according to the number of nitritation bacteria, because nitratation bacteria are chemical autotrophs using nitrite ion as a sole reductant. Thus, the direct effects of the solvents on nitratation bacteria are not examined in these experiments.

The number of denitrification bacteria significantly decreased in the presence of 10 mg/g (dry soil) of the solvents. The medium for denitrification bacteria contained NO_3^- as a sole nitrogen source, thus, the decrease of denitrification bacteria had no relation with that of nitratation bacteria. As a result, denitrification bacteria are highly susceptible to the solvents.

Filamentous fungi were also susceptible to high concentrations of the solvents.

Solvents. The inhibitory effects of the three solvents on soil nitrogen metabolism and microbial counts are almost equal, similarly to those on soil respiration and microbial biomass (Kiyota *et al.* 2006), even though their solubility in water is different.

The overall results indicate that an extremely high concentration of solvents in groundwater, sometimes detected around factories, will show significant inhibitory effects on soil microbial activities of neighbouring paddy fields.

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