

represented by a visible band in the mb661 DGGE in one of the replicates at all different sites (band1). Band 3-5 of the mb661 DGGE represented a *pmoA* sequence, which indicates for as-yet uncultivated methanotrophs (related to methanotrophic Gammaproteobacteria). The amino acid identity was below 92% to uncultivated bacteria represented by “clone Zhenjiang 5” and “clone Beijyuan 2” (Hoffmann *et al.* 2002) and to the cultivated methanotrophs of the genera *Methylococcus* and *Methylocaldum*. This *pmoA* sequence was present in all samples that were analyzed with mb661. With primer A682b, it was detected at site CF 3141 only (band 6).

3.6.4 Community composition as revealed by DGGE analysis

The DGGE banding patterns from the three different sampling sites indicated for a very similar community structure within one sampling site. But there were clear differences in the community composition of the different sampling sites. Based on the DGGE banding pattern of the two gels, a cluster analysis was performed with SYSTAT version 9 (SPSS Inc., Richmond, Calif.). The presence or absence of a band in each sample was used to calculate Jaccard dichotomy coefficients. These coefficients were used to calculate a hierarchial tree based on average linking and Euclidean distances.

The resulting tree confirmed the similarity of samples from one sampling site and differences between the different sampling sites (Fig. 29). All trees indicate for higher similarity between SK and AC than between SK and AC to CF. The tree includes all different detected sequences from each sampling site (with one exception, see above). Band names shown in capitals indicate for *pmoA* sequences obtained from the mb661 DGGE, while the others represent the bands of the A682b DGGE. There are three sequences added (methanotrophic isolates arc1, arc2, and sakb1), which belong to methanotrophic isolates, obtained from the soil samples. 3.6.1 DGGE.

The detection of the third cluster representing uncultivated bacteria, Cluster 2, has as yet been restricted to upland soils. Since the sequences of this branch are only distantly related to PmoA sequences of recognized genera of MB, it cannot be excluded that these sequences code for the AmoA of uncultivated ammonia-oxidizing bacteria. However, the growth of the organisms harboring these sequences was possibly stimulated in sieved soil samples after incubation under elevated methane mixing ratios (Knief, unpublished data). In this study, only *Methylocystis* spp. were isolated. These isolates probably did not represent the dominant *Methylocystis* spp. in the soil samples, since PmoA sequences

identical to those of the isolates were not detected by cultivation-independent methods in the soil samples.

The different land-use of the sampling sites was reflected by different methane uptake rates and different soil pHs, especially for the cornfield soil. The characterization of the methanotrophic community indicated that the community composition reflected the land use change as well. The *pmoA* sequences of USC α and Cluster 5 were detected in samples from sites SK and AC, but not in the soil samples from the cornfield. The absence of USC α at site CF is in agreement with the results of other studies, in which the methanotrophic community of some farmland soils was analyzed and in which USC α sequences were not detected, although they were present in adjacent grassland and forest soils (Knief et al., 2003). The pH shift in the farmland soil is probably not the reason for the disappearance of USC α sequences at this sampling site. The organisms harbouring USC α sequences, although they appear to occur more frequently in acidic than in pH-neutral soils, do occur in soils of a wide pH range (4.0 – 8.0). Further sequences that led to the different community compositions of the different sampling sites were those of *Methylocystis* spp., Cluster 2 and of ammonia-oxidizing bacteria (Table 4). Sequences of *Methylobacter* and Cluster 3 were detected at all sampling sites. A cluster analysis was used to compare the degree of similarity across sites among the communities of MB plus ammonia-oxidizing bacteria. The resulting tree confirmed the high similarity of replicate samples from one sampling site and differences between the different sampling sites (Fig. 29). The differences within the replicate samples from one site were due to faint DGGE bands visible in one sample but not in the other ones. Thus, *pmoA* sequences with abundance near the detection limit of the methods were mainly responsible for the dissimilarity of duplicate samples. The main purpose of this cluster analysis was the comparison of the different sampling sites. The community composition of SK and AC sites were more similar to each other than either was to CF site. This finding is in accordance with the methane uptake rates and pHs of the sampling sites, which also indicated a high degree of dissimilarity of site CF compared to sites SK and AC.

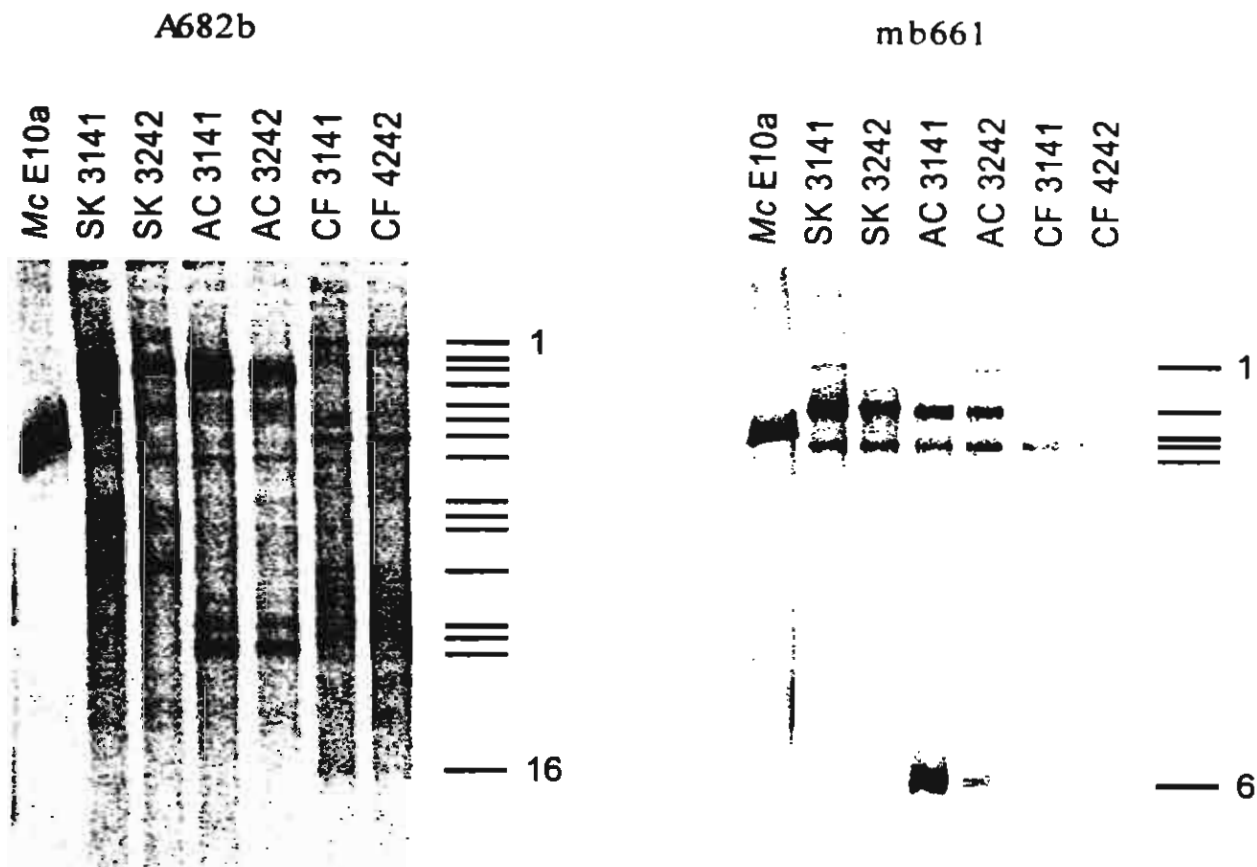
These data demonstrate clearly that land use changes are not only connected to changes in the methane uptake capacity of soils, but also to changes in the community composition of methane-oxidizing bacteria. The change in the community composition may be an important factor leading to the general decrease in the methane uptake rate of farmland soils. The methanotrophic communities of replicate samples within one sampling

site were highly similar, although methane uptake rates varied over space and time. However, several other factors influence the methane uptake rate of a soil. The water content is an important factor, since it heavily influences gas diffusion in the soil (Keller et al., 1993). Whether and how the community of MB responds to changes in the water content of a soil, remains to be studied.

The community composition of the reforested site AC represents a kind of intermediate state between the communities at sites SK and CF. Since it is unknown how the community composition and methane uptake activity of the reforested site AC looked before the site was reforested, we cannot conclude whether the community we see today has redeveloped or whether the community structure was not as much changed after deforestation. During this period this site was a farmland like site CF, so it is likely that the MB community was different at this time.

Table 4. Detected taxa of methanotrophic bacteria in soils taken from different land use.

	SK 3141 and SK 3242		AC 3141 and AC3242		CF 3141 and CF 3242	
	A682b	mb661	A682b	mb661	A682b	mb661
<i>Methylocystis</i> sp.			+	+	+	
USC α	+		+			
related to USC α		+		+		
<i>Methylobacter</i> sp.		+		+	+	+
related to Gammaproteobacteria.		+		+		+
PmoA / AmoA	+				+	
nitrifier (<i>amoA</i>)					+	



A682 bands (top down):

- 1 no PmoA or AmoA (CF)
- 2 PmoA / AmoA and *Methylocystis* (CF)
- 3 PmoA / AmoA (SK) and USC α (SK + AC)
- 4 no PmoA or AmoA (AC)
- 5 USC α (SK)
- 6 nitrifier, USC α and related to Gammaproteobacteria (CF, 2 bands)
- 7 PmoA / AmoA and *Methylocystis* (CF)
- 8 USC α (SK) and *Methylocystis* (AC)
- 9 PmoA / AmoA (SK)
- 10 PmoA / AmoA (SK)
- 11 PmoA / AmoA and USC α (SK)
- 12 USC α (SK), PmoA / AmoA, *Methylocystis* and nitrifier (CF)
- 13 *Methylocystis* (CF, 2 bands)
- 14 nitrifier (AC)
- 15 *Methylocystis* (AC)
- 16 *Methylocystis* (AC)

mb661 bands (top down):

- 1 *Methylobacter* (SK, AC, CF)
- 2 related to USC α (SK, AC)
- 3 related to Gammaproteobacteria (SK)
- 4 related to Gammaproteobacteria (SK, AC, CF)
- 5 related to Gammaproteobacteria (CF)
- 6 *Methylocystis* (AC)

Fig. 27 DGGE bands obtained with the primer A6682 and mb661.

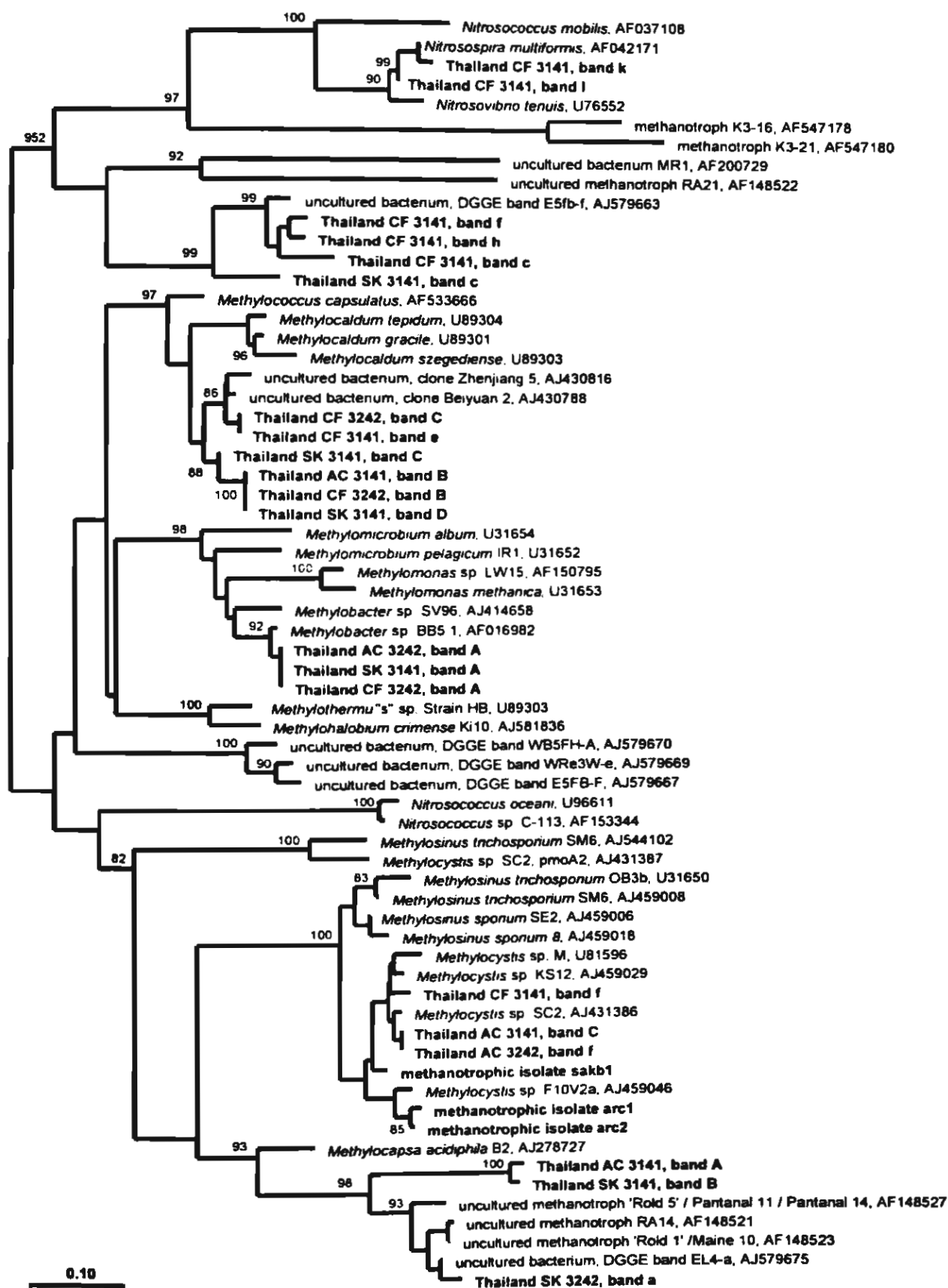


Fig. 28 Phylogenetic tree constructed from amino acid sequences of partial *pmoA* retrieved from native forest, forest-replanted (*A. magnum*) and corn cultivated soils and public-domain *PmoA* and *AmoA* sequences. The scale bar indicates the estimated numbers of base change per nucleotide sequence position.

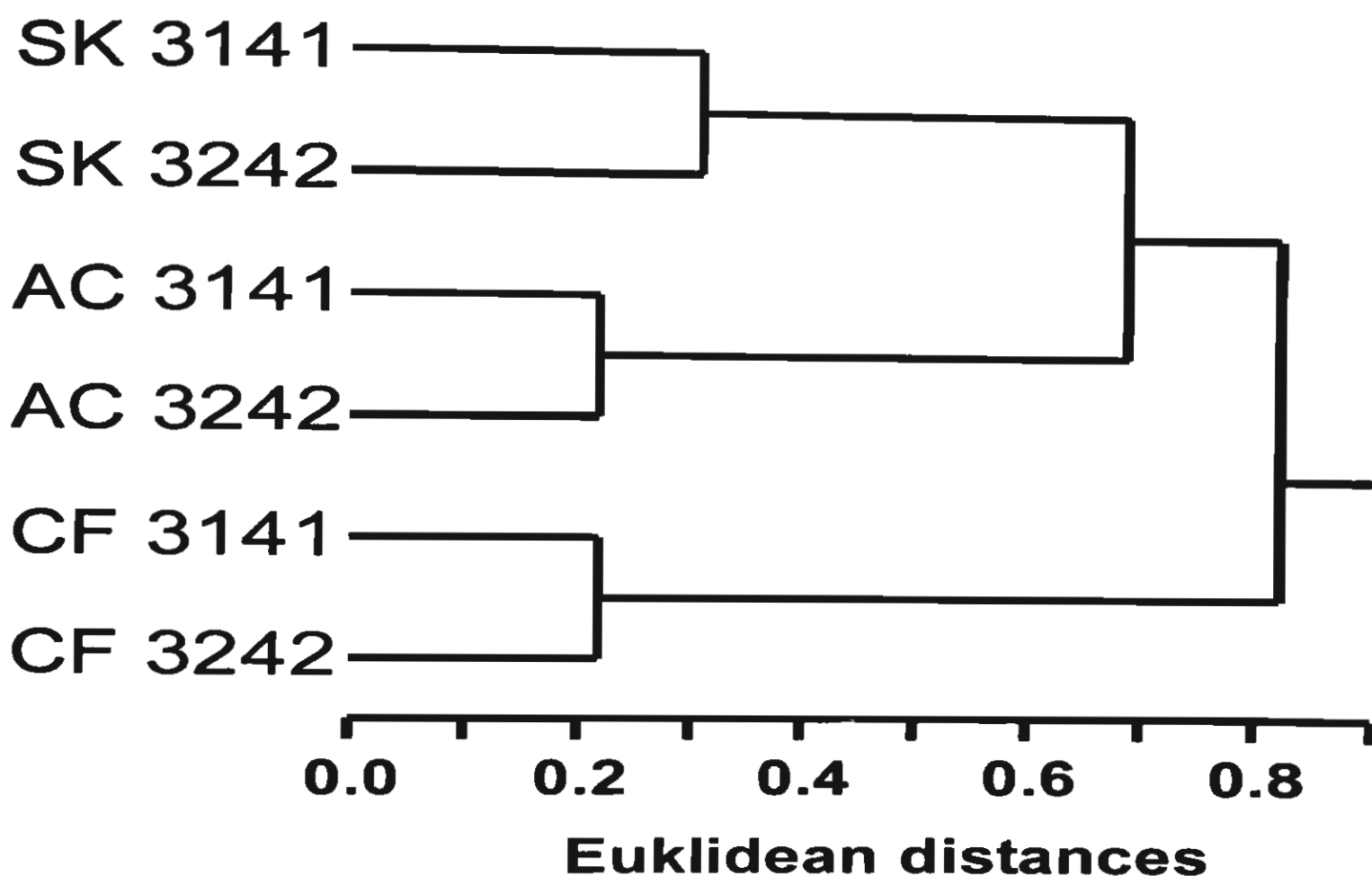


Fig. 29 Cluster analysis based on the presence or absence of 21 bands of DGGE gels from PCR products obtained with primer systems A189-GC/A682 and A189-GC/mb661. Jaccard similarity coefficients were calculated and a hierarchical tree was constructed based on average linking and Euclidean distances.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 Effects of land use on methane oxidation

The results obtained in this study clearly show that capacity of methane oxidation is strongly affected by land use type. Although characteristics of soil such as soil texture, bulk density and pH affects methane oxidation, but land use could mask the effects of such intrinsic soil properties. For example, soil pH (mild acidic) and soil texture (Sandy soil) in CF soil is supposed to be more suitable for methane oxidation than forest soils (strongly acidic with high clay content). However, methane oxidation rates in both forest soils are significantly higher than in CF soils. Thus, effects of intrinsic soil properties on methane oxidation may be comparable only among the same land use type, such as in forest of SK and AC soils. The reason for such differences in methanotrophic activity upon changing land use can be traced back to the fundamental unit responsible for dynamics of methane in the ecosystems; the activity and composition microbial community.

Three main groups of microorganisms involve in the dynamics of methane in a given ecosystem. These include methane-oxidizing bacteria (methanotrophs), methane-producing bacteria (methanogens) and nitrifying bacteria. Interactions among these groups of bacteria determine whether the ecosystem under consideration is the net sink or source of methane. In upland soil with high water draining characteristics, methanotrophs become dominant and such ecosystem usually acts as the net atmospheric methane sink. However, fertilizing the soil under crop cultivation usually lead to the dominant of nitrifying bacteria and thus resulting in loss of methanotrophic characteristics. Such ecosystem could become either sink or source of methane depending on the environment factors. In the poor-drained soil, methanogenesis prevails and the system could become the net sources of atmospheric methane. Which bacterial group will become dominant is, thus, largely affected by the pattern of land use and land management.

Changes of some soil physical and chemical properties upon changing land use such as water permeability may induce anaerobic condition thereby methanogenesis develop. This is especially obvious in the raining season in CF soil. It is apparent that high water content was found mainly in CF soil especially in the subsurface. Such

conditions lead to methane production and the soil become the net source of atmospheric methane; namely methanogenesis become the dominant process in determining methane dynamics at that time.

Within the similar land use type such as between SK and AC soils, the results show that methanotrophic activity is similar. However, here the effects of soil characteristics become obvious. More sand fraction in AC soil relatively to SK soil (thus higher water permeability) may result in a consistent characteristics of net methane sink at this site. However, during the dry months (Jan-April) SK soil was the most active methane oxidation and methane oxidation in AC soil was less than SK by 10-36%. However, when the rain season began, AC took over oxidation and in some months SK turns into net methane source (June and July). Presumably, the high clay content of SK soil with poor water permeability allowed anoxic conditions to be developed within the soil profile. From the result of kinetic study it is reveal that if concentration of methane is too high, SK site cannot oxidize it. Methanogenic activity may have been started in such condition, thereby produced high about of methane that exceeded the oxidizing capacity of SK soil. Thus, while SK is active methane sink during the summer months, disturbances and changes in environment conditions could easily change its characteristic as either source or sink. This indicates how delicate the natural forest soil is regarding to dynamics of methane. On the other hand, experiencing both human disturbances and recovering on the way to return to natural forest, the re-grown AC forest may have higher buffer capacity to encounter various levels of methane concentrations. It is thus assumed that such soil may have ability to oxidize both methane at low and high levels. It is noted that although AC soil shows the active atmospheric methane oxidation, but its kinetic characteristics are far different from SK soil. Thus, AC still inherits soil characteristics of cultivated soil once it had been about 16 years ago. Biphasic characteristics of reaction kinetics shown in Fig. 25 indicate the presence of both high and low affinity of methanotrophic communities. Thus, affinity of methanotrophs to methane changes according to environmental conditions. In the other words, threshold concentration of methanotrophic bacteria is changed by land use change.

It is interesting to note that in forest soils the active layer of methane oxidation lies below the surface. High concentration of inorganic nitrogen compounds in the topsoil may be the reason. However, rate of methane oxidation in the subsoil would be limited by diffusion of both methane and oxygen. It is thus assumed that methanotrophs in the subsoil are substrate limited and oxidation should increase upon exposing to high

concentration of methane. This is true for AC soil but surprisingly rate of methane oxidation in SK soil does not increase at elevated concentration of methane.

Methanotrophs present in subsoil may well adapt to low methane concentration, making them good at consuming low methane concentration and easily saturated with methane at high concentration as observed in the forest soils.

4.2 Role of forest in Thailand as methane sink

Assuming that the annual average of methane oxidation obtained in the present study is valid and a rough representative for methane oxidation capacity of forest soil in Thailand, total atmospheric methane consumption by forest areas in Thailand can be estimated. To do this, the average oxidation rate from both SK and AC sites is multiplied with the total area of forest in Thailand in 1985 and 1995. This estimation indicates that total methane uptake by forest areas in Thailand (natural and regrown forests) is estimated at 61.12 Gg yr⁻¹ in 1995. About 10% reduction in total methane uptake due to decrease in forest area between 1985 and 1995 (Table 5) was observed. Although the estimated amount of methane oxidation by forest soils makes up a small portion of total emission in Thailand in 1994 (which is 3.17 Tg), methane uptake by forest could cancel out the amount emitted from forestry section such as by biomass burning (which is estimated at 59.57 Tg in 1994, Ministry of Science, Technology and Environment, 2000). The estimated uptake by Thai forest, in addition, is more than two times higher than the amount of methane emitted by waste section (35.22 Gg in 1994). Thus, methane oxidation by forest soil alone could help improve the net methane released by Thailand. It is noted that this is only a preliminary estimate and accuracy of such estimate cannot be assessed due to small number of measurements and large temporal and spatial variations of methane emission. To improve accuracy and reliability of methane sink estimate by biological oxidation, more studies on relationship between environmental factors and methane oxidation rate, variations of oxidation in different types of forests and other aspects are necessary.

Table 5 Estimate of methane uptake by forest soil in Thailand in 1985 and 1995 using the averaged uptake rate of natural and re-grown forest obtained in the present study.

Year	Forest area ($\times 10^6$ ha)	Methane uptake (GgCH ₄ yr ⁻¹)*
1985 ¹	15.09	66.75
1995	13.83	61.17
-natural forest ¹	13.15	58.16
-reforestation (1997) ²	0.68	3.01

¹Royal Forest Department, 1997

²Luangjame, 1997 (cited in Thailand's National Greenhouse Gas Inventory 1994)

* Using the average uptake rate of 1.21 mgCH₄ m⁻² day⁻¹

4.3 Conclusions

The results reveal that forest soils in Thailand have high potential to oxidize atmospheric methane. The oxidation rates fall within the range reported earlier and generally higher than those observed in European forests (Table 1). The oxidation varied according to seasons, land use types and sampling locations. The main findings can be concluded as followed.

1. High rate of methane oxidation was found during the summer months. On the other hand, low oxidation rate was found during the rainy months. Natural forest soils show the high oxidation capacity in summer but sometimes it becomes net carbon sources in wet season. In corn cultivated soil, however, only occasionally that net methane consumption was observed. From these results it can be concluded that (1) undisturbed forest has the highest atmospheric methane oxidation capacity, (2) reforestation to *Acadia* recovers the methane oxidation capacity of soils, and (3) land-use change to agriculture (corn plantation in this case) results in loss of the methane oxidation capacity of soil.

2. Methane oxidation in all three sampling sites is associated with large seasonal variations. Similarly, variations in oxidation rates among different chambers during each sampling time were also large, especially at CF site where both net emission and oxidation were observed.

3. Along the soil profile of forest soils, the most active oxidation layer in most cases is between 15-40 cm. Results of kinetic study reveal that soil at SK has highest affinity for atmospheric methane, followed by soil at AC and CF sites, respectively.

4. The phylogenetic tree illustrating composition of methanotrophic community was constructed based on the partial sequences of amino acid of *pmoA* gene (encoding the α subunit of pMMO which is present in all methanotrophs) and of *amoA* gene (encoding the subunit of AMO). The *PmoA* sequences related to the nitrifier *Nitrosospira multiformis* was only detected in CF soil. The *PmoA* sequences of methanotrophs those closely related to the genera *Methylocystis*, *Methylobacter*, *Methylococcus*, and distantly related to *Methylocapsa*, were detected. In addition, the results show that there is a clear difference in methanotrophic community according to land use type. For example, *Methylocystis* was detected in only CF and AC soils but not SK soil. The USC α (a novel sequence cluster usually called the "forest sequence cluster") is present only in SK and AC soil samples. All trees indicate for higher similarity between SK and AC than between SK and AC to CF.

5. Some chemical and physical characteristic of soil such as nitrogen content, soil pH along with soil profile, seasonal and spatial variations in soil moisture along soil profile and during the study period, soil temperatures at 2 and 5 cm and air temperature at all sampling sites were measured. High content of nitrate and ammonium was observed in the top layers of both forest soils and their concentrations in the soil below 20 cm were low. Soil pH under forest (native and replanted) exhibited the highly acidic properties, with the pH in the surface soil as low as 2.4. Contrarily, pH of corn-cultivated soil was higher than forest soil but still in the acidic region. In addition, different land use types significantly affect the water regime and temperature characteristics of soils.

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Abstract for Conference

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Diversity of methanotrophic bacteria in tropical upland soils under different land use

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Abstract

Three upland soils from Thailand; a natural forest, a reforested site, and an agricultural field, were studied with regard to methane uptake and the community composition of methanotrophic bacteria (MB). Methane oxidation rates were lower in the agricultural soil than in the native forest or reforested sites. Sites also differed in the community composition of MB, which was characterized by denaturing gradient gel electrophoresis (DGGE) of *pmoA* gene fragments (encoding for a subunit of particulate methane monooxygenase) and PCR-amplified from total soil DNA extracts. Cluster analysis based on the DGGE banding patterns indicated that the MB community of the forested and reforested sites were similar to each other but different from that of the farmland site. Sequence analysis of excised DGGE bands indicated that *Methylobacter* spp. and *Methylocystis* spp represented genera of cultivated MB at the sampling sites. Sequences of upland soil cluster α (USC α), that has been suggested to represent organisms involved in atmospheric methane consumption in diverse soils, were detected in the native forest and reforested site. Such sequences formed a separate branch related to USC α . Other sequences that indicated the uncultivated groups of potential MB were related to methanotrophic Gammaproteobacteria or an unknown sequence cluster that may represent either *pmoA* or *amoA* (coding for a subunit of the ammonia monooxygenase of ammonia oxidizing bacteria).

Introduction

The current atmospheric mixing ratio of the greenhouse gas methane (CH_4) is 1.75 ppmv [1]. An estimated 30 Tg of CH_4 year⁻¹ is consumed via microbiological oxidation in upland soils, accounting for about 6% of the global methane sink [2]. Atmospheric methane oxidation has been detected in many different upland soils; ranging from arctic and sub-arctic tundra soils, grasslands and arable soils of the temperate zone, to tropical forests, savannahs and even arid desert soils [3-6]. While most of studies have been performed to estimate the methane uptake capacity of upland soils of the temperate zone, only few data are available for tropical and subtropical soils [7-13]. These data indicate that methane uptake rates of tropical and subtropical forest soils are comparable to those in forest soils of the temperate zone, although higher oxidation rates have been reported for some tropical soils [14].

Among upland soils, forest soils are much more efficient methane sinks than are cultivated soils [15-17]. Changes in land use, especially cultivation of formerly undisturbed soils, reduce the sink strength for atmospheric methane by 60 – 90% [14, 18, 19]. Such reduction has been reported for tropical soils [8, 10], but the methane oxidation rate seems to recover much faster after abandonment of agricultural activities [11], compared to systems of the temperate zone. Recovery of the oxidation rate to predisturbance levels takes > 100 years for soils of the temperate zone [14].

Methane oxidation rate of tropical soils is subjected to strong seasonal variations. Minimum uptake rates were observed during the rainy season and maximum rates during the dry period in several sites in India [13]. Keller *et al.* [20] reported a 2 – 3 fold increase in the uptake rates of soils in Costa Rica during the dry season. The seasonal variation in methane uptake was related to the moisture content of the soils, a trend commonly observed because soil moisture controls the diffusion rate of methane and oxygen in the soil [21]. Even a net methane emission was observed for tropical pasture sites during the wet season [20].

Methane oxidation in upland soils is mediated by methanotrophic bacteria (MB). Seven recognized genera of MB belong to the group of type I methanotrophs (Gammaproteobacteria), while the group of type II methanotrophs consists of 4 genera of MB (Alphaproteobacteria). MB catalyze the first step in methane oxidation through activity of

the enzyme methane monooxygenase (MMO). With the exception of *Methylocella* [22-24], all MB possess the particulate form of this enzyme (pMMO). The *pmoA* gene, encoding the α subunit of the pMMO, is therefore used as a marker gene for methanotrophs and has often been targeted by cultivation-independent methods to characterize the community of MB in different upland soils. Sequences closely related to those of 7 different genera of MB have been detected by these methods in upland soils [25-31]. Additionally, some *pmoA* sequences closely related to cultivated MB have been detected, indicating that uncultivated MB are present in these soils. Uncultivated organisms that harbour *pmoA* sequences of upland soil cluster α (USC α) or upland soil cluster γ (USC γ) are most likely involved in atmospheric methane oxidation in upland soils [26, 28]. With exception of one soil sample from a rainforest in Brazil, the characterization of soil methanotrophic communities has been restricted to upland soils from the temperate zone. The only detected *pmoA* sequence type in this rainforest soil sample belonged to USC α [26].

In the present study, we compared the methane oxidation activity and methanotrophic community composition of different land-use, including a natural forest site, a reforestation site and a corn field. The methane oxidation activity and the community composition of these soils were analyzed and compared with other data available from temperate soils.

Materials and Methods

Sampling sites. Three adjacent sampling sites located in Amphur Wang Nham Keaw, Nakorn Ratchasima province in Thailand. The forest site (SK) was a natural dry evergreen forest within the Sakerat Experimental Station. The dominant tree species were *Hopea ferrea*, *Pterocarpus marcrocarpus*, *Xylia xylocarpar*, *Dalbergia cochinchinensis*, *Lagerstroemia duppereana*, and *Shorea henryana*. The reforestation site (AC site) located about 5 km away from the SK site. It was planted with fast-growing, nitrogen-fixing tree species in 1988. Studies were performed on a plantation of *Acacia mangium*. Before reforestation, this site was used as a farmland (corn and cassava). The third sampling site was a cornfield (CF), situated adjacent to the restoration area (2 km away). It was deforested more than 40 years ago and corn has been continuously cultivated at this site during the last 16 years. Soil samples for molecular analysis were taken from 10 – 20 cm soil depth on 27th and 28th July 2003. Soils at both SK and AC sites are highly acidic (pH = 4.2, 1:1 in water) while pH at CF site was 5.6. Soil texture at SK site is Clay (clay content = 51%) while at both AC (clay content = 30%)

and CF (clay content = 21%) sites is Sandy Clay Loam. Soil preparation for corn plantation at CF site started in the mid of June, 2003 and seeds of corn were sown on July 10, 2003. On this date, chemical fertilizer (16-20-0, N-P₂O₅-K₂O) was applied at the rate of 125 kg ha⁻¹.

Methane oxidation activity. The net methane flux across the soil surface was determined in March and July 2003 by using the closed-static chamber method. Ten chambers were used at SK site, while 5 chambers were used at AC and CF sites, respectively. Chambers consisted of a top made of transparent acrylic glass (30 cm width × 30 cm length × 15 cm height) that could be attached to a fixed collar made of stainless steel. The collar was inserted into between 10 – 15 cm soil-depth and remained there during the whole study period. During measurement, the about 1 cm of chamber top was inserted into a water-filled channel of the fixed collar. Six or ten gas samples of 25 – 30 ml were taken within 1 h by a 30-ml syringe through a silicon stopper in the chamber top. Methane mixing ratios of the gas samples were determined on a Shimadzu gas chromatograph (Model 14B), equipped with a flame ionization detector (FID), and an Unibead C packed column (injection temperature, 120 °C; oven temperature 100 °C; detector temperature 300 °C). The first-order uptake rate constant was estimated from the exponential decrease of methane over time. The methane oxidation rate was calculated from the first-order uptake rate constant by multiplication with the initial methane mixing ratio in the chamber headspace.

DNA extraction. DNA was extracted from 0.5 g of soil using the Fast DNA SPIN Kit (BIO 101, La Jolla, CA, USA), according to the manufacturer's instructions with modifications as described elsewhere [32]. DNA extracts were purified with polyvinylpolypyrrolidone and a QIAquick PCR Purification KitTM (Qiagen, Hilden, Germany) [28].

PCR amplification and DGGE. A partial fragment of the *pmoA*-gene was PCR-amplified with primer A189 with GC-clamp [33] and primer A682 of Holmes *et al.* [34] and primer mb661 of Costello and Lidstrom [35]. Composition of the PCR reactions and the PCR program are described elsewhere [32]. A fragment of the *mmoX* gene encoding the active-site subunit of the soluble MMO was amplified with forward primer mmoXf945 with GC-clamp [32] and reverse primer mmoXB1401 of Auman *et al.* [36] using the PCR program of Knief *et al.* [32].

Mixed *pmoA*-PCR products were separated by DGGE. Visible bands were excised and reamplified. PCR products from excised DGGE bands were purified and sequenced exactly as described in Knief *et al.* [28].

Phylogenetic sequence analysis. Phylogenetic tree reconstructions were based on deduced amino acid sequences of partial *pmoA* and *amoA* sequences and were performed with the ARB software package [37]. Original tree construction included sequences of all DGGE bands, all available *pmoA* sequences in the GenBank database (December 2003), and selected public-domain *amoA* sequences. A selection was then made of 24 representative sequences from this study plus 38 public-domain sequences.

Isolation of methanotrophic bacteria. Diluted nitrate mineral salts medium at slightly acid pH (5.8) was used to enrich for MB as described by Dunfield *et al.* [24] from soil samples of the forested sampling sites SK and AC.

Nucleotide sequence accession numbers. Representative *pmoA* nucleotide sequences obtained during this study have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under the accession numbers XXXXXXXXX – XXXXXXXXX.

Results

In situ atmospheric methane oxidation

We measured methane oxidation activity twice at each sampling site, one in March and another in July 2003, or at the same day when soil samples were taken. Net atmospheric methane consumption was observed for both dates at SK and AC sites. However, only in March that we observed net oxidation at CF site (Fig. 1). Large spatial variations typically associated with net methane flux at all sites and not all chambers showed net consumptions. Likewise, in some chambers neither net emission nor consumption was observed. In this study, thus, only in the chambers with significant changes in headspace methane concentration ($p < 0.05$, fitted by linear regression) were taken into account for flux estimate. Only six chambers showed significant changes in headspace methane concentration over sampling time ($n = 6$) in both months at SK site. For example, at SK site the net methane flux

1 ranged from -4.5 to -0.6 $\text{mgCH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in March and -0.5 to -1.2 $\text{mgCH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in July
2 (negative flux indicates a net consumption while positive flux indicates a net emission). For
3 AC site, net methane flux ranged from -0.9 to -1.8 $\text{mgCH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in March ($n = 4$) and
4 $+2.7$ to -5.6 $\text{mgCH}_4 \text{ m}^{-2} \text{ day}^{-1}$ ($n = 3$), and for CF site it ranged from -1.4 to $+1.4$ $\text{mgCH}_4 \text{ m}^{-2}$
5 day^{-1} ($n=3$) in March and $+0.3$ to $+1.4$ $\text{mgCH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in July. Fig. 1 shows the average
6 values of net methane fluxes taken from these chambers.

8 **Characterization of the methanotrophic communities.**

10 Using cultivation-independent methods, the communities of MB were characterized in soil
11 samples taken from 10 – 20 cm soil depth during the second half of the wet season. Duplicate
12 soil samples were taken from each sampling site. The *pmoA* gene fragment, used as a
13 functional marker for the characterization of the methanotrophic community, was successfully
14 PCR-amplified from all soil DNA extracts. A gene fragment of the *mmoX* gene could not be
15 amplified. Two different reverse primers were used for the amplification of the *pmoA* gene
16 fragment. Primer A682 is the less specific of the two, since it was designed to amplify both
17 *pmoA* and *amoA* gene fragments [34]. Primer mb661 was designed to amplify *pmoA* gene
18 fragments specifically, excluding amplification of *amoA* gene fragments [35].

20 The recovery of PmoA sequences very closely related to sequences from isolates of
21 *Methylocystis* and *Methylobacter* indicated that well-known cultivated genera of MB were
22 present in the soil samples of the three sampling sites (> 98 % identity on amino acid level)
23 (Table 1). Sequences from uncultivated bacteria of USC α were detected at SK and AC sites
24 (98 % identity), but not in the soil samples from the cornfield (CF). Likewise, *pmoA*
25 sequences in a closely related, but clearly distinct group from previously detected USC α
26 sequences (Cluster 5) were detected in these two soil samples, but not at CF site. Cluster 5
27 sequences AC-A and SK-B, which are shown in the phylogenetic tree (Fig. 1), showed a
28 maximum identity of 83 % to sequences of USC α , represented by DGGE band E L4-a in
29 Figure 1. While USC α sequences were obtained only when using primer A682 in PCR
30 reactions, this novel sequence type was detected only when using primer mb661. Another
31 PmoA sequence type indicative for a second taxon of uncultivated MB was detected at all
32 sampling sites. PmoA sequences of this "Cluster 3" formed two separate branches, which
33 were related to sequences of methanotrophic Gammaproteobacteria. The amino acid identity
34 of PmoA sequences of these branches was 95 – 98 % to the uncultivated bacteria represented

by "clone Zhenjiang 5" and "clone Beijyuan 2" and 91 – 92 % to the cultivated methanotrophs of the genera *Methylococcus* and *Methylocaldum*. Since the amino acid identity of these novel sequences was approximately equal to PmoA sequences from both of these genera, the phylogenetic relationship could not be resolved and is represented by a multifurcation in the tree (Fig. 1). Sequences with a phylogenetic position intermediate to those of clearly definable AmoA or PmoA sequence types were detected in soil samples SK and CF. These sequences of "Cluster 2" had 87 – 97 % identity to a sequence previously recovered by cultivation-independent methods from a temperate forest soil, represented by DGGE band E 5FB-f [28]. Sequences that were phylogenetically most closely related to *amoA* genes of ammonia-oxidizing bacteria were detected only in soil sample CF.

Isolation of MB from forested sites. The methanotrophic isolate sakb1 was isolated from site SK, while two isolates (arc1 and arc2) were obtained from site AC. Based on the partial *pmoA* gene sequence, these isolates were identified as *Methylocystis* spp. (Fig. 1). This result was confirmed by 16S rRNA sequence analysis and by the morphology of the isolates. The *pmoA* sequences of the isolates from site AC were not identical to the *pmoA* sequences that were detected in this soil by cultivation independent methods. At site SK *pmoA* sequences indicating the presence of *Methylocystis* spp. were not detected at all.

Relationship between sampling site and community composition of methane-oxidizing bacteria. The DGGE banding pattern of PCR products from both backward primers indicated clear differences in the community composition of methane-oxidizing bacteria at the different sampling sites. The analyzed duplicates from each sampling site were very similar to each other. This impression was confirmed by the results of a cluster analysis (SYSTAT version 9; SPSS Inc. Richmond, Calif.), which was performed based on the DGGE banding pattern (Fig. 2). The duplicates of all soil samples formed separate clusters. The MB community similarity between sites SK and AC was higher than the similarity of either to site CF.

Discussion

In recent years, several studies have examined the methane oxidation activity together with the structure of the methanotrophic community of diverse upland soils [26-29, 31]. With the exception of Brazilian rainforest soil [26], all data on methanotrophic community structure originate from upland soils of the temperate zone of the northern hemisphere. Because some

tropical sites have much higher rates of methane oxidation than temperate sites [12-14], it is interesting to know the MB community composition in these areas. This is the first study to analyze the methanotrophic community composition of a tropical soil under different land use.

The measured methane oxidation rates of the soils corresponded quite well to those published in several other studies for upland soils (up to $7 \text{ mg CH}_4 \text{ m}^{-2} \text{ d}^{-1}$), including tropical and subtropical forest soils [14], but were not as high as the rates reported for some tropical forest soils (up to $14 \text{ mg CH}_4 \text{ m}^{-2} \text{ d}^{-1}$) [12, 13]. In all land use types examined in this study, the methane oxidation rates were higher in March than in July. Our long interval of measurement (once a month) does not permit us to discuss the reason of such methane oxidation differences. However, we speculated that difference in soil moisture between these two months might have some influences on oxidation activity. Rainy season in the study site normally begins in May. Unusual for March, in 2003 we encountered the heavy rainfall before gas sampling date. As a result, when compares the moisture content of soil (at 6 cm depth) the results do not significantly difference ($23 \pm 4\%$ by vol in March and $20 \pm 3\%$ in July for to SK site, $15 \pm 4\%$ in March and $15 \pm 5\%$ in July for AC site, and $22 \pm 2\%$ in July (no data in March) for CF site, unpublished data). As described above, even within a single day large spatial variations among chambers were found. Thus, further study is needed to understand the correlation between environmental factors and methane oxidation activity in the field.

At CF site the methane oxidation activity was always lower than in the forested sites, and net methane production was observed in July. Thus, some parts of the soil profile might have been saturated with water to the level that methanogenesis developed and took over the net oxidation. In addition, field preparation and fertilization in July may inhibit methane oxidation and allow the net flux to shift to emission. The inhibitory effects of fertilizer especially nitrogen fertilizer are well understood [4, 7, 17].

In general, the oxidation capacity decreased in the order of forest site (SK), reforestation site (AC), and cornfield (CF). This agrees well with the general observation that the conversion of natural forest sites to farmland leads to a reduction of 60 – 90% of the atmospheric methane uptake rate, but that the activity recovers in reforested sites [10, 14, 18, 19]. It has been found that the oxidation rate of tropical soils recovers much faster after abandonment of agricultural activities, compared to temperate systems [11, 14]. This may explain why the methane

oxidation rate of the reforestation site AC was comparable to the rate in site SK, although site AC was reforested only 16 years ago.

Fragments of the *pmoA* gene were amplified from all soil DNA extracts, but *mmoX* genes were not recovered from the soil samples. This is in accordance with results from upland soils from Central Europe [28] and suggests two different explanations. Either the primer systems used are not very sensitive for the detection of *mmoX* genes, or the dominant MB in upland soils do not possess *mmoX* genes. The latter is reasonable considering that MB in upland soils have to live on very low substrate concentrations. Under these oligotrophic conditions it should be more preferable to use pMMO over sMMO, since it has a lower energy demand for the oxidation of methane to methanol. MB expressing pMMO have higher growth yields and show a higher affinity for methane [38].

The only known genera of MB detected in the Thai soil samples were those of *Methylocystis* and *Methylobacter*. While *Methylocystis* has been detected in several different upland soils [25, 26, 28-30], *Methylobacter* has only been detected in an acidic heath-land soil [25]. Unusual sequences obtained from several DGGE bands indicated that different taxa of uncultivated putative methanotrophs were also present in the soil samples. Organisms harbouring *pmoA* sequences of USC α have been detected in several upland soils before, including acidic tropical forest soils [26]. They were present at sampling sites SK and AC, as were sequences that are represented by Cluster 5, which is closely related to USC α . Cluster 3 sequences represent a group of uncultivated type I methanotrophs related to *Methylocaldum* and *Methylococcus*. A further branch of PmoA sequences within this broad *Methylococcus-Methylocaldum* cluster is formed by sequences that were detected in different rice field soils (clone Beiyuan 2, clone Zhenijang 5) [39]. Thus, different uncultivated taxa of MB seem to exist, which have – on PmoA level – a phylogenetic position between *Methylocaldum* spp. and *Methylococcus* spp.

The detection of the third cluster representing uncultivated bacteria, Cluster 2, has as yet been restricted to upland soils. Since the sequences of this branch are only distantly related to PmoA sequences of recognized genera of MB, it cannot be excluded that these sequences code for the AmoA of uncultivated ammonia-oxidizing bacteria. However, the growth of the organisms harboring these sequences was possibly stimulated in sieved soil samples after incubation under elevated methane mixing ratios (Knief, unpublished data). In this study, only

Methylocystis spp. were isolated. These isolates probably did not represent the dominant *Methylocystis* spp. in the soil samples, since *PmoA* sequences identical to those of the isolates were not detected by cultivation-independent methods in the soil samples.

The different land-use of the sampling sites was reflected by different methane uptake rates and different soil pHs, especially for the cornfield soil. The characterization of the methanotrophic community indicated that the community composition reflected the land use change as well. The *pmoA* sequences of USC α and Cluster 5 were detected in samples from sites SK and AC, but not in the soil samples from the cornfield. The absence of USC α at site CF is in agreement with the results of other studies, in which the methanotrophic community of some farmland soils was analyzed and in which USC α sequences were not detected, although they were present in adjacent grassland and forest soils [28]. The pH shift in the farmland soil is probably not the reason for the disappearance of USC α sequences at this sampling site. The organisms harbouring USC α sequences, although they appear to occur more frequently in acidic than in pH-neutral soils, do occur in soils of a wide pH range (4.0 – 8.0) [28]. Further sequences that led to the different community compositions of the different sampling sites were those of *Methylocystis* spp., Cluster 2 and of ammonia-oxidizing bacteria (Table 1). Sequences of *Methylobacter* and Cluster 3 were detected at all sampling sites. A cluster analysis was used to compare the degree of similarity across sites among the communities of MB plus ammonia-oxidizing bacteria. The resulting tree confirmed the high similarity of replicate samples from one sampling site and differences between the different sampling sites (Fig. 2). The differences within the replicate samples from one site were due to faint DGGE bands visible in one sample but not in the other ones. Thus, *pmoA* sequences with abundance near the detection limit of the methods were mainly responsible for the dissimilarity of duplicate samples. The main purpose of this cluster analysis was the comparison of the different sampling sites. The community composition of SK and AC sites were more similar to each other than either was to CF site. This finding is in accordance with the methane uptake rates and pHs of the sampling sites, which also indicated a high degree of dissimilarity of site CF compared to sites SK and AC.

These data demonstrate clearly that land use changes are not only connected to changes in the methane uptake capacity of soils, but also to changes in the community composition of methane-oxidizing bacteria. The change in the community composition may be an important factor leading to the general decrease in the methane uptake rate of farmland soils. The

methanotrophic communities of replicate samples within one sampling site were highly similar, although methane uptake rates varied over space and time. However, several other factors influence the methane uptake rate of a soil. The water content is an important factor, since it heavily influences gas diffusion in the soil [21]. Whether and how the community of MB responds to changes in the water content of a soil, remains to be studied.

The community composition of the reforested site AC represents a kind of intermediate state between the communities at sites SK and CF. Since it is unknown how the community composition and methane uptake activity of the reforested site AC looked before the site was reforested, we cannot conclude whether the community we see today has redeveloped or whether the community structure was not as much changed after deforestation. During this period this site was a farmland like site CF, so it is likely that the MB community was different at this time.

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Table 1: PmoA sequence types detected in the different sampling sites. Phylogenetic positions of the different taxa are given in Figure 1. (Designation of Clusters 2, 3, and 5 according to Knief *et al.* [32].), SK = natural forest site, AC = reforested site and CF = comfield site.

Detected taxa	SK		AC		CF	
	A682b	mb661	A682b	mb661	A682b	mb661
Ammonia oxidizer (AmoA)					+	
Cluster 2 (PmoA/AmoA)	+				+	
<i>Methylobacter</i>		+		+	+	+
Cluster 3		+		+	+	+
<i>Methylocystis</i>			+	+	+	
USC α	+		+			
Cluster 5		+		+		

Legends

Figure 1 In situ net methane flux. The values are the average of measurements from 6, 4 and 3 chambers for SK, AC and CF sites, respectively.

Figure 2 Phylogenetic tree of deduced amino acid sequences of partial *pmoA* and *amoA* sequences. The tree includes representatives of different PmoA sequences that were detected at the different sampling sites. Sequences recovered from PCR products that were amplified using the A682 reverse primer are shown in small letters while capitals indicate sequences from PCR products that were obtained using the mb661 reverse primer. PmoA sequences of three methanotrophic isolates, obtained from soils SK and AC, are also included. Tree calculation was based on 130 amino acid positions using the treepuzzle algorithm with the Jones-Taylor-Thornton evolutionary model [40]. The position of recovered PmoA and AmoA sequences in this tree was confirmed by the topology of a maximum-likelihood and a neighbor-joining tree. Black circles indicate branches that were present in 90 % of 25,000 reconstructed treepuzzle trees, white circles indicate branches present in 80 % of the trees. AmoA sequences of ammonia-oxidizing bacteria were set as an outgroup. The bar represents 0.10 change per amino acid position.

Figure 3: Cluster analysis based on the presence or absence of 21 bands of DGGE gels from PCR products obtained with primer systems A189-GC/A682 and A189-GC/mb661. Jaccard similarity coefficients were calculated and a hierarchical tree was constructed based on average linking and Euclidean distances.

Figure 1

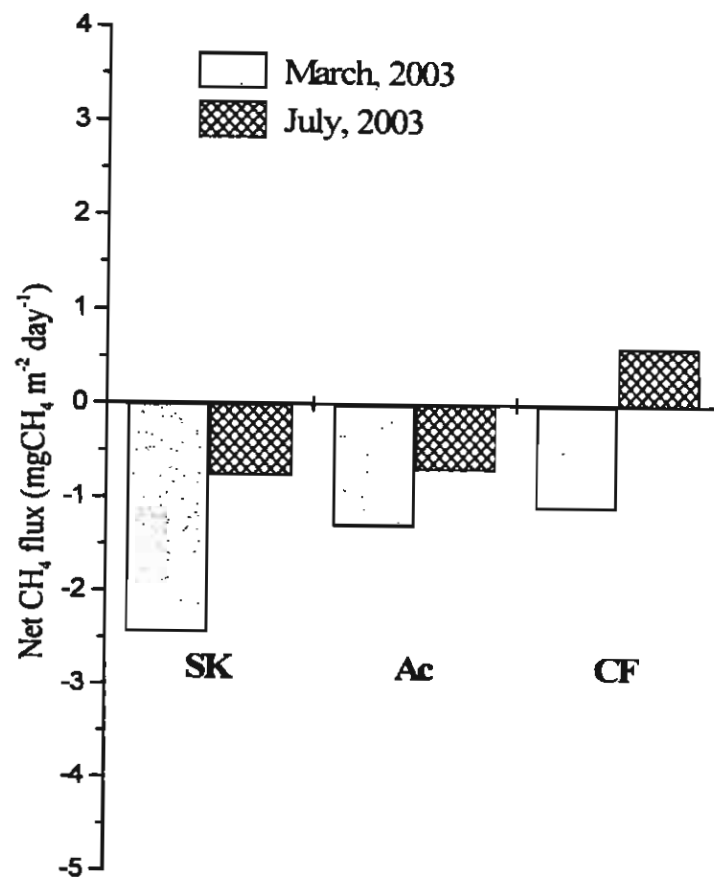


Figure 2

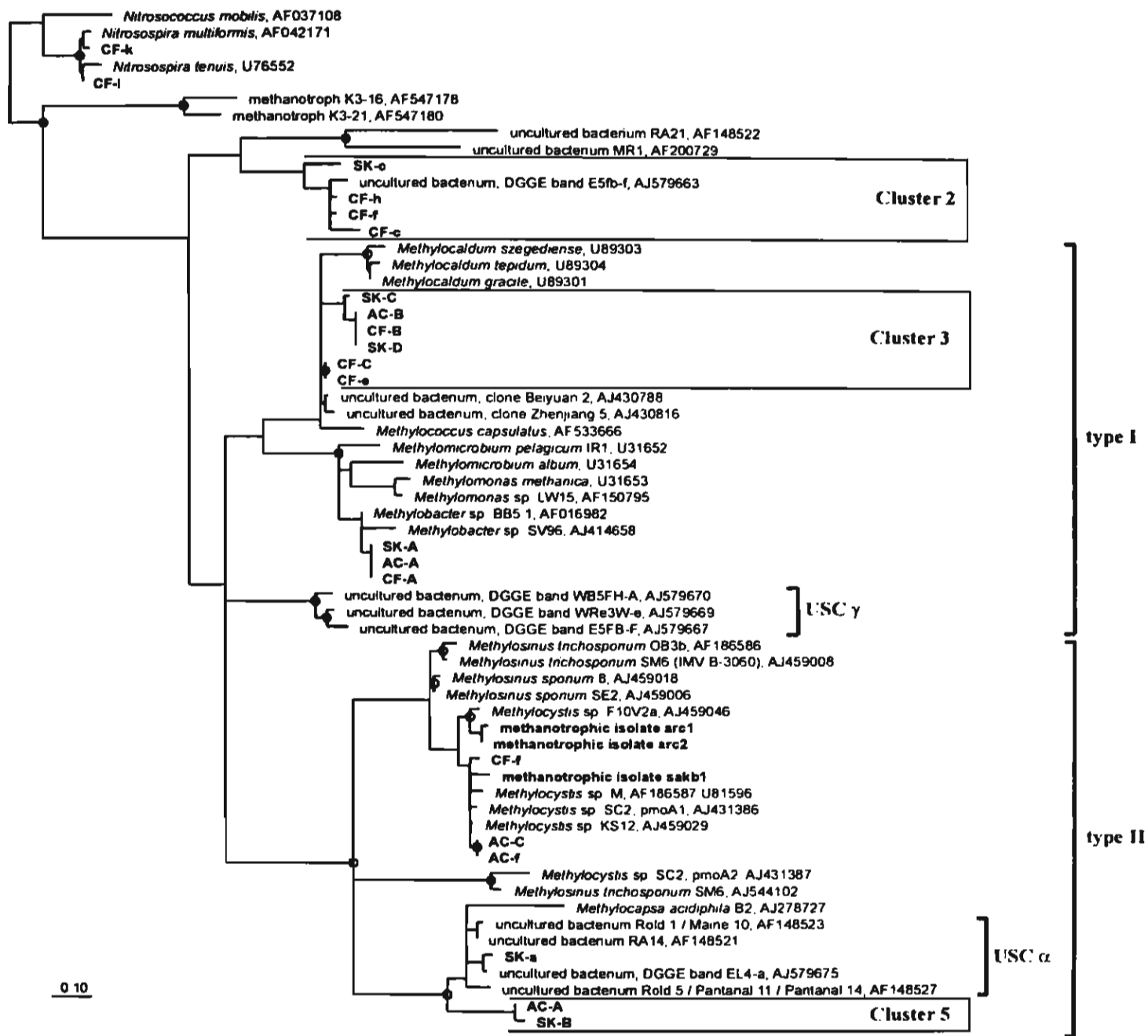
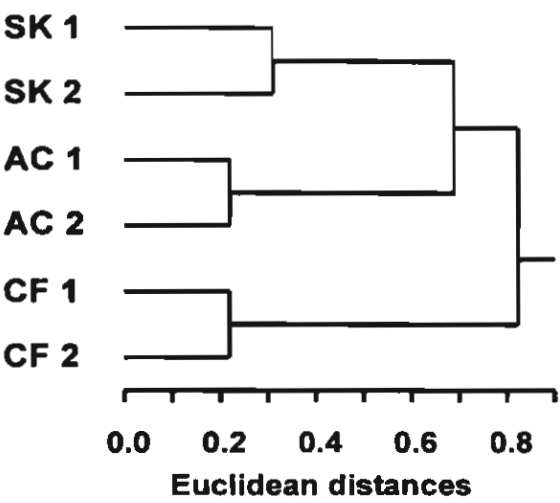


Figure 3



Draft:

Kinetics of methane oxidation in tropical upland soils under different land use

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Abstract

Three upland soils from Thailand; a natural forest (SK soil), a reforested site (AC soil), and an agricultural field (CF soil) were studied for their methane oxidation potential and kinetics of methane oxidation. In SK and AC soils a clear zonation for active methane oxidation layer was detected along the soil depth. The most active oxidation layers in SK and AC soils lied between 15 cm and 40 cm while in CF soil no clear active layer was observed. Stratification of active oxidation zones coincides with the trends of inorganic nitrogen content profile. In SK and AC soils, high concentration of inorganic nitrogen compounds (usually $> 100 \text{ mg NO}_3^- \text{ or NH}_4^+ \cdot \text{kg soil}^{-1}$) was detected in the top 15-cm soil while there was no clear distribution trend found in CF soil. It was assumed that such high concentration of inorganic nitrogen in the topsoil inhibited the activity of methane oxidizing bacteria thus only in the subsoil that methanotrophs were active. Examining kinetic coefficients of these active layers revealed that soil at SK site had high affinity for methane (K_m of 52 ppmv) but rather low methanotrophic capacity ($V_{\max}$ of $0.82 \text{ nmol} \cdot \text{g soil}^{-1} \cdot \text{h}^{-1}$). Soil at AC and CF sites, on the other hand, showed low affinity for methane (K_m of 724 ppmv and 1454-2362 ppmv, respectively). However, soils at these two sites were capable of oxidizing high concentration of methane ($V_{\max}$ about $10 \text{ nmol g soil}^{-1} \text{ hr}^{-1}$). These results indicate that land use type significantly affects depth distribution of methane oxidation and kinetics of methanotrophic community in tropical soils.

1. Introduction

Microbial uptake in upland soils represents the major terrestrial sink of atmospheric methane. This process consumes about 20-60 Tg of CH₄ annually, accounting for about 6% of the global methane sink [Houghton et al., 2001; Adamsen and King 1993; Reeburgh et al., 1993]. Atmospheric methane oxidation has been detected in many different upland soils; ranging from arctic and sub-arctic tundra soils, grasslands and arable soils of the temperate zone, to tropical forests, savannahs and even arid desert soils (Keller et al., 1983; Mosier et al., 1991; Seiler et al., 1984; Whalen and Reeburgh, 1990). While most of studies have estimated the methane uptake capacity of upland soils of the temperate zone, only few data are available for tropical and subtropical soils (Castro et al., 1994; Goreau et al., 1988, Keller et al., 1986, 1990, Keller and Reiners, 1994).

In many oxic soils, biphasic methane oxidation kinetics were observed and this is suggested to be attributed to the presence of two methanotrophic communities (Bender and Conrad 1992). The first group are the methanotrophs that have high capacity (high V_{max}) and low affinity (high K_m) for methane. They are capable of oxidizing methane at elevated concentrations (>1000 ppmv). The second group exhibits high affinity for methane and are able to consume methane at atmospheric concentration. Most of the cultured and isolated methanotrophs belong to the first group. However, the presence and role of methanotrophs in the second groups have been confirmed by culture-independence techniques (Holmes et al., 1999; Henkel et al., 2000 Knief et al., 2003). Recently, the methotrophs able to oxidize methane at atmospheric levels have been isolated from a humisol soil (Dunfield et al., 1995, Dunfield et al., 2003).

Among upland soils, forest soils are more efficient methane sinks than are cultivated soils (Ambus and Christensen, 1995; Born et al., 1990; Hütsch et al., 1994). Changes in land use, especially cultivation of formerly undisturbed soils, usually results in reduction of sink strength for atmospheric methane by 60 – 90% (Smith et al., 2003). This has been attributed to inhibition by nitrogen fertilizers applied during crop cultivation (Mosier et al., 1991, Hütsch 1994). Inorganic nitrogen, especially ammonium (NH₄⁺) significantly inhibits methane oxidation, presumably through the competition with methane as a substrate. It has also been suggested that changes in soil water regime, soil pH, some physical properties

such as water permeability and gas diffusion rate induced by change in land use significantly affect on methane oxidation capacity of soils.

The most remarkable change in land use in the past decades is deforestation in the tropics. It is estimated that deforestation rates in the tropic are as rapid as 2% year⁻¹ (Keller and Reiners, 1994). Such land use change leads to a reduction of methane oxidation and thus may have implicated the global methane by contribution to increasing atmospheric concentrations. However, recently reforestation in the abandoned lands has been promoted in many tropical countries. It is not known whether or not such activity could recover methanotrophic capacity of soil.

Here we report the kinetics of methane oxidation in soils taken from different land use in Thailand. Our main objective is to assess how oxidation kinetics of tropical upland soils are affected by converting natural forest into cultivation land, and by converting agricultural soil back into planted forests.

2. Materials and Methods

2.1 Soil sampling sites

Soil samples were taken from three adjacent sampling sites located in Amphur Wang Nham Keaw, Nakorn Ratchasima province in Northeast Thailand. The forest site (SK) was a natural dry evergreen forest located in the Sakerat Environmental Research Station. The dominant tree species were *Hopea ferrea*, *Pterocarpus macrocarpus*, *Xylia xylocarpus*, *Dalbergia cochinchinensis*, *Lagerstroemia duppureana*, and *Shorea henryana*. The reforestation site (AC) located about 5 km away from the SK site was planted with fast-growing, nitrogen-fixing tree species *Acacia mangium* in 1988. The third sampling site was a cornfield (CF), situated adjacent to the restoration area (2 km away). It was deforested more than 40 years ago and corn has been continuously cultivated at this site during the last 16 years. Soil texture at SK site is Clay (clay content = 51%) while at both AC (clay content = 30%) and CF (clay content = 21%) sites is Sandy Clay Loam. Soil preparation for corn plantation at CF site started in the mid of June, 2003 and seeds of corn were sown on July

10, 2003. On this date, chemical fertilizer (16-20-0, N-P₂O₅-K₂O) was applied at the rate of 125 kg ha⁻¹.

2.2 Kinetic study

The air-dried and 2-mm sieved soil was used. Firstly, oxidation activity at different soil depth was determined. Soil layers were divided according to soil depth of 5 cm intervals. 30 g of soil samples were weighed into the vials and then they were incubated at room temperature (25-26 °C). Concentration of methane in the vial's headspace was determined. Decrease in headspace methane concentration was then plotted against incubation time and rate constant of methane consumption was determined from the slope of log-transformation plot. Oxidation rate was calculated by multiplying the rate constant with the initial methane concentration.

The most active oxidation layer was then selected for further determination of kinetic coefficients. 30 g of 2-mm sieved soil were weighed and put into the 100-mL vials. Soil moisture was adjusted to 25% by weight. The vials were then closed with a rubber stopper and the headspace methane concentrations were adjusted to the range from ambient to 1800 ppmv. The vials were incubated at room temperature (26°C) and headspace methane concentration was determined at appropriate time intervals to follow a decrease in methane concentration with times. Incubation of duplicate samples for each soil layer was made. Kinetic coefficients ($V_{\max}$, K_m value) associated with CH₄ uptake were estimated from the plot between the initial CH₄ concentrations versus oxidation rates. Such plot was fitted to a Michaelis-Menten hyperbolic model of pseudo-first-order enzyme kinetics using the least-square iterative fitting procedures of Origin 5.2 (Microcal Software, Inc., Northampton, Maine).

2.3 Chemical analysis

A Shimadzu Gas Chromatograph (Model 14B) equipped with FID detector and “methanizer” was used to measure CH₄ in the present study. The GC operating conditions are: FID temperature: 300°C, Injection temperature; 120°C, Column temperature; 100°C,

Carrier gas; Helium (99.99% purity), Carrier gas flow rate; 65 mL/min, Column; Unibead C packed column.

Inorganic nitrogen contents (NH_4^+ , NO_3^- , NO_2^-) were determined by Ion Chromatograph (Dionex DX600, USA) equipped with an electrochemical detector. Ten grams of 2-mm sieved soil from each layer were weighed and placed into an Erlenmeyer flask and 70 mL of deionized water was added. Extraction was carried out in triplicates for each soil layer. The flasks were shaken on a shaker for 45 min and the supernatant was filtered through a 0.45 μm cellulose acetate filter membrane. Deionized water was used as a blank control. Concentrations of inorganic nitrogen in filtered liquid were then determined and expressed as mg of an inorganic nitrogen compound per kg of dry soil.

3. Results

3.1 Soil pH

Soil pH was measured in deionized water (soil : water = 1:1) after drying the soil samples at room temperature. Forest soils (both at SK and AC sites) were acidic, especially in surface layers (Fig. 1). The SK soil at surface had the pH of about 2.0 while at AC site the pH at surface was 3.5. However, pH values at both forest sites increased to about 4 at 10 cm and then stayed fairly constant at this value throughout a 50-cm profile. Contrarily, soil under corn plantation showed a different pH profile. In cornfield soil, the highest pH was found at surface (pH 6.2) and pH decreased to and stayed constant around 5.2 below 5 cm.

3.2 Concentration of inorganic nitrogen compounds

Inorganic N content (NO_2^- , NO_3^- and NH_4^+) of all soil was measured along the soil depth. Results are given in Fig. 2A-C. The top layer of natural forest soil (SK) showed the highest nitrate content (about 260 mg $\text{NO}_3^- \text{ kg soil}^{-1}$, Fig. 2A). High level of nitrate was detected down the profile to 10-15 cm and below this layer nitrate content was below 25 mg kg soil^{-1} . On the other hand, nitrate content throughout soil profile at both AC and CF sites was fairly constant around 50-75 mg kg soil^{-1} . However, very low concentration of nitrate

was found below 30 cm in AC soil. No clear trend along the profile was observed for both AC and CF sites.

In contrast to nitrate, nitrite content along the soil profile and across the sampling sites was not different. Compared to nitrate, nitrite content was approximately more than 10 times lower (Fig. 2B). It was lower than $3 \text{ mg kg soil}^{-1}$ below 10 cm in SK site and below 15 cm in AC site. NO_2^- was detected across the soil profile at CF site, although its concentration was generally lower than $10 \text{ mg kg soil}^{-1}$.

Ammonium content in all sites is given in Fig. 2C. Similar to the trends found in nitrate content, natural forest soil at SK site had the highest ammonium content. The highest concentration at SK was found in the top 10 cm surface layer ($173 \text{ mg kg soil}^{-1}$ at 0-5 cm depth). In both forest sites, ammonium concentration trended to decrease as soil depth increased and below 10 cm the typical concentration was below $50 \text{ mg kg soil}^{-1}$. In contrast to the forest site, a fairly constant amount of ammonium was observed throughout the soil profile at CF site. However, the concentration at CF site at all depths was below $50 \text{ mg kg soil}^{-1}$ and it trended to increase in the deeper layers.

3.2 Methane oxidation rate along soil profile

Soils at different depths taken from all three sampling sites were studied in the laboratory. Soils from SK and AC sites were incubated under ambient methane concentration and then consumption was followed over time. Since soil from CF site shows low oxidation activity, incubation was carried out under 5 ppmv methane. Most of soil layers exhibited the methane oxidation activity. However, the most active methane oxidation layers for SK site was between 15 and 30 cm (Fig. 3A). For AC site clear oxidation activity was found only at 10-15 and 20-30 cm (Fig. 3B) and changes in concentration of methane in the incubation vials were erratic. Repeated experiments gave the similar results. Similarly, all layers of soil from CF site show oxidation activity (Fig. 3C). There was no oxidation observed at 5-10 cm. However, the most active layer was in the subsurface at 0-5 cm and 30-40 cm.

3.3 Kinetics of methane oxidation

Methane oxidation rate at each concentration was estimated by linear regression of CH_4 mixing ratios versus time. A plot of initial methane concentration and methane oxidation rate is given in Fig. 4. The initial methane concentrations used in the experiment were 5, 10, 20, 50, 100, 200, 300, 700, 800, 1000 and 1800 ppmv. For SK site, the plot between consumption rate and substrate concentration followed an expected shape of enzymatic kinetics with initially increase oxidation rate paralleling increase concentration, followed by a rate that no longer shows concentration dependence. This is true even at low methane concentration. However, in AC and CF soil it seemed that at concentration below 50 ppmv, there was no clear correlation between oxidation rate and methane concentration as suggested by pseudo-first order kinetic model of Michaelis-Menten. Increase of oxidation rate in these two sites was observed at concentration above 50 ppmv (Fig. 4).

From Fig. 5, it is clear that at methane concentration below 250 ppmv, rate of methane oxidation in SK soil was higher than at both AC and CF site. Thus, soil at SK can oxidize methane at low concentration better than soils at AC and CF site. However, above about 200 ppmv, the oxidation rate becomes concentration independence at SK site. Thus, methane oxidation rate in SK soil will not increase significantly if its methane concentration is too high. In AC and CF sites oxidation rate still increased and then stabilized around 800 and 1000 ppmv, respectively. At ambient and 50 ppmv concentration, however, soil at AC site shows higher oxidation rate than at CF site, though the difference was not clear.

The estimated kinetic coefficients associated with methane oxidation as affected by different land use are given in Table 1. The highest affinity for methane was found in SK soil ($K_m = 52$ ppmv). Soil from AC and CF show lower methane affinity compared with SK site (K_m of 724 ppmv and 155-2362 ppmv, respectively). The lowest V_{max} was found in SK soil. However, there was no significant difference in V_{max} between AC and CF soils.

4. Discussion

Results from previous studies indicate that methane oxidation in soils is affected by several factors. Among these, land use type and mode agricultural practices are among the most influencing ones (Hütsch, 2001). Conversion of natural forest to agriculture usually accompanies with partial loss of methanotrophic activity. Powlson et al. (1997) reported that continuous cultivation of 150 years for arable crops led to a reduction of methane consumption rate by 85%, compared to the soil under woodland. Several explanations have been suggested for the loss of methane oxidation associated with land use change.

Repeated application of nitrogen fertilizers have been shown to inhibit methane oxidation (Powlson et al., 1997; Mosier et al., 1991; Hütsch, 1994). Among type of nitrogen compounds, ammonium seems to have the strongest effects. Although nitrite is directly toxic to methanotrophs, its low concentration and highly unstable in nature make it less important than ammonium. Inhibitory mechanisms on methane oxidation has been suggested to be through the competition between ammonium ions and methane for the binding site of methane monooxygenase, the key enzyme that catalyzes the first step of methane oxidation by methanotrophs (Dunfield and Knowles, 1995). However, Gullledge and Shimel (1998) showed that besides the competitive mechanism, salting effects on methane oxidation accompanied with fertilization was also important. They showed that application of nitrogen fertilizer in form of ammonium chloride had stronger effects on methane oxidation than ammonium sulfate. They also showed that ammonium concentration of $94 \text{ mg kg soil}^{-1}$ (as Ammonium sulfate) inhibited methane oxidation by 67% in temperate hardwood forest. On the other hand, nitrate becomes inhibitory to methane oxidation only at high concentration (usually $> 10 \text{ mM}$ dissolved nitrate, Adamsen and King, 1993).

Results in the present study agree well with the finding in the previous studies that when high concentration of inorganic nitrogen present (Fig. 2), low methane oxidation was observed. However, concentrations of all nitrogen species were higher in SK and AC soils than in CF soil and such levels of ammonium might be sufficiently high to inhibit methane oxidation. Nevertheless, relatively high oxidation rate was observed at both SK and AC

sites. Thus, nitrogen content alone in surface soil cannot explain the oxidation rate differences between forests (SK and AC) and agriculture soil (CF) in this case.

It was observed that the distribution pattern of nitrogen content along the soil profile differed among three sites. In SK and AC soils, high content of inorganic N were observed only in the top 10-15 cm and significantly lower concentrations were found below this depth. If the inhibition of such nitrogen compound is considered, oxidation of methane may occur at depth below 15 cm in SK and AC site. On the other hand, soil at CF site showed a fairly uniform distribution of inorganic N along soil depth and a slight increasing trend along the soil profile was observed. Such distribution pattern in CF soil may be resulted from mixing between top and subsoil by cultivation practices during corn plantation. Leaching of nitrogen from the topsoil to subsoil may explain the increasing trends along the soil profile found at CF site. Such distribution of N and at level of concentration found may lead to a relatively uniform inhibition of methane oxidation throughout the CF soil profile (Fig. 3), while such inhibitory effects of N is not prevail in SK and AC soils due to their relatively low concentration in subsoil. As a result, net methane oxidation was observed in SK and AC site throughout almost most of the time.

From these results, it is thus concluded that the active layers for methane oxidation lie below the soil surface in forest soils. The reason for this is not clearly known. However, it is typical that oxidation in the organic layers of forest soils is usually lower than the mineral soil layers (Roslev et al., 1997; Bradford et al., 2001; Reay et al., 2001). This is due to the fact that mineralization in organic layers release inorganic nitrogen species that exert the inhibitory effects on methane oxidation. The depth profile of nitrogen content especially ammonium and nitrate confirms that relatively low methane oxidation in surface layers of forest soil is indeed due to high ammonium and nitrate contents (Fig. 2). In addition, the pH at the surface layers is very low in the present study (Fig. 1). Thus, The soil at the surface layers may be too acidic for methane oxidation.

Hütsch (2001) suggested that repeated application of ammonium could result in change in the kinetics of methatrophic bacteria with consequences for the threshold value (concentration below which no oxidation takes place). Although incubation was not long enough to allow estimate of threshold value, kinetic coefficients clearly indicated that no methane oxidation was observed over 200 ppmv in SK soil, and seemed that no oxidation

occurred below 50 ppmv in AC and CF soils. Nesbit and Breitenbeck (1992) reported that cultivated soil consumed methane only when it was exposed to relatively high concentration of methane (>1000 ppmv) and no oxidation was observed at ambient concentration. In Thai forest soil, the $V_{\max}$ and the K_m are within the range found in temperate forests. For example, the K_m for forest soils reported by Bender and Conrad (1994) was 22 ppmv and the $V_{\max}$ associated with this methane oxidation was $3.6 \text{ nmol g}^{-1} \text{ hr}^{-1}$. Environmental factors and land use, thus, lead to change in kinetics of methane oxidation. Such change in kinetics may be associated with changes in microbial community. However, it is noted that only apparent kinetic coefficients are measured in a study with a mix microbial community such as in the current soil incubation study. Thus, it cannot be assumed that these represent true enzyme properties.

Due to the porous nature of soil, it is reasonable to assume that methane oxidation in subsurface layer is limited by gas diffusion (oxygen and methane) from the atmosphere. Thus, methane oxidation of such soil is controlled mainly by substrate availability. Methanotrophic community in SK subsoil may have experienced and well adapted to such limitation. Therefore, SK soil develops high affinity to methane but oxidation rate could not increase beyond certain levels.

On the other hand, land use usually leads to changes in various chemical, physical and biological properties of soil, such changes may in turn affect methane oxidation activity. Change in gas diffusibility and water regime may be also altered by land conversion from forest to agriculture (Boeckx et al., 1997). On the other hand, low affinity for methane in CF soil may be due to the existence of different methanotrophic community which is classified as the first ground as mentioned above. Methanotrophic community in CF soil may have experienced high concentration of methane during certain period of crop cultivations. Since corn plantation has been continuous for over 16 years, such cultivation practices may help establish the different methanotrophic community that well adapt to high concentration of methane. It is interesting to note that kinetic coefficients for AC soil come in the middle between SK and CF soil, as for the history of land use. This may indicate that soil at AC site is in the transient period from previously disrupted by human activity (agriculture) towards the natural conditions. Methanotrophic community in AC soil, thus, may be the mixture of those found in SK and AC sites, possibly means it could oxidize both

methane at low (as observed in SK site) and high methane concentration (as observed in CF site).

In conclusion, kinetics of methane oxidation is strongly depended on land use type. In tropical upland soil, methanotrophic community with high affinity for methane establishes in undisturbed-natural forest soils. Conversion of such forest soils into agriculture leads to the loss of soil capacity to oxidize atmospheric levels of methane. However, it seems that when agricultural land is converted back to forest, methane oxidation capacity and potential of soil can be somehow recovered. Present study shows that after planting for 16 years to *A. mangium*, oxidation rate and kinetics of soils are still not yet fully returned to the previous levels. However, such forest plantation improves methane oxidation capacity substantially compared with agricultural lands.

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Table 1 Kinetic coefficients measured for methane oxidation by soil samples taken from different land use types.

Site	$V_{\max}$ (nmol g ⁻¹ hr ⁻¹)	K_m (ppmv)
SK (10-20 cm)	0.82	52.02
AC (10-20 cm)	5.72	723.96
CF		
0-5 cm	10.66	1454.88
30-40 cm	9.97	2361.76

Figure Legends

Fig. 1 Soil pH along soil depths at SK, AC and CF sites (measured in H₂O)

Fig. 2 Nitrate (A), nitrite (B) and ammonium (C) contents along the soil profile in different land use.

Fig. 3 Oxidation rate of methane along the soil profile and in different land use types.

Fig. 4 A plot between initial methane concentration vs methane oxidation rate. Soil samples used in experiments at SK and AC sites were taken from 10-20, 5-10 cm depth, respectively, while at CF site two depths (0-5 and 30-40 cm) were examined.

Fig. 5 Exponential plot of relationship between oxidation rate and methane concentration below approximately 350 ppmv.

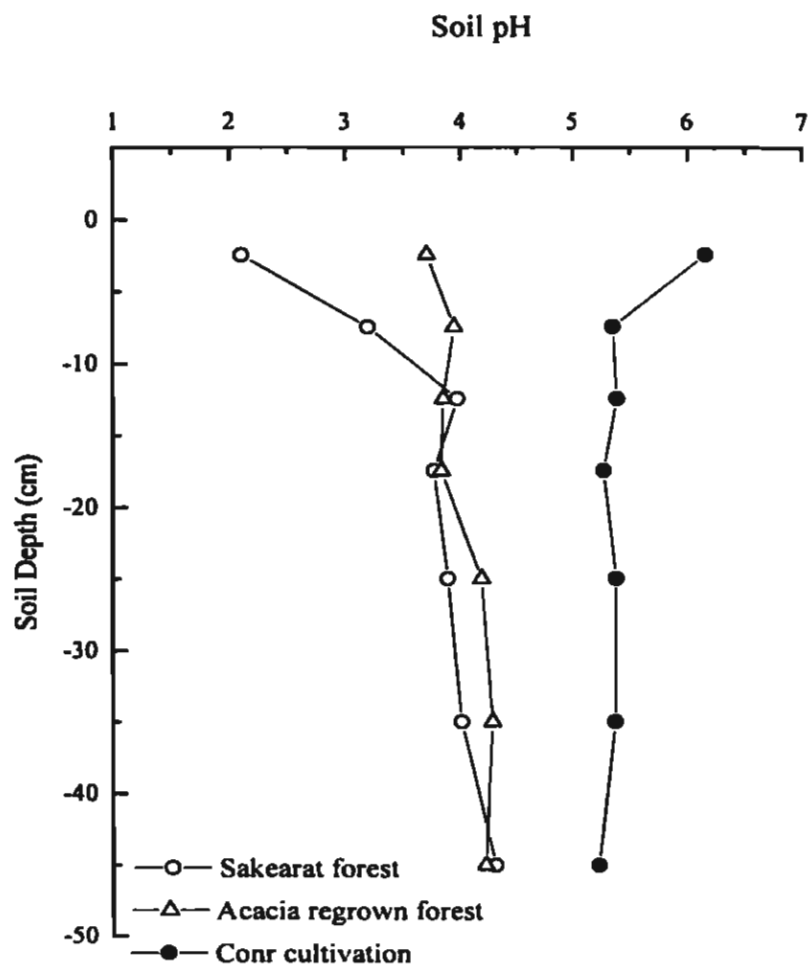


Fig. 1

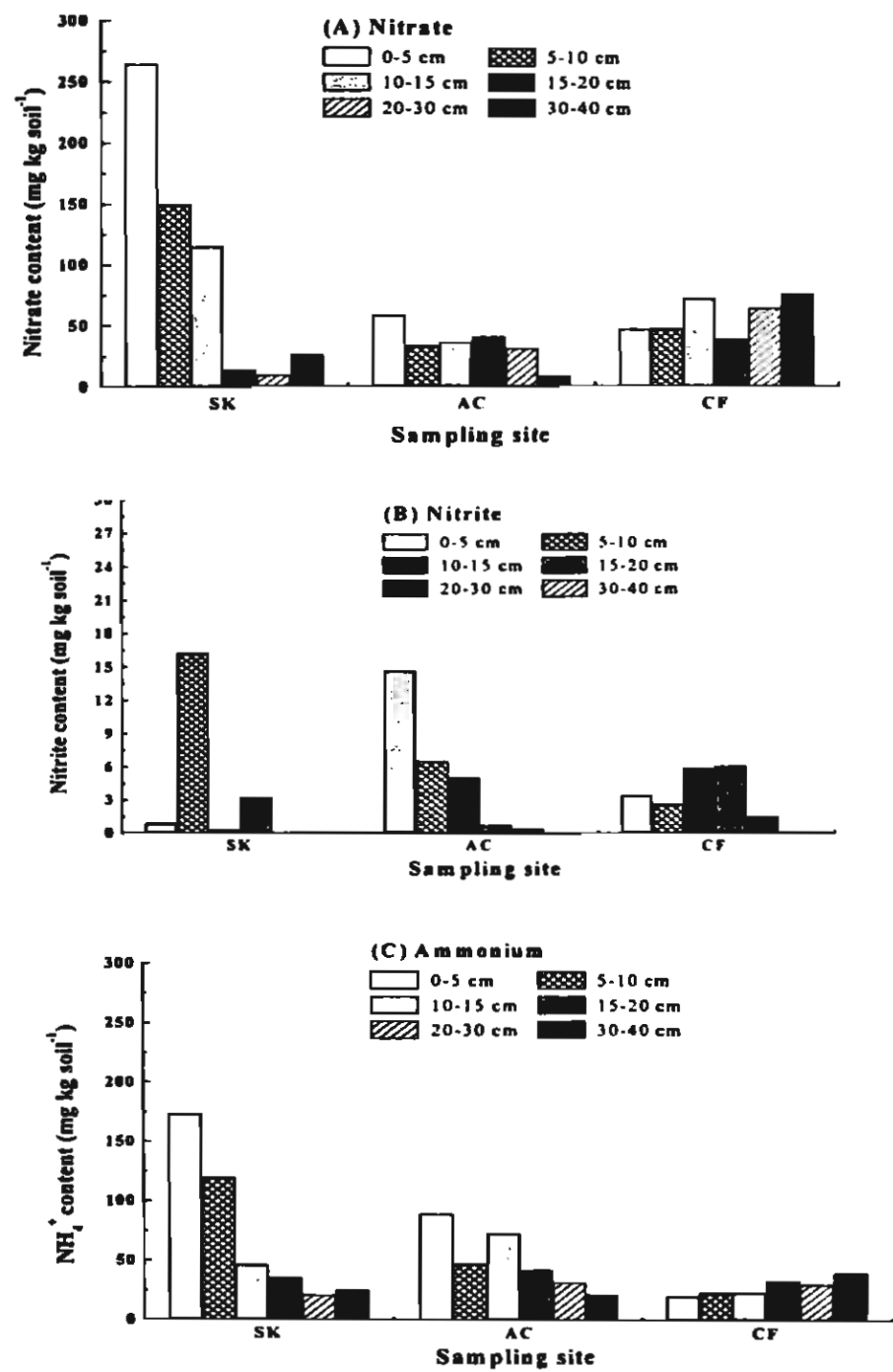


Fig. 2

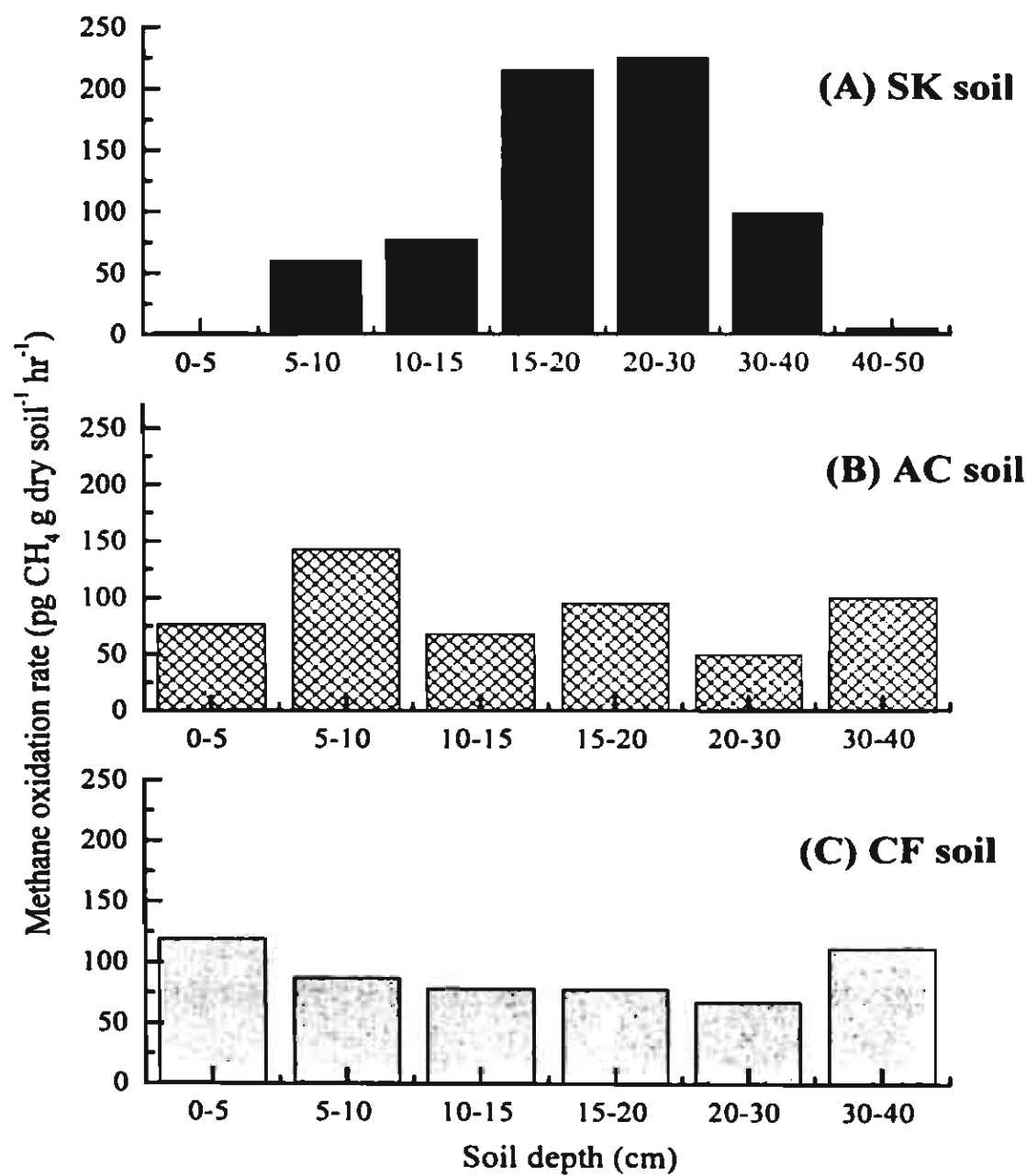


Fig. 3

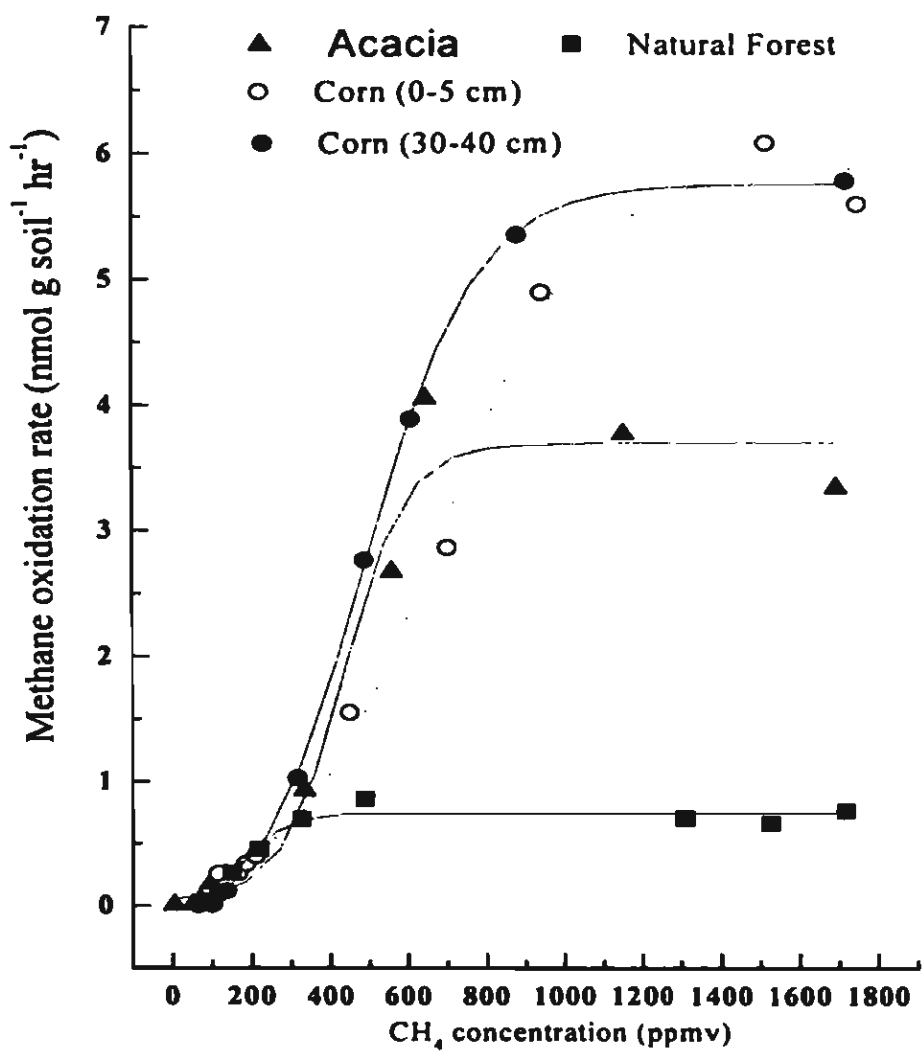


Fig. 4

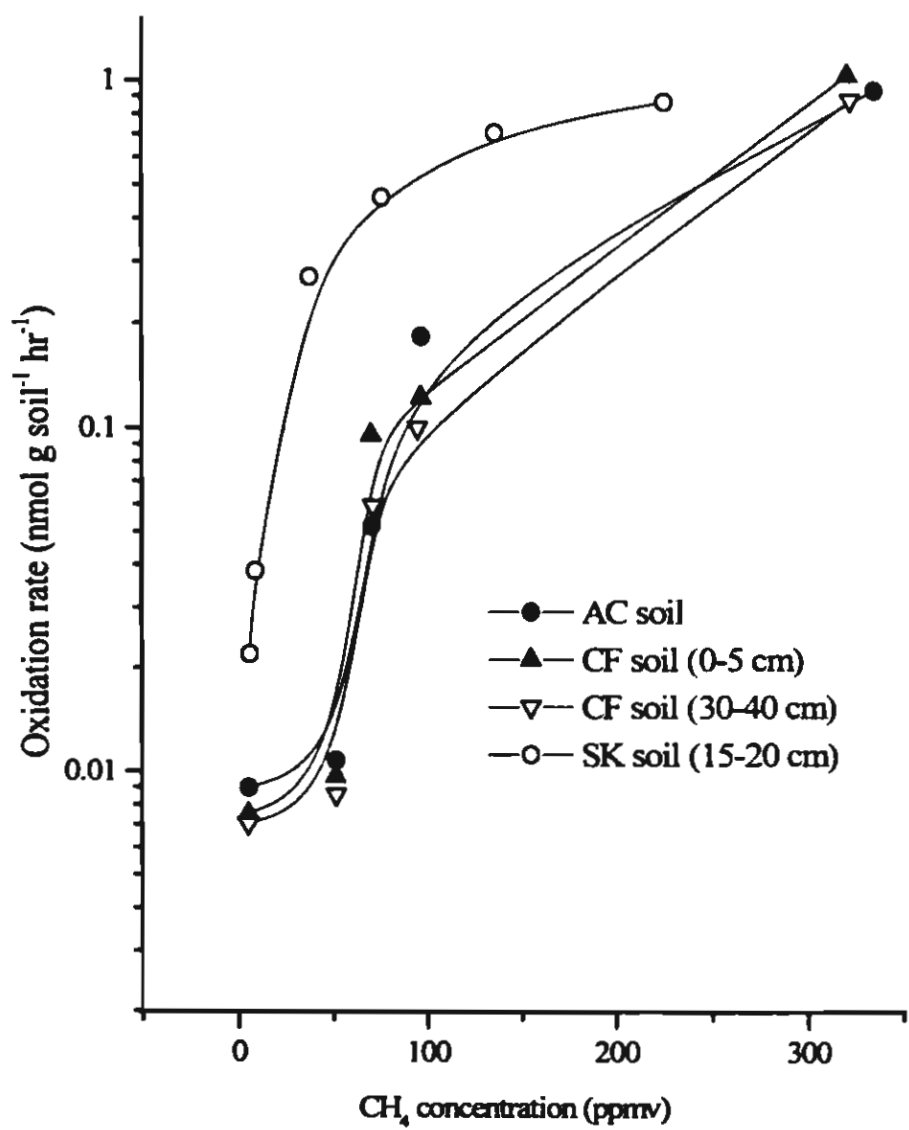


Fig. 5

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Effects of Land Use on Methane Oxidation in Tropical Upland Soils

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Abstract

Methane is an important greenhouse gas and methane oxidation in soil represents a significant sink, accounting for approximately 6% of the global methane sink. Land use is one of influencing factors that contributes to variations of methane oxidation by soils. In this study we investigated methane oxidation activity in different land use in Thailand. Three different land uses included dry evergreen forest, reforestation (a 16-year old *Acacia mangium*), and cultivation (corn field) that were located in Nakorn Ratchasima province Thailand were selected. Methane oxidation rates were measured during January – December 2003 by using a close chamber technique. Results reveal that methane oxidation in forest soil and reforestation soils were significantly higher than cornfield soil. The average methane oxidation from forest soil, reforestation soil, and cornfield soil were 1.50 ± 0.89 , 1.17 ± 0.48 , and -12.87 ± 48.65 mg CH₄/m²/d respectively (negative sign designates net methane emission). Soil chemical and physical property were also observed in all of land uses. Methane oxidation rates were higher during wet season, indicating that soil moisture is one of influencing factors on methane oxidation by soils. High soil moisture might limit gas diffusion (oxygen and methane) from atmosphere, thus, reduce soil oxidation rate in soil. In addition, application of nitrogen fertilizer during corn cultivation may also cause the lower oxidation rate than other sites. Inorganic nitrogen compounds especially ammonia is known for their inhibitory effects on methane oxidation, presumably through competition with methane for the binding site of methane oxidation-catalyzing enzyme. The results indicate that methane oxidation is strongly affected by land use types and high oxidation rate is usually found in undisturbed-natural forest soils. Reforestation, however, help recover methane oxidation activity.

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Other output:

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