



รายงานวิจัยฉบับสมบูรณ์

โครงการ "การประเมินศักยภาพของพื้นที่ป่าไม้ในประเทศไทย
ในการออกซิไดซ์ก๊าซมีเทน"

(Estimate of Methanotrophic Capacity of Tropical Forests in Thailand)

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ก๊าซมีเทนเป็นการเรือนกระจกที่สำคัญและการออกซิไดซ์มีเทนโดยแบคทีเรียในดินถือเป็นแหล่งดูดกลับก๊าซมีเทนจากอากาศที่สำคัญแหล่งหนึ่ง ประเทศไทย ได้มีการประมาณการปลดปล่อยก๊าซมีเทนจากแหล่งต่างๆไว้ค่อนข้างมาก แต่ที่ผ่านมายังไม่มีการศึกษาเกี่ยวกับแหล่งดูดกลับและปริมาณการดูดกลับโดยแหล่งต่างๆเหล่านั้น การศึกษานี้จึงได้วัดอัตราการออกซิไดซ์มีเทนโดยเปรียบเทียบในดินที่มีการใช้ประโยชน์แตกต่างกันในจังหวัดนครราชสีมา ได้แก่ ดินป่าธรรมชาติดิบแล้ง ดินป่าปลูกกระถินเทพา และดินที่ใช้ทำการเกษตร (ปลูกข้าวโพด) อัตราการออกซิไดซ์วัดโดยวิธี Close chamber method โดยทำการวัดทุกเดือนระหว่างมกราคม ถึง ธันวาคม 2546 นอกจากนี้ได้เก็บตัวอย่างดินเพื่อศึกษาลักษณะทางจุลศาสตร์ของการออกซิไดซ์ก๊าซมีเทนและลักษณะโครงสร้างประชากรของ methanotrophic bacteria โดยวิธี denaturing gradient gel electrophoresis (DGGE) จากชิ้นส่วนของยีนที่ควบคุมการทำงานของเอนไซม์มีเทนโมโนออกซิจีเนส ผลการศึกษาพบว่า ดินทั้งสามแห่งสามารถออกซิไดซ์ก๊าซมีเทนระดับความเข้มข้นตามธรรมชาติได้ แต่อัตราการออกซิไดซ์แตกต่างกันไป ขึ้นอยู่กับปัจจัยต่างๆ เช่น ฤดูกาล ลักษณะการใช้ที่ดิน และจุดที่ทำการเก็บตัวอย่าง ในฤดูแล้งอัตราการออกซิไดซ์ก๊าซมีเทนจะสูงกว่าในฤดูฝน นอกจากนี้ในฤดูฝนบางครั้งยังมีการปลดปล่อยก๊าซมีเทนออกมา แสดงว่าความชื้นของดินเป็นปัจจัยหลักอันหนึ่งในการควบคุมอัตราการออกซิไดซ์ก๊าซมีเทน จากการเปรียบเทียบ พบว่าการออกซิไดซ์ก๊าซมีเทนในป่าธรรมชาติและป่าปลูกในประเทศไทยอยู่ในระดับที่พบในดินแถบอบอุ่น จากค่าเฉลี่ยต่อปีพบว่า ดินป่าเป็นแหล่งดูดกลับก๊าซมีเทนสุทธิ (1.50 และ 1.17 มิลลิกรัมต่อตารางเมตรต่อวัน ในป่าธรรมชาติและป่าปลูก ตามลำดับ) สำหรับในดินที่ใช้ทำการเกษตร การปลดปล่อยก๊าซมีเทนโดยเฉพาะในช่วงฤดูฝนทำให้เฉลี่ยทั้งปีดินนี้เป็นแหล่งปลดปล่อยสุทธิ

ในดินป่าทั้งสองแห่ง พบว่าชั้นดินระหว่าง 15-40 ซม. เป็นชั้นที่มีอัตราการออกซิไดซ์มีเทนสูงสุด ในขณะที่ในดินที่ใช้ทำการเกษตร ความแตกต่างในการออกซิไดซ์มีเทนตามความลึกไม่ชัดเจนนัก ความเข้มข้นที่สูงของไนเตรท ไนโตรเจนและแอมโมเนียในดินชั้นบนอาจมีผลยับยั้งการเจริญของแบคทีเรียที่ออกซิไดซ์มีเทน ทำให้ไม่พบการออกซิไดซ์ในดินชั้นบนนี้ จากการศึกษาทางจุลศาสตร์ของการออกซิไดซ์มีเทนพบว่าดินป่าธรรมชาติมีความจำเพาะต่อมีเทนสูง (ค่า $K_m = 52$ ppmv) แต่ศักยภาพในการออกซิไดซ์ต่ำ (ค่า $V_{max} = 0.82$ นาโนโมลต่อกรัมดินต่อชั่วโมง) ในขณะที่ดินจากป่าปลูกมีความจำเพาะต่อมีเทนสูงรองลงมา ($K_m = 723$ ppmv) และดินจากพื้นที่เกษตรมีความจำเพาะต่อมีเทนต่ำสุด ($K_m = 1454-2362$ ppmv) ฉะนั้น ดินป่าสามารถออกซิไดซ์มีเทนที่ความเข้มข้นต่ำได้ดีกว่าดินที่ใช้ทำการเกษตร ลักษณะทางจุลศาสตร์นี้สัมพันธ์กับลักษณะโครงสร้างประชากรของแบคทีเรียในดิน กล่าวคือผลของ DGGE ชี้ว่าโครงสร้างประชากรของ methanotroph ในดินป่าทั้งสองแห่งมีความคล้ายคลึงกัน และแตกต่างดินที่ใช้ทำการเกษตร ทั้ง *Methylobacter* spp. และ *Methylocystis* spp. เป็นกลุ่มแบคทีเรียหลัก นอกจากนี้ ยังพบ methanotroph ที่จัดอยู่ใน cluster α (USC α) ซึ่งเชื่อว่าเป็นกลุ่มหลักที่สามารถออกซิไดซ์มีเทนที่ระดับความเข้มข้นตามธรรมชาติ ในดินป่าทั้งสองแห่ง และพบลำดับเบสของกลุ่มแบคทีเรียที่ยังไม่เคยพบมาก่อนที่อาจมีความสัมพันธ์กับแบคทีเรียในกลุ่ม Gammaproteobacteria หรือกลุ่มอื่นๆที่มียีนควบคุมการทำงานของเอนไซม์มีเทนโมโนออกซิจีเนส ของแบคทีเรียที่ออกซิไดซ์แอมโมเนีย จากผลที่กล่าวมาแสดงให้เห็นว่า นอกจากปัจจัยแวดล้อมเช่นความชื้นแล้ว ความแตกต่างด้านโครงสร้างประชากรของ methanotroph ที่พบในดินที่มีการใช้ประโยชน์ต่างกัน ก็เป็นเหตุผลที่สำคัญประการหนึ่ง อันนำไปสู่ความแตกต่างในศักยภาพและจุลศาสตร์การออกซิไดซ์มีเทนดังที่กล่าวมาแล้ว

ผลการศึกษาครั้งนี้ แสดงให้เห็นว่าลักษณะการใช้ประโยชน์ที่ดินมีผลอย่างมากต่ออัตราการออกซิไดซ์มีเทน การเปลี่ยนจากป่าไปเป็นพื้นที่เกษตรส่งผลให้ดินสูญเสียคุณสมบัติการออกซิไดซ์ก๊าซมีเทน อย่างไรก็ตาม การปลูกป่าก็ช่วยทำให้ดินกลับมาออกซิไดซ์มีเทนได้อีกครั้ง การสูญเสียความสามารถในการออกซิไดซ์ก๊าซมีเทนนี้มีผลมาจากการเปลี่ยนแปลงโครงสร้างประชากรของ methanotroph และคุณสมบัติทางจุลศาสตร์ของการออกซิไดซ์มีเทนของแบคทีเรียเหล่านั้น จากการประมาณการในเบื้องต้นพบว่า ดินป่าไม้ในประเทศไทยมีศักยภาพสูงในการออกซิไดซ์มีเทนที่ความเข้มข้นระดับธรรมชาติ การช่วยรักษาพื้นที่ป่าจึงมีความสำคัญในการรักษาลักษณะที่เป็นแหล่งดูดกลับมีเทนสุทธิของป่าเอาไว้

คำหลัก: มีเทนออกซิเดชัน ดินป่าไม้ในประเทศไทย การเปลี่ยนแปลงการใช้ประโยชน์ที่ดิน แบคทีเรียกลุ่ม methanotroph, DGGE

Abstract

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Methane is an important greenhouse gas and oxidation in soils represents a significant sink. In Thailand, estimate of methane emission is well established but there has been no estimate available for methane sink. In this study, methane oxidation fluxes were measured with the closed chamber method in different land use types; natural forest (dry evergreenforest; SK site), re-grown forest (*Acacia mangium*, AC site) and corn cultivation (CF site). Monthly *in situ* fluxes were monitored during January – December 2003 in Nakornratchasima Province, Northeast Thailand. Soil samples were taken for kinetic study in the laboratory and for analysis of methanotrophic bacteria (MB) community by denaturing gradient gel electrophoresis (DGGE) of *pmoA* gene fragments (encoding for a subunit of particulate methane monooxygenase) that were PCR-amplified from total soil DNA extracts. Results reveal that methane oxidation occurred in all land use types but oxidation rate varied according to season, land use types, and sampling spots. Both SK and AC forests showed the oxidation rates comparable to that found in temperate forests. High rate of methane oxidation was found during the summer months. In raining season, net methane emission was occasionally observed, indicating the importance of soil moisture as the controlling factor. On one-year average basis, soil at both SK and AC forests were the net methane sinks (1.50 and 1.17 mg CH₄ m⁻² day⁻¹, respectively). On the other hand, high methane emission during raining season made soil at CF site became a net methane source on annual average basis.

In SK and AC soil a clear zonation for active oxidation layer was between 15–40 cm while in CF soil no clear active layer was observed. This was possibly due to high concentration of inorganic nitrogen compounds (NO₃⁻, NO₂⁻ and NH₄⁺) in the topsoil that inhibited the activity of methane oxidizing bacteria. Examining kinetic coefficients of these active layers revealed that soil at SK site had low methanotrophic capacity (V_{max} of 0.82 nmol·g soil⁻¹·h⁻¹) but relatively high affinity for methane (K_m of 52 ppmv). Soil at AC and CF sites 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 while soil at SK site it was not. Difference in kinetic characteristics among these three sites is also reflected in structure of methanotrophic community. Cluster analysis based on the DGGE banding patterns indicated that the MB community of SK and AC sites were similar to each other but different from that of the CF 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). It is thus concluded that besides the environmental factors such as water content, different community composition of methanotrophic bacteria under different land use is one of the important factors leading to different in methanotrophic capacity and kinetic characteristics of methane oxidation.

In conclusion, the results obtained in this study indicate that land use change from forest to agriculture significantly reduces methanotrophic capacity of soils. However, conversion of agriculture back to forest and preserving the natural forest conditions can maintain high methane oxidation capacity. Loss of methanogenic capacity upon changing land use is associated with altering bacteria community and their characteristics of kinetic methane uptake. Preliminary estimate indicates that Thai forest soils have high capacity for atmospheric methane sink and thus maintaining forest area is important if their sink characteristics are to be preserved.

Keywords: Methane oxidation, Thai forest soil, Land use, Methanotroph, DGGE

TABLE OF CONTENT

CONTENT	PAGE
Summary (Thai)	i
Summary (English)	ii
CHAPTER 1 INTRODUCTION	
1.1 Background	1
1.2 Literature review	2
1.3 Objectives	6
CHAPTER 2 MATERIALS AND METHODS	
2.1 Study sites	7
2.2 Construction of chambers and chamber base	8
2.3 Gas sampling procedures	8
2.4 Calculation of methane flux	9
2.5 Chemical analysis	10
2.6 Kinetics of methane oxidation	12
2.7 Analysis of methanotrophic structure	12
CHAPTER 3 RESULTS AND ANALYSIS	
3.1 Results of soil analysis	21
3.2 <i>In situ</i> methane oxidation	26
3.3 In situ soil moisture	35
3.4 Soil temperature	39
3.5 Results from laboratory study	
3.5.1 Oxidation profile	40
3.5.2 Kinetic study	45
3.6 Molecular biology aspects of methanotroph	

3.6.1 DGGE	48
3.6.2 Amplification of the <i>pmoA</i> gene	48
3.6.3 Detected taxa in soils from different land use	49
3.6.4 Community composition as revealed by DGGE analysis	50

CHAPTER 4 GENERAL DISCUSSION AND COCLUSIONS

4.1 Effects of land use on methane oxidation	56
4.2 Role of forest in Thailand as methane sink	58
4.3 Conclusions	59

REFERENCES	61
-------------------	-----------

Lists of Figures

Figure	Page
1. Pathway of methane oxidation in methanotroph and ammonia oxidation in ammonia oxidizer, PQQ is pyrroquinoline quinone, X and XH_2 are the oxidized and reduced forms of an unknown electron donors (after Hütsch, 2001).	3
2. Effects of ammonium and nitrate-based fertilization on oxidation of CH_4 by soils (after Hütsch, 2001).	6
3. Map showing the study areas; 1) Sakaerat Forest Research Station, 2) Reforested areas under the management by Royal Forest Department, Ministry of Natural Resources and Environment, and 3) agricultural area in which corn plantation has been continuous for more than 16 years.	15
4. Setups for sampling the chamber headspace gas	16
5. Stainless steel chamber base used as the basement for chamber placement.	17
6. Chromatogram and peak identification of gas sample components. The GC operating conditions are as indicated in the text. The sample type shown here is the ambient air (1.7 ppmv CH_4 concentration) with injection volume of 5 mL.	18
7. Example of calibration curve for calculating CH_4 concentration in the samples.	19
8. Changes in bulk density along the soil depths and comparison among different land use. Error bars indicate S.D. of three soil core measurements.	21
9. Soil pH along soil depth at SK, AC and CF sites.	24
10. Nitrate (A), nitrite (B) and ammonium (C) contents along the soil profile in different land use.	25
11. The monthly average of methane oxidation rates in different land use types.	31

12. Variations in methane oxidation rates among ten chambers at SK site. (CH1 to CH10 designate Chamber No. 1 to Chamber No. 5). In some months, results from certain chambers are omitted due to lack of significant changes in headspace concentration of methane.	32
13. Variations in methane oxidation rate among chambers at AC site. (CH1 to CH5 designate Chamber No. 1 to Chamber No. 5). In some months, results from certain chambers are omitted due to lack of significant changes in headspace concentration of methane.	33
14. Variations in methane oxidation rate among chambers at CF site. (CH1 to CH5 designate Chamber No. 1 to Chamber No. 5). In some months, results from certain chambers are omitted due to lack of significant changes in headspace concentration of methane.	34
15. Soil moisture at 6 cm soil depth in different land use type. Each point is the average from 10 measurements and errors bars indicate S.D.	35
16. Temporal and spatial variations in soil moisture along soil profile at SK site.	36
17. Temporal and spatial variations in soil moisture along soil profile at AC site.	37
18. Temporal and spatial variations in soil moisture along soil profile at CF site.	37
19. Fig. 19 Relationship between soil moisture and methane oxidation rate at SK site (A) and AC site (B).	37
20. Seasonal variations in soil temperature at 2 and 5 cm and air temperature in 2003.	38
21. Oxidation of methane by different soil layers taken from SK site and incubated under ambient methane concentration.	41
22. Oxidation of methane by different soil layers taken from AC site and incubated under ambient methane concentration.	42
23. Oxidation of methane by different soil layers taken from CF site, incubated under 5-6 ppmv of methane.	43
24. Oxidation of methane along soil profile and in different land use type.	44
25. Kinetic plots for initial methane concentration vs methane	46

oxidation rate constant. 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.

26. Exponential plot of relationship between oxidation rate and methane concentration below approximately 350 ppmv. 47
27. DGGE bands obtained with the primer A6682 and mb661. 53
28. Phylogenic tree constructed from amino acid sequences of partial pmoA retrieved from native forest, forest-replanted (*A. magium*) 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. 54
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. 55

List of Tables

Table	Page
1 Geographical variations of CH ₄ oxidation rates under different field condition	20
2 Soil texture along the soil depth at three different land use types	23
3. Kinetic coefficients measured for methane oxidation by soil samples taken from different land use types	46
4. Detected taxa of methanotrophic bacteria in soils taken from different land use	53
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	59

CHAPTER 1

INTRODUCTION

1.1 Background

Although greenhouse gases (CO_2 , CH_4 , N_2O , O_3 and CFCs) make up less than 1% of the atmosphere (99.9% of the atmosphere consists of N_2 , O_2 and Ar), they play the important roles in global energy balance. The presence of these trace gases keeps the surface global temperature warm (through greenhouse effect phenomenon), thus allows lives to thrive. Living organisms especially human being, in turn, can interact and exert a strong influence on trace gas concentrations and their spatial and temporal fluctuations. In fact, increasing concentration of trace gases in the atmosphere over the past centuries has been closely related with increasing human population, energy consumption and other human activities. This continuously increasing concentration causes an additional greenhouse effect and eventually the undesirable global warming.

The two most important greenhouse gases are CO_2 and CH_4 . These two gases combined contribute approximately 70% of current global warming (Hütsch 2001). Annual increasing rate of CH_4 is about 1%, with current atmospheric concentration of 1.75 ppmv (Dlugokencky et al., 2003). The main sources of CH_4 include wetlands, rice paddies, livestock, landfills and biomass burning. All sources combined emit CH_4 about 515 Tg (10^{12} g) per year. On the other hand, the main CH_4 sinks are removal in the reaction initiated by hydroxyl radical in the troposphere (about 470 Tg per year), and removal by soils (30 Tg per year). Balancing the sources and sinks results in the atmospheric increase of about 32 Tg per year. Methane oxidation in aerobic soils is the only known net biological CH_4 sink, where CH_4 is oxidized to CO_2 and H_2O , or assimilated into microbial biomass. Thus, oxidation is environmentally beneficial, since CO_2 has much lower radiative absorbing potential when compared with CH_4 (32 times lower than CH_4). Although the soil sink strength for CH_4 is relatively small (30 Tg per year or about 6% of global methane sink), its absence would cause the atmospheric methane concentration to increase at approximately 1.5 times the current rate (Duxbury, 1994).

Thailand signed the United Nation Framework Convention on Climate Change (UNFCCC) at the United Nations Conference on the Environment and Development in Rio

de Janeiro on 12 June, 1992 and ratified the Convention in December 1994. The Convention became effective for Thailand in March 1995. As for the Non-Annex country, Thailand has committed to meet certain voluntary obligations. These include the issue such as; (1) submitting the national greenhouse gases inventory carried out with the support of UNFCCC to the Intergovernmental Panel on Climate Change (IPCC) and (2) cooperating with international community to reduce greenhouse gas emission based on its own capacity.

Current estimate of CH_4 emission from Thailand is 3.17 Tg (66.6 Tg CO_2 -equivalent or approximately 23.3% of total greenhouse gas emission in Thailand in 1994 (Ministry of Science, Technology and Environment, 2000). The main source (94%) is from agricultural sector such as rice paddies and livestock production. As mentioned above, methane is also taken by soil. Therefore, the net estimate of methane emission should take into account the sink strength. However, there is still no data on methane sink in Thailand. It is, therefore, important to study methane oxidation capacity in various potential ecosystems in Thailand. Knowing this will be possible when the data such as soil and land use classification are integrated with the results of methane oxidation measurements. Comparing the results from different ecosystems may be useful in term that we can know how and identify where to maintain and preserve methane oxidation capacity as the net CH_4 sink.

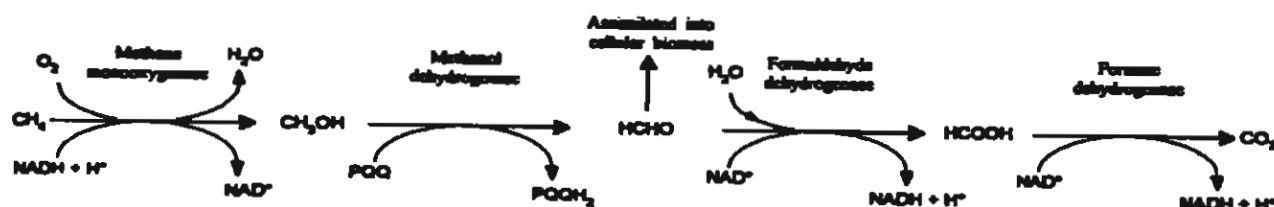
1.2. Literature Reviews

1.2.1 Basic processes involving biological CH_4 oxidation

In aerobic habitats, CH_4 is oxidized to CO_2 by the so-called methanotrophic bacteria (methanotrophs or CH_4 oxidizing bacteria, MOB, Conrad 1996; Hansan and Hanson, 1996). They are thus unique in their ability to utilize CH_4 as a sole carbon and energy source. These bacteria are ubiquitous in nature. The key enzyme involved in oxidizing CH_4 ; the methane monooxygenase, exhibits the remarkably low substrate specificity (especially in its soluble form, sMMO). Thus, MOB can use a wide range of substrates including those are xenophobic. The ability of MOB to oxidize ammonia is of ecologically significant because it serves as the linkage between carbon and nitrogen cycles in nature. The general pathways of methane and ammonia oxidation are given in Fig. 1. All methanotrophs are able to oxidize ammonia to nitrite, but the specific rate of

oxidation is much lower than that of ammonia oxidizers. Thus, it is not surprising that in the presence of ammonia, rate of methane oxidation by MOB is reduced.

Methane oxidation in methanotrophs



Ammonia oxidation in ammonia oxidizers

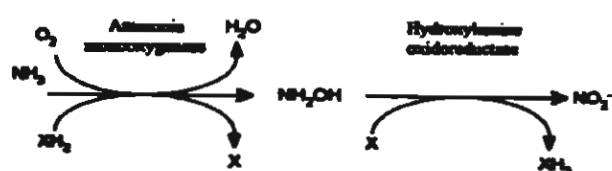


Fig. 1. Pathway of methane oxidation in methanotroph and ammonia oxidation in ammonia oxidizer, PQQ is pyrroquinoline quinone, X and XH_2 are the oxidized and reduced forms of an unknown electron donors (after Hütsch, 2001).

Methanotrophs are generally classified into two types according to their carbon assimilation pathways. Type I methanotrophs assimilate cell carbon entirely from CH_4 through the RuMP pathway while type II methanotrophs use the serine pathway to assimilate cell carbon. For type II methanotrophs, 50-70% of the cell carbon is derived from CH_4 , and the rest (30%) is from CO_2 (Hanson and Hanson 1996). The presence of methane monooxygenase (MMO) renders the molecular detection of methanotrophic community in natural samples possible. This can be achieved by targeting the gene encoding the MMO (*pmoA* gene, Henckel *et al.*, 2000). Analysis of *pmoA* gene libraries from forest soils has demonstrated the existence of a new group of methanotrophs (Henckel *et al.*, 2000, Homes *et al.*, 1995). Knowing the diversity of methanotrophs in various ecosystems is necessary if we want to understand the process of methanotrophy.

1.2.2 Factors affecting methane oxidation

Temperatures Most of culturable methanotrophs are mesophiles. However, oxidation of methane have been reported in wide range of temperature; ranging from about 0 °C in Arctic tundra (Whalen and Reeburgh, 1990; Reeburgh *et al.*, 1997, Dunfield *et al.*, 1993) to 35°C (King, 1992). In laboratory, some strains of methanotrophs are capable of growth up to 50°C. These data indicate that populations of methanotrophs can adapt to and maintain activity in the wide range of temperatures. The optimum temperature of methane oxidation in general is 25 °C.

Soil pH Oxidation of methane has been detected in a board range of pH, from acidic to neutral pH in most case. Born *et al.* (1990) reported high oxidation rate at pH values of 3.5 and 7.7 in sandy forest soils. Bender and Conrad (1995) observed CH₄ oxidation activity at pH as low as 2.3 in forest soil. In reviewing methane fluxes in central Europe (50 sites), Dörr *et al.* (1993) found no significant correlation between CH₄ uptake and pH. However, Brumme and Borken (1999) investigated seven forest ecosystems and concluded that there was a strong positive correlation between annual CH₄ oxidation and soil pH ($r^2 = 0.77$).

Nitrogen fertilization Ammonium fertilization inhibits methane oxidation both in field and laboratory study of pure culture of methanotrophs (Hanson and Hanson 1996). Thus, chemical fertilization is one of the key factors limiting CH₄ oxidation by soils. Mosier *et al.* (1991) were the first who reported the inhibition of CH₄ uptake by N fertilization. An annual fertilization of 22 kg N ha⁻¹ as NH₄NO₃ reduced CH₄ oxidation by 41% when compared to the unfertilized control. Hütsch (2001) pointed out that N fertilization has both long term and immediate effects on CH₄ uptake. Immediate effects involve the interference of NH₄⁺ with the methanotrophic enzyme system (MMO). Due to a low substrate specificity of MMO, methanotrophs metabolize NH₃ as soon as it is applied to the soil. Thus, NH₄⁺ acts as a competitive inhibitor of the methanotrophic bacteria and the effect was not fully reversible. However, it is noted that inhibition of CH₄ oxidation by NH₄⁺ is quite complicated. For example, in rice fields, landfills and perhaps in some soils, NH₄⁺ becomes limited for growth of MOB. In this case, CH₄ oxidation is stimulated by small amounts of NH₄⁺. CH₄ oxidation is not inhibited before a certain NH₄⁺ concentration

is reached; namely when $\text{NH}_4^+/\text{CH}_4$ ratio gets higher than a particular value (Conrad, personal communication).

The long-term effects result from repeated N applications in the long run cause changes in microbial ecology and probably a shift in microbial population (Adamsen and King, 1993). Hütsch *et al.* (1996) investigated the long-term effects of N fertilization in the “Broadbalk Wheat Experiment” at Rothamsted, UK. They found that the highest CH_4 oxidation rate was observed in the no nitrogen treatment, and the rates declined progressively for increases in mineral N application up to 144 kg N ha^{-1} per year. They concluded that CH_4 oxidation was greatly decreased in soils with a history of inorganic N application. On the other hand, Hütsch *et al.* (1994) and Willison *et al.* (1996) demonstrated that inhibition of methane oxidation by N fertilization was in fact due to the presence of NH_4^+ , but not NO_3^- . Fig. 2 shows an example of one of their results in the experiment receiving the same amount of N and similar pH. The CH_4 oxidation rates were 0.008 and $1.46 \text{ mg CH}_4 \text{ m}^{-2}$ per day in the presence of NH_4^+ and NO_3^- , respectively. They explained how the complete loss of CH_4 oxidizing ability after long-term NH_4^+ application that this could be due to a change in the kinetics of methanotrophic bacteria in relation to the threshold value. The threshold value is the CH_4 concentration below which no oxidation occurs and is a critical determinant for the total CH_4 uptake. Application of NH_4^+ for decades could have shifted the threshold to the much higher values.

Application of organic manure Hütsch *et al.* (1994; 1996) demonstrated that long term application of farmyard manure resulted in inhibition of CH_4 oxidation, although the effect was less strong than application of mineral N application. This can be explained by the fact that much of N especially in form of NH_4^+ can be absorbed in organic complexes, therefore, makes it only partly accessible to methanotrophs and other microorganisms.

Land use Changes in land use, especially cultivation of the formerly undisturbed soils, strongly reduced the soil's sink strength for atmospheric CH_4 (Keller *et al.* 1990, Ojima *et al.* 1993, Reay *et al.* 2001). Measurements of CH_4 uptakes usually reveal several times higher CH_4 uptake rates in the undisturbed forests than in regularly cultivated arable lands. Dobbie *et al.* (1996) found that mean reduction resulting from conversion of land to agriculture in Europe was 60%. This probably results from the disturbance of the original soil structure by cultivation (Conrad 1996, Bender and Conrad 1994). According to Conrad (1996), trace gas metabolism occurs in certain unites in aerobic soils, either

individual soil crumbs, non-aggregated soils, or sand grains covered by microbial films. Agricultural practices such as plowing of soils may easily destroy some of these “activity centers”.

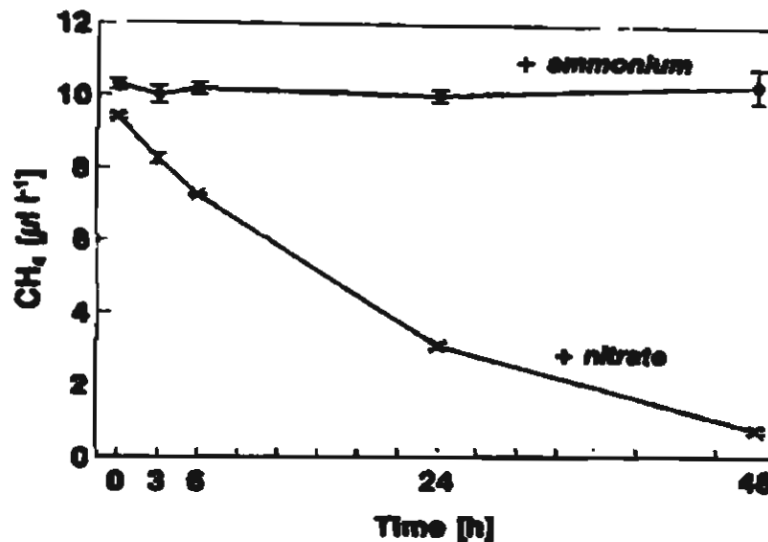


Fig. 2. Effects of ammonium and nitrate-based fertilization on oxidation of CH₄ by soils (after Hütsch, 2001).

1.3 Objectives

The objectives of current study are;

1. To quantitatively estimate methane oxidation capacity of forest and some selected arable lands in Thailand, thereby providing the preliminary information for adjusting emission inventory of the country.
2. To study the effect of agricultural practice that affects methane oxidation capacity.
3. To study the kinetics of methane oxidation under tropical Thailand ecosystem.
4. To investigate community structure and diversity of methanotrophic bacteria in Thai forest soil.
5. To know and compare geographical distribution of methane oxidation capacity and methanotrophic community structure.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study Sites

The survey of study sites was carried out on November 17, 2002. Besides checking the site of evergreen forest at Sakaerat Experimental Station, some areas nearby were also surveyed to use as reference site (agricultural area, Fig. 3). Accordingly, three sites with different land-use types were selected. Details for each site are described below.

Sakaerat Experimental Research Station (SK site) This was the primary sampling site in this project (Fig. 3). It is located in Amphur Wang Nham Keaw, Nakorn Ratchasima province and belonged to Ministry of Science and Technology. Majority of the area is made up of gentle slope mountains of between 10 and 30%. The average elevation is between 730 m and 760 m above the mean sea level. This experiment station was established in 1967, covering the area of 50 km². The sampling plots were selected in area of dry evergreen forest because it covers the majority (60%) of the forest area in this site. Under the monsoon climate, the annual temperature at the site is 26 °C with average annual rainfall of 1240 mm. Dry season in this area covers the period from December to February during which monthly rainfall is usually less than 50 mm (Sakurai *et al.*, 1998; Wacharakitti *et al.*, 1980) and wet season from mid of May until the end of September. Average relative humidity is around 75%. Soil at this site is classified as Phra Wihan formation of the Korat group. The soil is low in fertility by nature. Some physical and chemical characteristics of soils were also investigated in during the course of this project study.

The natural evergreen forest at SK site comprises of various native species (Sakurai *et al.*, 1995). Some of the dominant species include *Hopea ferrea*, *Pterocarpus macrocarpus* Kurz, *Xylia xylocarpar*, *Dalbergia cochinchinensis*, *Lagerstroemia duppereana*, *Shorea henryana*.

Re-afforestation Research and Training Station (AC site) This site is maintained and managed by the Royal Forest Department, Ministry of Natural Resources and Environment. It is the forest restoration area after deforestation about 20 years ago, planted with the fast growing, and nitrogen fixing tree species such as: *Acacia mangium*,

Pterocarpus marcrocarpus, *Xylia xylocarpa*, *A. auriculaeformis*. In this study, the area planted to *A. mangium*, a nitrogen fixing and fast growing tree was selected. This *A. mangium* (age 16 years old) plantation is the main type of reforestation at the AC site.

Cornfield (CF site) The site is situated next to Re-afforestation Research and Training Station. This area was selected due to its proximity to other sites but with different land use type. Thus, all sites are located within the same climates and some other variables can be removed. Corn cultivation has been continuously practiced for more than 16 years after deforestation. In 2003 when this project study was initiated, soil preparation for corn plantation started in the mid of June and seeds of corn were sown on July 10, 2003. On this date, fertilizer (16-20-0) was also applied at the rate of 20 kg per Rai (1 Rai = 0.2 ha).

2.2 Construction of chambers and chamber base

Close-static chamber method was applied to quantify the rate of methane oxidation in this study. Chamber was made of a transparent-acrylic glass with the dimension of 30 cm x 30 cm x 15 cm (width x length x height). On top side of each chamber, two small holes were made; one for inserting thermometer in order to monitor the temperature during gas sampling and another for facilitating gas sampling by using a syringe and needle inserting through the silicon stopper (Fig. 4). Small capillary tube was inserted during air sampling to allow pressure equilibration. The chamber base was made of stainless steel and custom constructed with an upper trough that exactly fits the size of the chamber (Fig. 5). The bases were inserted into the sampling location at 10-15-cm deep into the soil and were remained in the same proximal and distal locations during the entire study period. Before using, all possible leakages were checked to ensure the complete air-seal during gas sampling. When gas samples were taken, the chamber was placed on the water-filled channel of this chamber base to prevent gas exchange between inside and outside of enclosure.

2.3 Gas sampling procedures

For the gas sampling in January and February 2003, ten gas samples at 0, 5, 10, 15, 20, 25, 30, 35, 45 and 60 min after the enclosure were taken. However, for the remaining

months, six gas samples at 0, 10, 20, 30, 45 and 60 min were collected, since oxidation was not rapid and six sampling points are considered enough to follow the decreasing concentration in the chamber headspace. For each sampling time, 25-30 mL of gas was taken from the headspace by using a 30-mL gas syringe and then transferred immediately to vials. The vials were prepared in the laboratory prior to the field trip. They were filled with the autoclaved-saturated NaCl solution. The gas sample taken from the chamber's headspace was injected into the vials to replace the NaCl solution. Together with gas collection, other environment parameters such as soil and air temperatures, soil moisture along soil depth to 1 m were also recorded. After being taken, the gas samples were kept in a cool box to maintain the stable temperature. Upon returning to the lab, the concentration of CH₄ was determined by GC as soon as possible following the procedures described below.

2.4 Calculation of methane flux

In situ biological methane oxidation rate was estimated from the first order rate constant multiplied by initial concentration of methane, according the equation 1 below;

$$F = k_1[\text{CH}_4] \quad (1)$$

where

F is methane oxidation rate (ppm CH₄ min⁻¹)

k_1 is the methane oxidation rate constant (min⁻¹)

$[\text{CH}_4]$ is the initial methane concentration in the chamber headspace (ppm)

The methane oxidation rate constant (k_1) was calculated from the plot between methane concentration and sampling time as shown equation 2.

$$\ln[\text{CH}_4]_t = -k_1 t + \ln[\text{CH}_4]_0 \quad (2)$$

where

$[\text{CH}_4]_t$ is the headspace methane concentration at time t (ppm)

$[\text{CH}_4]_0$ is the initial methane concentration in headspace (ppm)

t is sampling time (min)

k_1 is oxidation rate constant (min⁻¹).

When there is a significant correlation between increasing methane concentration and sampling time in the chamber headspace, methane emission rate was also determined. The flux (F) is calculated from the change of the methane concentration inside the chamber over sampling time as:

$$F = \rho(V/A)dC/dt \quad (3)$$

where

ρ is the density of methane at pressure and temperature inside the chamber (mg m^{-3})

V is the headspace volume of the chamber

A is the area of surface that cover by the chamber

dC/dt is the increase of methane concentration inside the chamber as a function of time determined from linear regression (ppm min^{-1}).

2.5 Chemical analysis

Gas Chromatograph (GC) setup A Shimadzu Gas Chromatograph (Model 14B) equipped with FID detector and Methanizer was used to measure CH_4 in the present study. The GC operating conditions were: 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 . After being separated by column, CO and CO_2 in the samples were converted to CH_4 by the Methanizer and thus enabling them detectable by FID. With the Methanizer and Unibead C column, this GC setup can detect three main gas components in the samples: CO, CH_4 and CO_2 (Fig. 6) with retention time at 3.6 min, 7.0 min and 13.9 min, respectively. When determining CH_4 concentration for each set of measurement, new calibration curve was made. The example of typical calibration curve is given in Fig. 7.

Soil texture Soil texture was determined by using hydrometer method (Bouyoucous Particle Size Analysis) on air-dried soils that were passed through a 2-mm sieve to remove small rock, roots, pebbles, and debris followed by wet sieving to separate sand fraction. Sand, silt, and clay were expressed as a percentage of oven dry weight. This method determines the physical proportions of three sizes of primary soil particles as determined by their settling rates in an aqueous solution using a hydrometer. The hydrometer method

of estimating particle size analysis (sand, silt and clay content) is based on the dispersion of soil aggregates using a sodium hexametaphosphate (calgon: $(\text{NaPO}_3)_6$) solution and subsequent measurement based on changes in suspension density (Gee et al., 1982).

Soil bulk density Determinations of bulk density were done by measurement of soil volume and weight. Soil bulk density is the ratio of the mass of dry solids to the bulk volume of the soil (Blake and Hartge, 1986). The bulk volume includes the volume of the solids and of the pore space. The termination of bulk density consists of drying (105°C) at less than 48 hours and weighing a soil sample, the volume of which is known (core method) or must be determined.

Soil moisture Soil moisture content was measured by using the automatic soil moisture sensor; Theta Probe type ML2x (Eijkelkamp, The Netherlands) for measuring soil moisture in the soil surface (at 6 cm from soil surface) and Profile Probe type PR1 for vertical soil moisture profile. A single probe can measure soil moisture at 6 different depths down to 1 meter. Moisture meter displays soil moisture in % water by volume, included is internal calibration for mineral and organic soils.

Soil pH Soil pH was determined in the laboratory with a glass-electrode and pH meter (HACH sensIon378, USA) using 1:1 ration of air-dried and 2-mm sieved soil : deionized water.

Determination of nitrate, nitrite and ammonium content Dissolved inorganic nitrogen compounds (NO_3^- , NO_2^- and NH_4^+) 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. The flask was shaken on a shaker for 45 min and the supernatant was filtered through a $0.45\ \mu\text{m}$ cellulose acetate filter membrane. Concentrations of inorganic nitrogen in filtered liquid were then determined and expressed as mg of an inorganic nitrogen compound per kg of dry soil.

2.6 Kinetics of CH₄ oxidation

Soil samples were collected on July 27, 2003 in the proximity of the gas sampling sites for kinetic study. Firstly, oxidation activity at different soil depth was determined. 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 15-30% by volume. The vials were then closed with a rubber stopper and the headspace methane concentrations were adjusted to the range from ambient to about 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 (see Section 2.4). 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).

2.7 Analysis of methanotrophic structure

2.7.1 DNA extraction

DNA was extracted from 0.5 g of soil using the BIO 101 “FastDNA SPIN Kit for Soil” (Carlsbad, CA, USA) with modifications. Cell lysis was performed in a cell disruptor (model FP 120 FastPrep, Savant Instruments Inc., Farmingdale, NY, USA) for 2×30 s at 6.5 m s^{-1} . After centrifugation (10 min, $20,000 \times g$) the supernatant was collected and the soil bead mixture was re-suspended in kit-supplied buffers for a second extraction. Proteins and debris were precipitated as described by the manufacturer instructions and 1 ml of Binding Matrix was added. For purification, the matrix-bound DNA was washed 3 times with 1 ml of 5.5 M guanidine-thiocyanate solution. After the last centrifugation step (1 min, $14,000 \times g$) the supernatant was removed and the Binding Matrix was resuspended in 600 μl guanidine- thiocyanate solution. The Binding Matrix was loaded onto the kit-supplied SPINTM Filters and further steps were carried out as described by the manufacturer instructions. The final elution of the DNA was performed twice with 100 μl

of DNase free water. DNA was further purified and concentrated with PVPP and the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany (Knief *et al.* 2003).

2.7.2 Amplification of functional marker genes

A partial fragment of *pmoA* was amplified with the primer sets A189f / A682b (these primer sets amplify both *pmoA* and *amoA* gene, McDonald and Murrell, 1997; Knief *et al.*, 2003) and A189f / mb661 (these amplify only *pmoA* gens, Costello and Lidstrom, 1999). The *pmoA* encodes part of the α subunit of pMMO which is present in all methanotrophs (except *Methylocella palustris* and *Methylocella silvestris*). Targeting *pmmoA* of pMMO rather than the gene encoding sMMO is advantageous, since only a few sequences are available and sMMO is presents only in some methanotrophs. For denaturing gradient gel electrophoresis (DGGE) a GC-clamp was attached to the 5' end of the A189f primer (Henckel *et al.*, 1999). A touchdown PCR program (Henckel *et al.*, 1999) with decreasing annealing temperatures from 64°C to 57°C was applied with 35 cycles. A fragment of the *mmoX* gene, encoding the active-site subunit of soluble methane monooxygenase (sMMO), was amplified using a newly designed forward primer (*mmoX* f945: 5'-TGGGGYGNAATCTGGAT-'3) with a GC-clamp published by Iwamoto *et al.* (2000) and the reverse primer of Auman *et al.* (2000). A touchdown PCR program with decreasing annealing temperatures from 63°C – 56°C within the first 15 cycles and 20 cycles with an annealing temperature of 56°C was applied. It consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 63°C – 56°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 7 min. The PCR reactions had a total volume of 50 μ l and contained 5 μ l of Accutaq-LA 10 $\times$ buffer (Sigma-Aldrich, Steinheim, Germany), 0.5 μ g μ l⁻¹ BSA (Roche Diagnostics, Mannheim, Germany), 3.5 mM MgCl₂, 200 μ M of each dNTP (Promega, Mannheim, Germany), 1 μ M of each primer, 0.05 U μ l⁻¹ REDAccuTaq LA DNA Polymerase (Sigma), and 1 μ l of template DNA. PCR reactions were performed on a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, Weiterstadt, Germany) or a Primus 96 Cycler (MWG Biotech, Ebersberg, Germany).

2.7.3 DGGE and Sequencing of excised DGGE-bands

Separation of mixed PCR products was done by DGGE as described previously (Henckel *et al.*, 1999; Knief *et al.*, 2003). 40 µl of PCR products were loaded onto the gel. Visible bands were excised, DNA was reamplified by PCR, and PCR products were purified for sequencing (Knief *et al.*, 2003). Reamplification of some bands lead to non-specific PCR products, which appeared in the agarose gel as a second band or were visible after sequencing. The former problem was solved by cutting the *pmoA* PCR product out of the agarose gel (Knief *et al.* 2003). Otherwise, PCR products of were separated by cloning using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). The *pmoA* PCR product of positive clones was sequenced. For some bands, primers mb661 and A650 (Bourne *et al.* 2001) were used for reamplification. These primers are more specific for *pmoA* and thus prevent amplification of non-*pmoA* targets.

2.7.4 Phylogenetic analysis

Phylogenetic tree reconstructions, based on deduced amino acid sequences of partial *pmoA* and *amoA* sequences, were performed using the ARB software package (Strunk *et al.*, 1998). Original tree construction included sequences of all DGGE bands, all available *pmoA* sequences in the GenBank database (www.ncbi.nlm.nih.gov/GenBank, 15 December 2003), and selected public-domain *amoA* sequences. A selection was then made of 24 representative sequences from this study plus 42 public-domain sequences. The presented tree was calculated based on 130 deduced amino acid positions using a neighbor-joining algorithm with a Kimura correction. Bootstrap values were generated by 1000 data reassemblings. The general tree topology was confirmed by a maximum-likelihood tree.

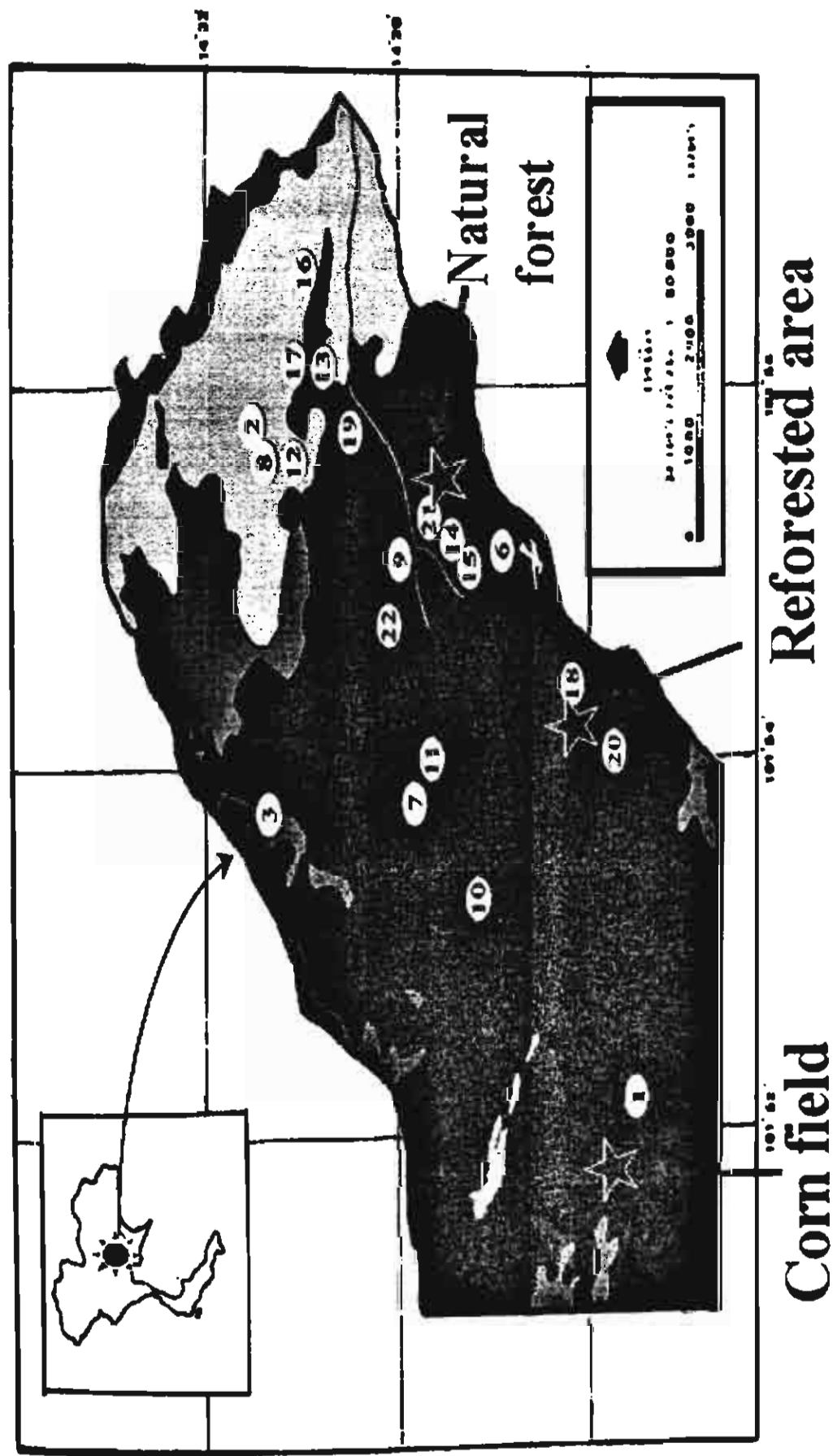


Fig. 3 Map showing the study areas; 1) Sakaerat Forest Research Station, 2) Reforested areas under the management by Royal Forest Department, Ministry of Natural Resources and Environment, and 3) agricultural area in which corn plantation has been continuous for more than 16 years.

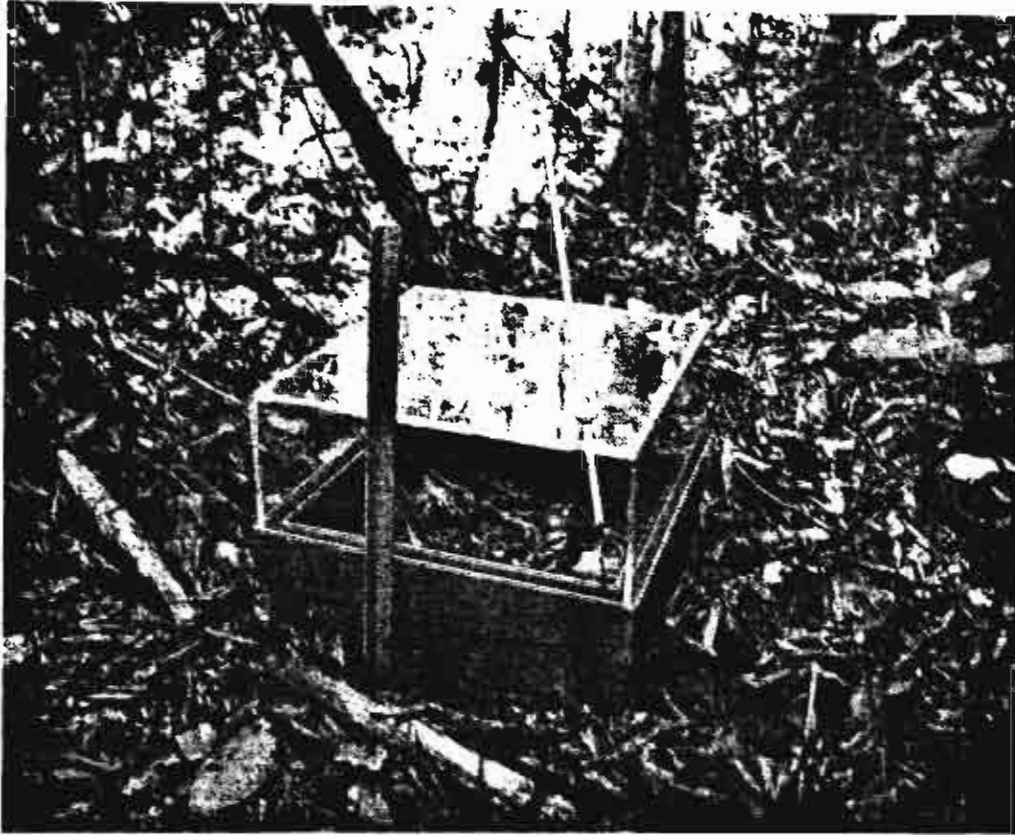


Fig. 4 Setups for sampling the chamber headspace gas.

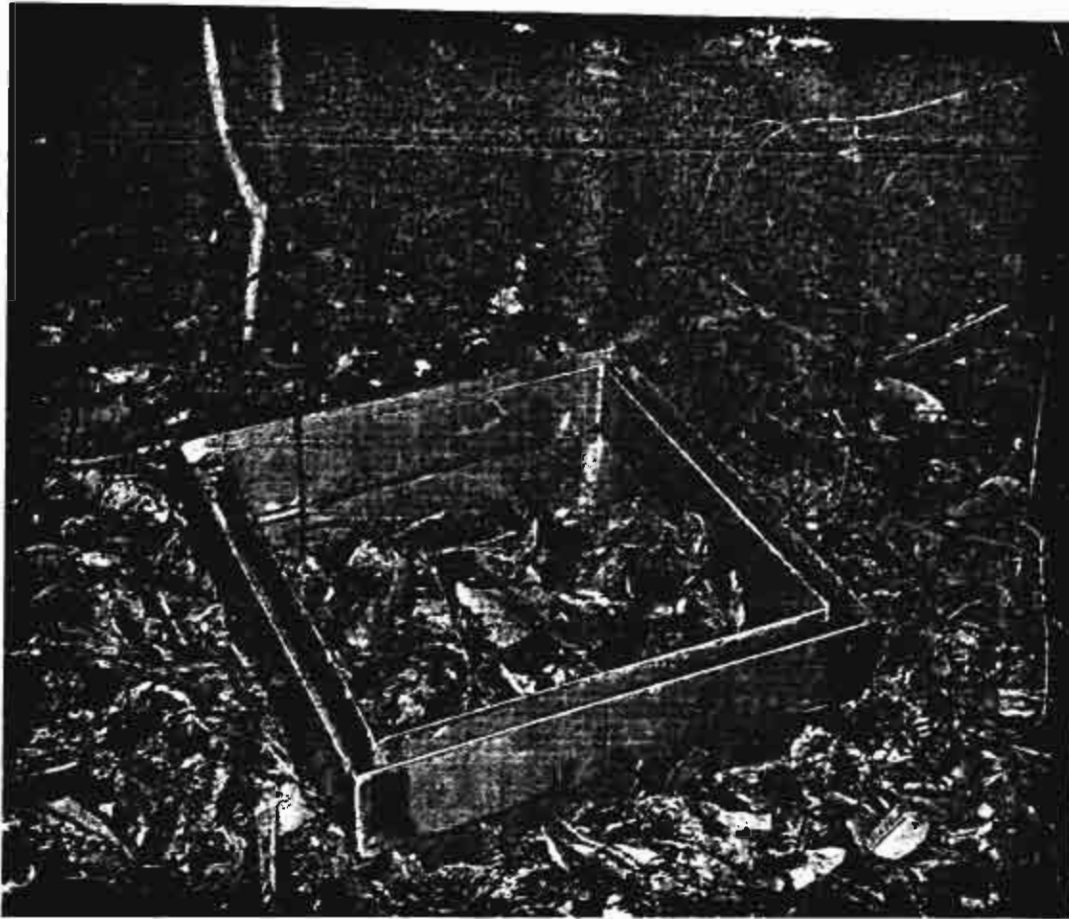


Fig. 5 Stainless steel chamber base used as the basement for chamber placement.

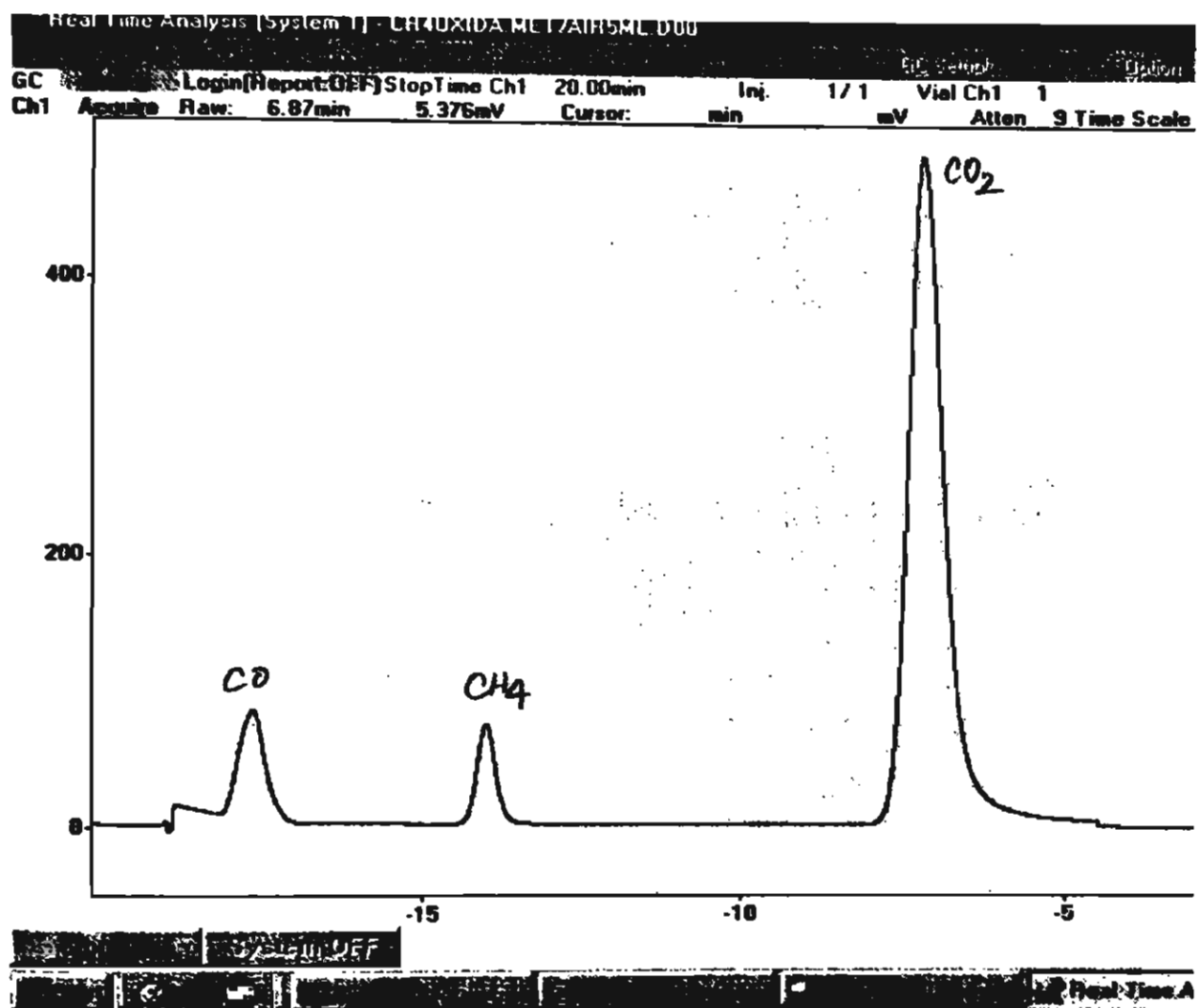


Fig. 6 Chromatogram and peak identification of gas sample components. The GC operating conditions are as indicated in the text. The sample type shown here is the ambient air (1.7 ppmv CH₄ concentration) with injection volume of 5 mL.

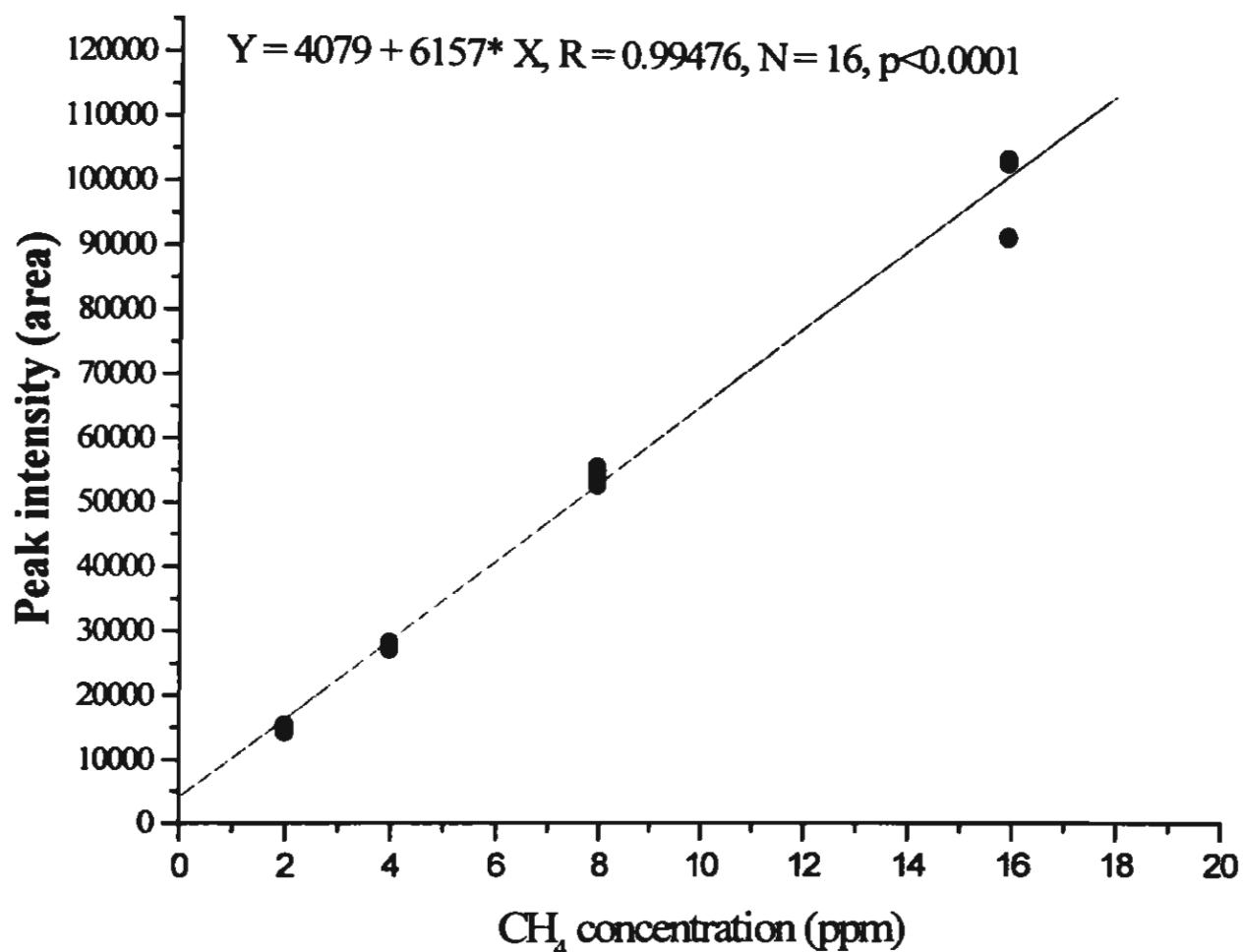


Fig. 7 Example of calibration curve for calculating CH₄ concentration in the samples.

Table 1 Geographical variations of CH₄ oxidation rates under different field condition (modified from Hütsch, 2001 and Ishizuka et al., 2000).

Location	Methane oxidation rate (mg CH ₄ m ⁻² per day)			References
	Arable	Grassland	Forest	
North America				
USA	0.17	0.35		Mosier et al., 1991
	0.03-0.20	0.35-0.84		Mosier and Shimel, 1991
			3.20-4.16	Steudler et al., 1989
			3.84-5.44	Castro et at., 1994
	0.91-1.03	1.16		Kassavalou et al., 1998
Canada			0.8-6.4	Castro et al., 1995
			2.1-6.9	Goldmann et al., 1995
	0-0.13		0.04-1.10	Lessard et al., 1994
			2.7	Adamsen and King, 1993
Central America				
Costa Rica			0.3-2.3	Keller and Reiners, 1994
Europe				
Denmark	0.04-0.08	0.03-0.25	0.14-0.33	Ambus and Christensen, 1995
	0.2		0.7	Dobbie et al., 1995
Germany			0.12-0.96	Butterbach-Bahl et al., 1998
	0.04-0.05			Ruser et al., 1998
	0.11			Flessa et al., 1996
			0.03-0.68	Brumme and Borken, 1999
	0.01-0.55		0.25-3.66	Born et al., 1990
UK	0-0.62		0-1.79	Koschorreck and Conrad, 1993
			2.19-2.97	Dobbie and Smith, 1996
			0.80-1.4	Sitaula et al., 1995
			0.86-1.06	Macdonald et al., 1996
			1.4	Dobbie et al., 1996
Norway			0.19-0.36	Macdonald et al., 1997
Scotland	0.02-0.12	0.85		Dobbie et al., 1996
	0.7			
Poland	0.2			Dobbie at al., 1996
Asia-Pacific				
New Zealand			0.04-0.05	Van der Weerden et al.,
1999				
India			6.12-17.0	Singh et al., 1997
Japan			0.69-10.7	Ishizuka et al., 2000

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Results of Soil Analysis

3.1.1 Soil bulk density

Bulk density of a natural forest, reforestation and corn cultivation soils is shown in Fig. 8. In the natural forest, reforestation and corn cultivation, the values ranged from 1.46 to 2.01, 1.56 to 2.04 and 1.62 to 1.94 g cm^{-3} , respectively. The bulk density tended to increase when the soil depth increased. Except at depth 15-20 cm, bulk density in corn cultivation soil was higher than both natural and re-grown forests. Relatively higher bulk density in cornfield soil is possibly due to field preparation and other cultivation practices. This may affect methane oxidation through limitation of atmospheric methane and oxygen diffusion into the oxidation layers as described below (Section 3.2 and 3.3).

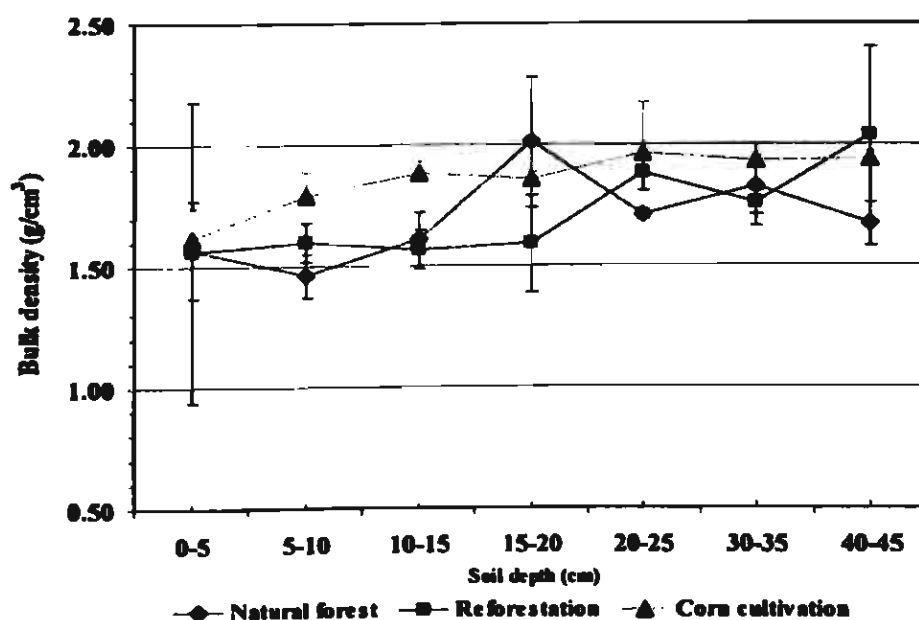


Fig. 8 Changes in bulk density along the soil depths and comparison among different land use. Error bars indicate S.D. of three soil core measurements.

3.1.2 Soil texture

Soil texture was determined based on the composition proportion of sand, silt and clay particles (Table 2). Throughout the 50-cm upper layer, soil at SK site contains more clay particle and soil at CF contains more sand particle than other sites. Thus, soil at SK is a clayey soil while at CF is a sandy soil. Soil texture at AC site comes in between SK and CF sites. Among them, the soils at 30-40 cm and 40-50 cm in natural forest show the highest clay content throughout the profile (60.08 %). Changes in soil particle composition and thus its texture after a long period conversion of natural forest soil to cultivated soil are possible under the intensive agriculture and high amount of rainfall (Phopinit and Limtrakul, 1999). This is due to the mixing of surface and subsurface soil during field preparations such as plowing. Such practices usually expose soil to leaching by rainfall. Clay particle is thus transported from upper to the deeper parts, resulting in an accumulation of clay particle as observed in this study (Table 2). On the other hand, the presence of forest and its dense network of roots and the litter layer in the forest floor prevent water transport of soil particle along the soil profile. Thus relatively large proportion of clay particle can be found near the soil surface when compared to the cultivated soils.

3.1.3 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. 9). The SK soil at surface was highly acidic with the pH of about 2.0 while at AC site the pH at surface is 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, land with corn plantation showed a different pH profile compared with forest soils. 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.

Table 2 Soil texture along the soil depth at three different land use types.

Site	Soil depth		Particle Size (%)			Texture
	(cm)		Sand	Clay	Silt	
CF	0-5		72.56	19.44	8.00	Sandy loam
	5-10		70.56	19.44	10.00	Sandy loam
	10-15		69.20	21.44	9.36	Sandy clay loam
	15-20		68.98	21.44	9.58	Sandy clay loam
	20-30		70.20	21.44	8.36	Sandy clay loam
	30-40		68.84	21.44	9.72	Sandy clay loam
	40-50		68.20	21.44	10.36	Sandy clay loam
SK	0-5		49.56	35.44	15.00	Sandy clay
	5-10		42.56	42.44	15.00	Clay
	10-15		37.98	47.44	14.58	Clay
	15-20		32.98	53.08	13.94	Clay
	20-30		32.56	57.08	10.36	Clay
	30-40		28.56	60.08	11.36	Clay
	40-50		30.56	60.08	9.36	Clay
AC	0-5		54.56	23.08	22.36	Sandy clay loam
	5-10		50.56	26.44	23.00	Sandy clay loam
	10-15		50.92	28.44	20.64	Sandy clay loam
	15-20		48.56	31.44	20.00	Sandy clay loam
	20-30		40.56	40.44	19.00	Clay
	30-40		39.56	44.44	16.00	Clay
	40-50		46.92	39.44	13.64	Clay

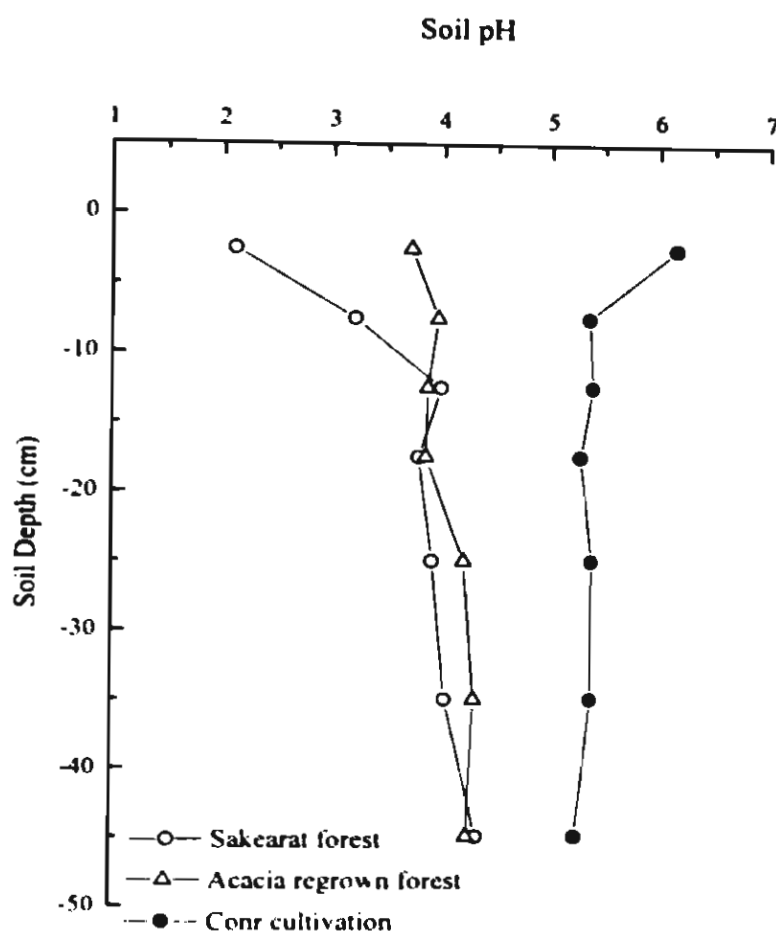


Fig. 9 Soil pH along soil depth at SK, AC and CF sites.

3.1.5 Inorganic N content

Inorganic N content (NO_2^- , NO_3^- and NH_4^+) of all soil was measured along the soil depth. Results are given in Fig. 10A-C. The top layer of natural forest soil showed the highest nitrate content (about $260 \text{ mg NO}_3^- \text{ kg soil}^{-1}$, Fig. 10A). High level of nitrate was detected down the profile to 10-15 cm and below this layer nitrate content was below $25 \text{ mg kg soil}^{-1}$. On the other hand, nitrate content throughout soil profile at both AC and CF sites was fairly constant around $50\text{--}75 \text{ mg kg soil}^{-1}$. 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. 10B). It was lower than $3 \text{ mg kg soil}^{-1}$ below 10 cm in SK site and below 15 cm in AC site. In contrast, 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. 10C. Similar to the trends found in nitrate content, natural forest soil at SK had the highest ammonium content. The highest concentration at SK was found in the top 10 cm surface layer (173 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 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 mg kg soil⁻¹ and it trended to increase in the deeper layers.

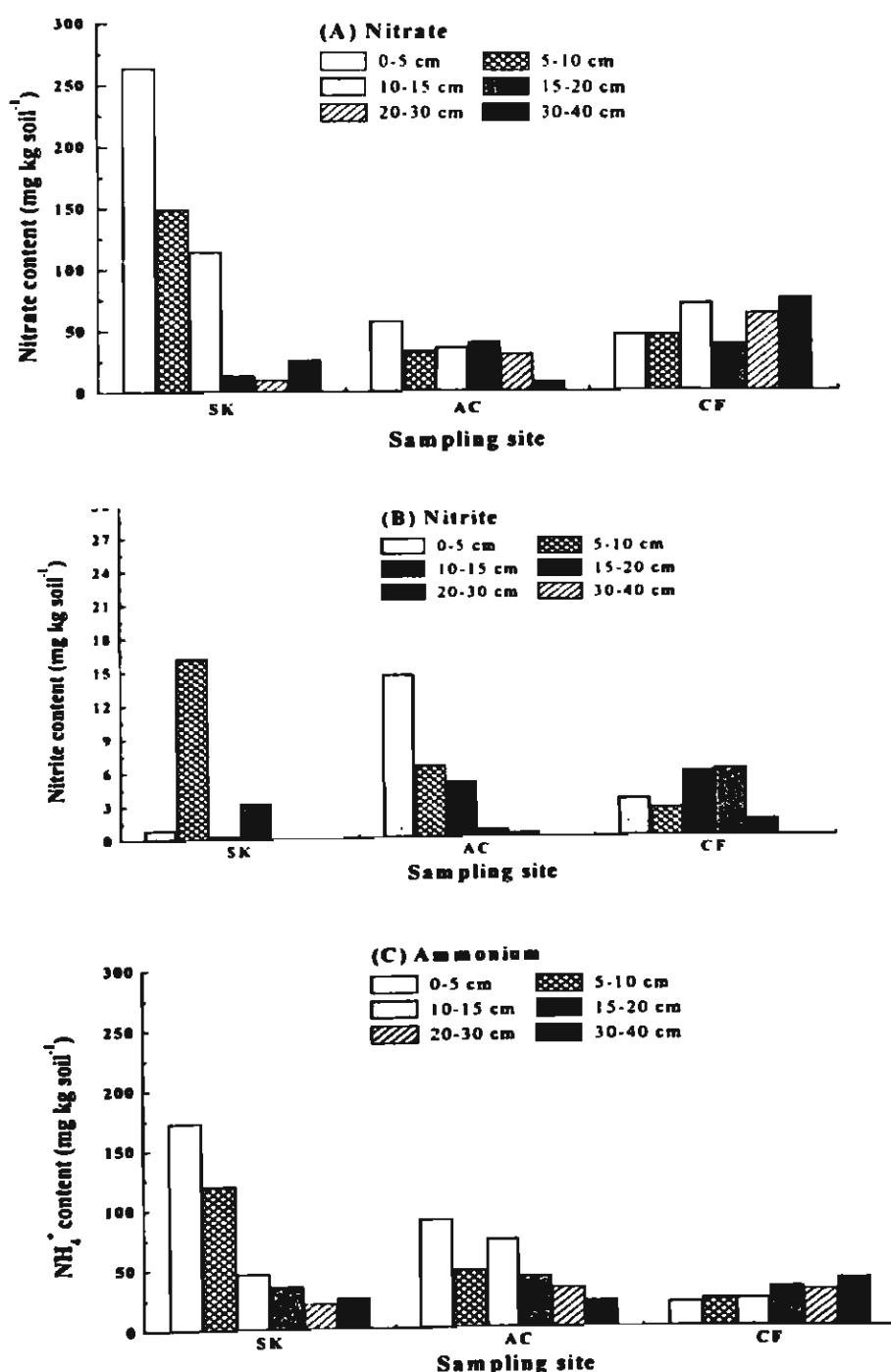


Fig. 10 Nitrate (A), nitrite (B) and ammonium (C) contents along the soil profile in different land use.

3.2 *In situ* CH₄ oxidation

3.2.1 *Seasonal variations of methane oxidation among different land use type*

Monthly methane flux was measured during January –December 2003. However there was no measurement made in September 2003. The results show that atmospheric methane oxidation varied both temporally and spatially. Type of land use strongly affects methane oxidation. On average over one year, the highest oxidation rate was observed in forest sites but rates were usually associated with large variations among chambers. Although the oxidation varies seasonally, at AC site net methane oxidation was observed consistently in almost all chambers measured. This is different from SK site where sometimes the positive methane flux (net methane emission) was observed. On the other hand, land use as corn plantation results in a drastically decrease in methane oxidation rate. Occasionally, net methane emission appeared, particularly when soil moisture content was high (see results below). Thus, soil at CF site soil could become either source or sink of methane depending largely on environmental conditions and cultivation practice such fertilization. Details of measurement results are described below.

a). Sakaerat forest site. At this site, ten chambers were used to monthly monitor the methane oxidation. However, not all chambers showed methanotrophic or methanogenic activity. Only those chambers showing a significant increase or decrease in headspace concentration over time were included in flux estimation. During January to December 2003 (no measurement was carried out in September), the highest average oxidation rate was found in February and November as 2.8 and 2.6 mg CH₄ m⁻² day⁻¹ respectively, Fig. 11). Except in June and July when net emission of methane was observed, forest soil acted as the net methane sink throughout the years. The relatively high oxidation rates appeared during the dry months and low rates were found during the raining season. The average value during one year measurements was 1.50 mg CH₄ m⁻² day⁻¹, indicating the fact that forest soil act as the net atmospheric methane sink although in some period of the year it may emit certain amount of methane.

It is noted that the rates of methane oxidation found in Thai forest soil are comparable to those found in other forests (Table 1). For example, methane oxidation rate of 0.3-2.3 mg CH₄ m⁻² day⁻¹ in Costa Rica (Keller and Reiners, 1994), 0.8-6.4 mg CH₄ m⁻² day⁻¹ in USA (Castro et al., 1995), 0.67-10.7 mg CH₄ m⁻² day⁻¹ in Japan (Ishizuka et al.,

2000) were reported. However, the rates obtained in the present study are usually higher than those measured in most European temperate forests. This indicates that soil under dry evergreen forest in Thailand possess a relatively high potential for oxidizing the atmospheric methane.

b). Reforested site (Acacia plant, AC site). Similar to the SK site, net methane consumption by AC forest was observed (Fig. 11). The rates of methane oxidation are similar to those observed for SK site, ranging from 0.5 to 2.1 mg CH₄ m⁻² day⁻¹. High oxidation rate was observed in dry season, the same trend observed in SK site. The highest methane oxidation rate was found in October (2.1 mg CH₄ m⁻² day⁻¹) followed by February (2.0 mg CH₄ m⁻² day⁻¹). Soil at AC site always showed the net methane sink throughout the year. The average oxidation rate of all months was 1.17 mg CH₄ m⁻² day⁻¹.

c). Agricultural site (Cornfield site, CF). CF soil acted as both methane sink and source throughout the year (Fig. 11). Even in dry months such as in January and February, net methane emission was observed. There was neither net methane emission nor consumption in March. However, CF soil turned to be the net methane sink during March to June. When cultivation of corn began in July, it became net methane source again and high amount of methane emission was observed in October, concomitant with heavy rainfall and sharply increased in soil water content (see below). Because of this emission peak, on the average basis throughout the measurement period the CF soil became a net methane source (+12.9 mg CH₄ m⁻² day⁻¹). Difference in emission or oxidation pattern in this site compared to forest soil site indicates that different factors may control methane production and oxidation. Even during the dry season, it seemed that some part of soil profile at CF site remained anoxic, allowing methanogenesis and emission of methane to occur as observed in the present study.

When the results of averaged oxidation rates from all of these three land-use types are compared, it was found that the oxidation capacity is in the order of Sakaerat forest (-1.50 mg CH₄ m⁻² day⁻¹) > Acacia reforest (-1.17 mg CH₄ m⁻² day⁻¹) > agricultural area (cornfield, +12.9 mg CH₄ m⁻² day⁻¹). Thus highly acidic pH in SK and AC soils (Fig. 9) is not the inhibitory to methane oxidation. On the other hand, soil texture (Table 2) which is one of the important parameters limiting gas diffusing seems not to relate much to the different oxidation rate among these three sites. The soils at SK and AC sites contain more

clay particle than at CF site but bulk density at SK and AC site is generally lower than at CF site, indicating that gas diffusion (O_2 and CH_4 from the atmosphere into soil layers) at SK and AC sites may be faster than at CF site. High bulk density at CF site may be resulted from soil cultivation practices.

Another factor that affects methane oxidation activity of soils is the presence of inorganic nitrogen species. Of these, ammonium and nitrite have been known for their strong effects on methane oxidation rates (Dunfield and Knowles, 1995). Ammonium acts as a competitive substrate for MMOs while nitrite is toxic to methanotrophic bacteria. Gullledge and Schimel (1998) showed that ammonium concentration of $94 \text{ mg kg soil}^{-1}$ (as Ammonium sulfate) inhibited methane oxidation by 67% in temperate hardwood forest. Nitrate becomes inhibitory to methane oxidation only at high concentration (usually $> 10 \text{ mM}$ dissolved nitrate, Adamsen and King, 1993). However, concentrations of all nitrogen species are 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 throughout the year 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 was 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 soil profile, 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. In addition, forms of ammonium present in the soil also have significant effects on methane oxidation. Gullledge and Schimel (1998) found in temperate hardwood forest soil that NH_4Cl inhibited methane oxidation by 88% while $(NH_4)_2SO_4$ only inhibited methane

oxidation by 67, compared to the control. Inputs of nitrogen through fertilization at CF site, thus, may lead to difference in nitrogen forms present in the CF soil from those found in SK and AC site. This may lead to inhibitory effects on methane oxidation although its concentration is not as high as that found in SK and AC soils.

Seasonal variations in oxidation found in all sites during the study period may be attributed mainly to changes in soil moisture. Soils with heavy texture such as SK and AC soils usually contain more water per soil weight than soil with light texture such as CF soil in the present study. However, it is likely that when moisture increases to certain level, soil texture plays only a minor role in regulating methane oxidation. Relatively low activity of methane oxidation observed in all sites during the raining months might have been caused by high soil moisture content (see Section 3.3). Difference in draining capacity of soil between at SK and CF might result in different in methane oxidation, especially in June. This is supported by the distinct soil texture between these two sites, especially in the top 20 cm.

From these results, some distinct characteristics of methane oxidation associated with different land use types can be concluded as followed;

1. methane oxidation capacity of soils is strongly affected by land use types and season-associated factors such as soil moisture and cultivation practices,
2. undisturbed forest has the highest atmospheric methane oxidation capacity,
3. methane oxidation capacity can be recovered by reforestation, but not yet fully the same level as in undisturbed forest,
4. land use change from forest to agriculture (corn plantation in this case) reduces methane oxidation activity significantly.

3.2.2 Spatial variations of methane oxidation

a). SK site. In this site although on average net methane consumption was observed but large spatial variations among chambers are noted (Fig. 12). For example, within each sampling time oxidation rate among all chambers could be as high as 86% above the average values (in February). However, sometimes such as in April the spatial variations of methane oxidation among chambers was as low as 13%. Similarly, if we follow the same chamber through out the study period, oxidation also varied seasonally. These results indicate that there are the true large variations in oxidation rates of methane in different locations of forest floor and this may be due to the heterogeneous nature of soils.

b). AC site. Methane oxidation rates among all five chambers at AC site are shown in Fig. 13. In contrast to SK site, except in July all chambers showed net methane consumption. However, variations in methane consumption rates among chamber are remarkable. For example, in January range of oxidation rate among five chambers is from 1 to about $2.7 \text{ mg CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ (Fig. 13) and in February is from about 2.7 to $3.7 \text{ mg CH}_4 \text{ m}^{-2} \text{ day}^{-1}$. Methane oxidation different among chambers was relatively small during March to June, and in August and November, respectively.

c). CF site. As expected, large variations among chambers was found at CF site. Almost in all cases during raining season net emission was observed. Net emission became obvious in July and this lasted until October. Net methane consumption in all chambers was found in only May, June and November. In all other occasions, at least one chamber showed the net methane emission (Fig. 14). It is noted that in October, three out of five chambers emitted more than $100 \text{ mg CH}_4 \text{ m}^{-2} \text{ day}^{-1}$. This was probably due high soil water content, especially below 40 cm where water content was always above 40% (Fig. 18).

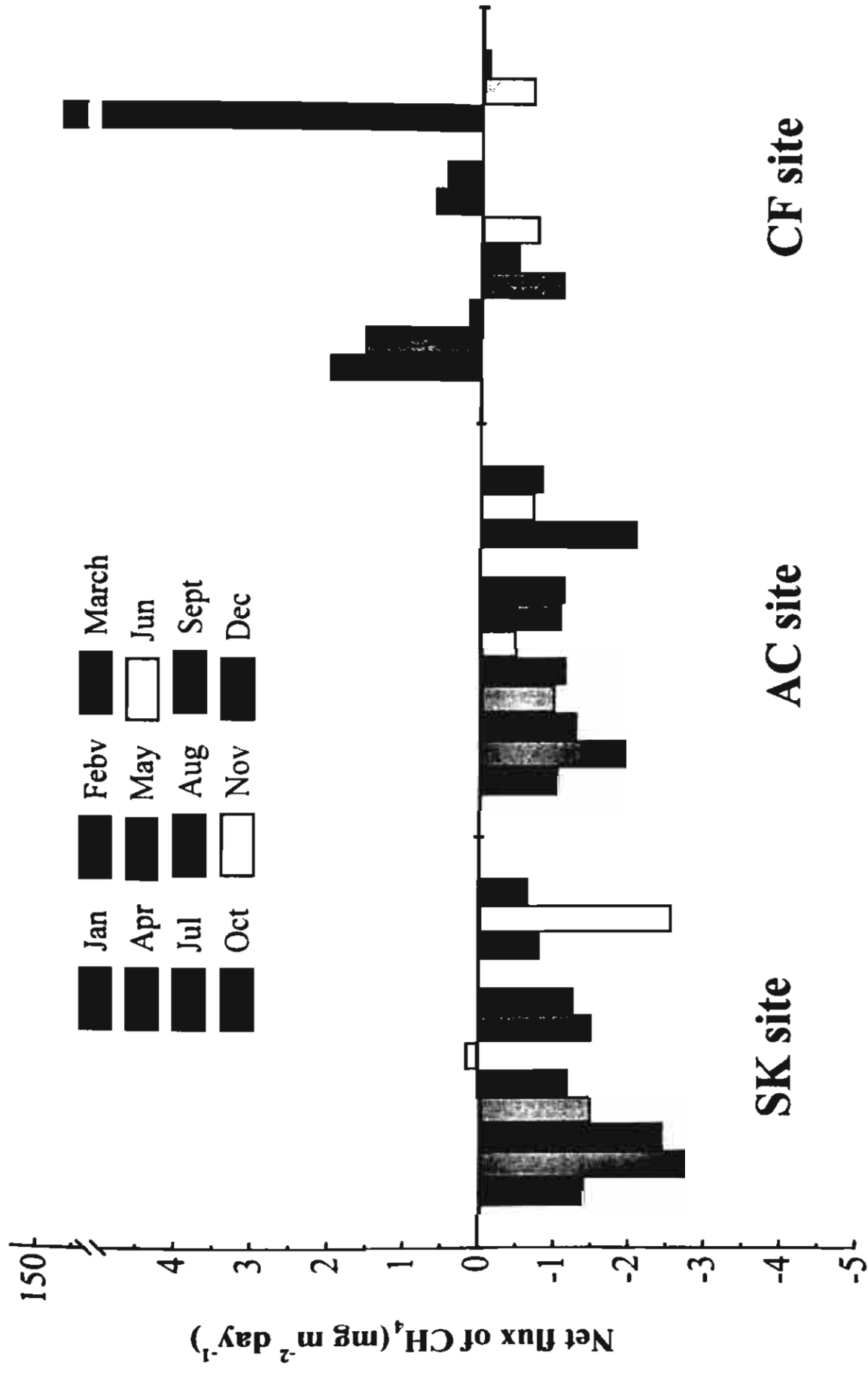


Fig. 11 The monthly average of methane oxidation rates in different land use types.

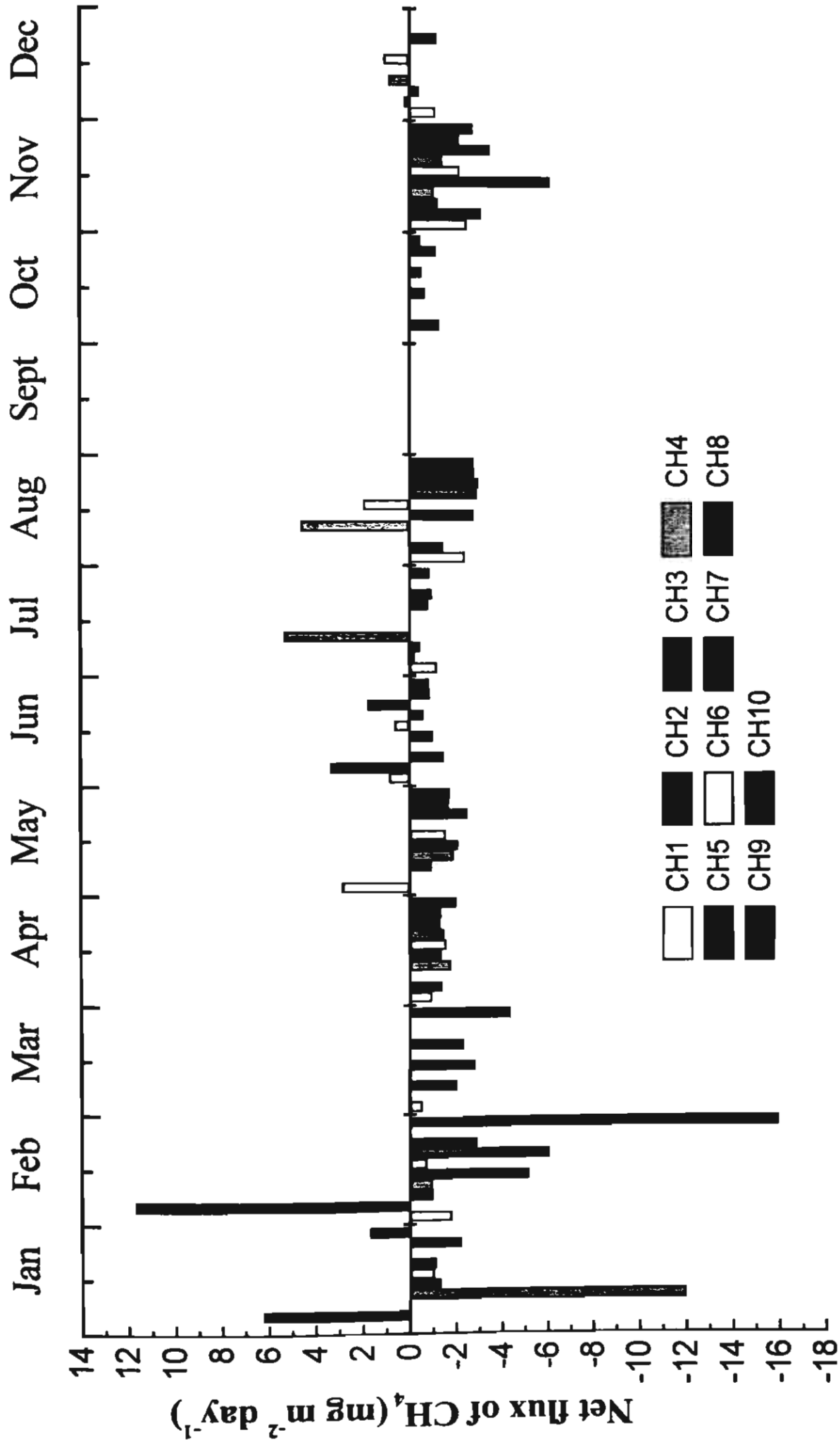


Fig. 12 Variations in methane oxidation rates among ten chambers at SK site. (CH1 to CH10 designate Chamber No. 1 to Chamber No.

5). In some months, results from certain chambers are omitted due to lack of significant changes in headspace concentration of methane.

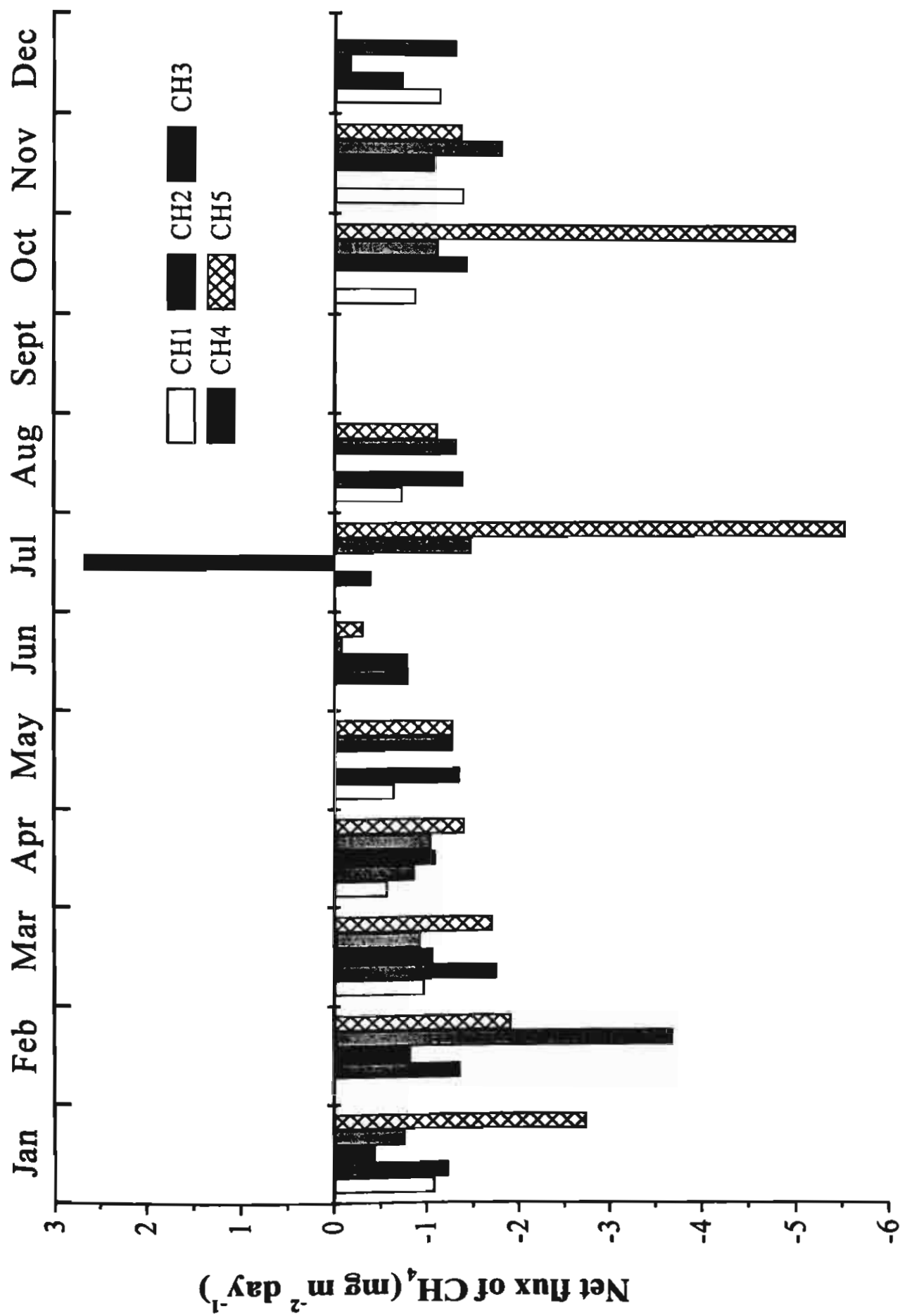


Fig. 13 Variations in methane oxidation rate among chambers at AC site. (CH1 to CH5 designate Chamber No. 1 to Chamber No. 5). In some months, results from certain chambers are omitted due to lack of significant changes in headspace concentration of methane.

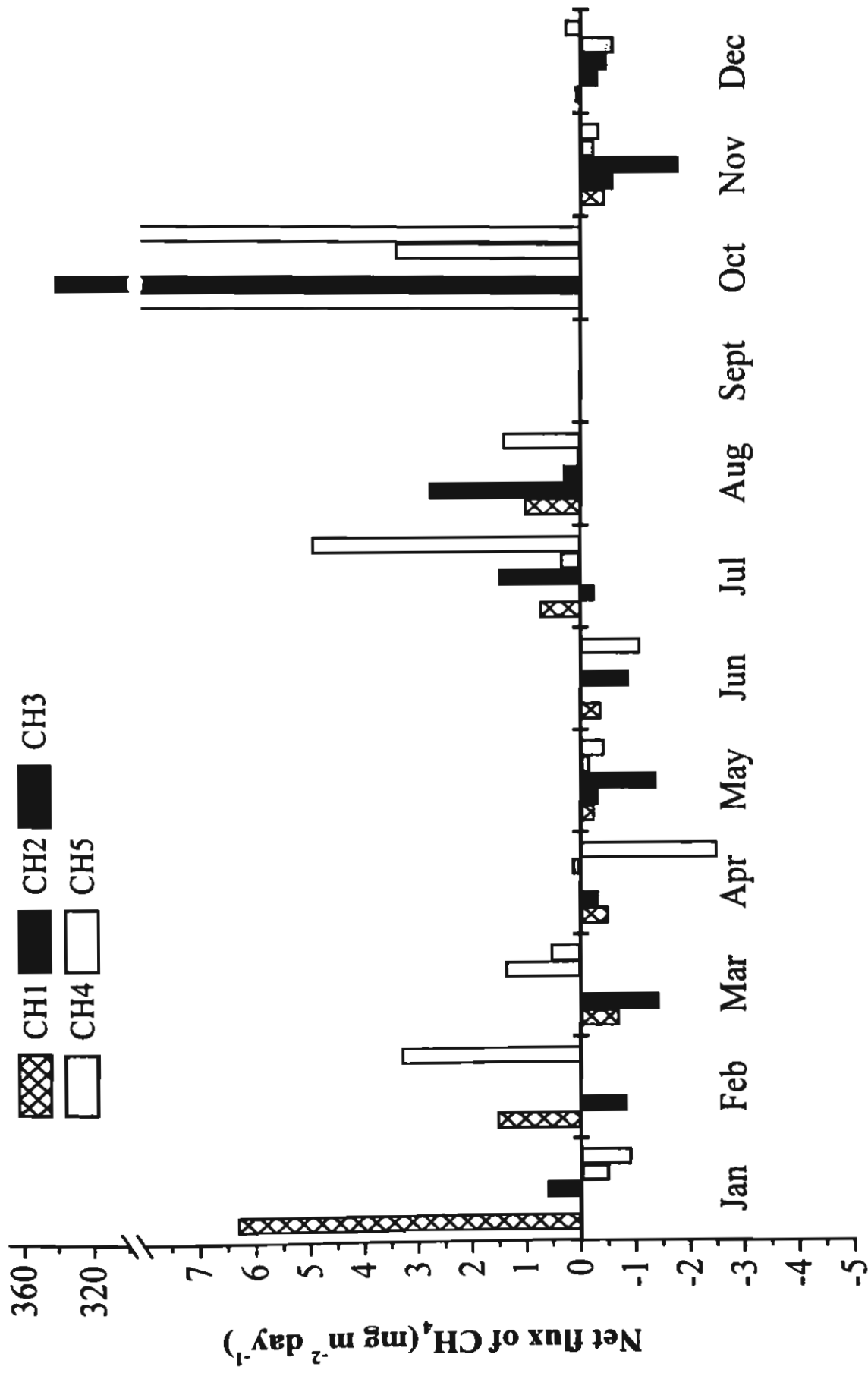


Fig. 14 Variations in methane oxidation rate among chambers at CF site. (CH1 to CH5 designate Chamber No. 1 to Chamber No. 5). In some months, results from certain chambers are omitted due to lack of significant changes in headspace concentration of methane.

3.3 *In situ* soil moisture

Soil moisture was measured during April 2003-Feb 2004. Earlier measurement was not carried out due to unavailability of instruments. Moisture was measure at 6 cm below soil surface and at every 10 cm after that down to 100 cm below the soil surface.

3.3.1 Soil moisture at 6 cm

At 6 cm depth, soil moisture in all sites ranged from about 4 to 30% by volume (of soil pore). Relatively higher seasonal variations were observed at CF site than in other sites. Almost in most cases, high soil moisture developed during July to October after the beginning of the raining season. Many occasions that soil moisture in CF site was below 10% or more than 20% while such were not usually observed in both AC and SK site.

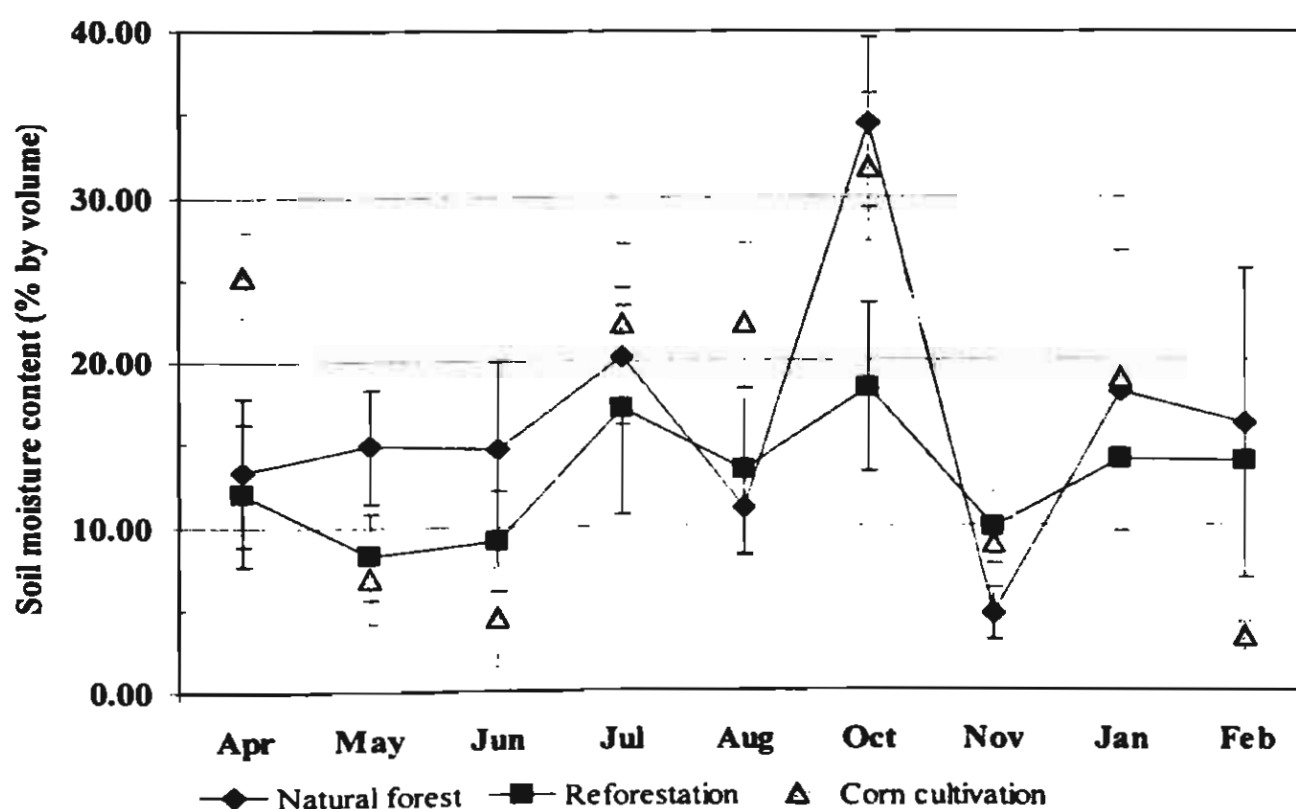


Fig. 15 Soil moisture at 6 cm soil depth in different land use type. Each point is the average from 10 measurements and errors bars indicate S.D.

3.3.2 Soil moisture along the soil profile

a) Sakaerat Forest (SK site). Except in October, soil moisture at this site stayed below 30% throughout the soil profile (Fig. 16). However, moisture tended to increase from January towards July and decreased again in August. It then increased and peaked in October when through the profile the moisture content was more than 80%.

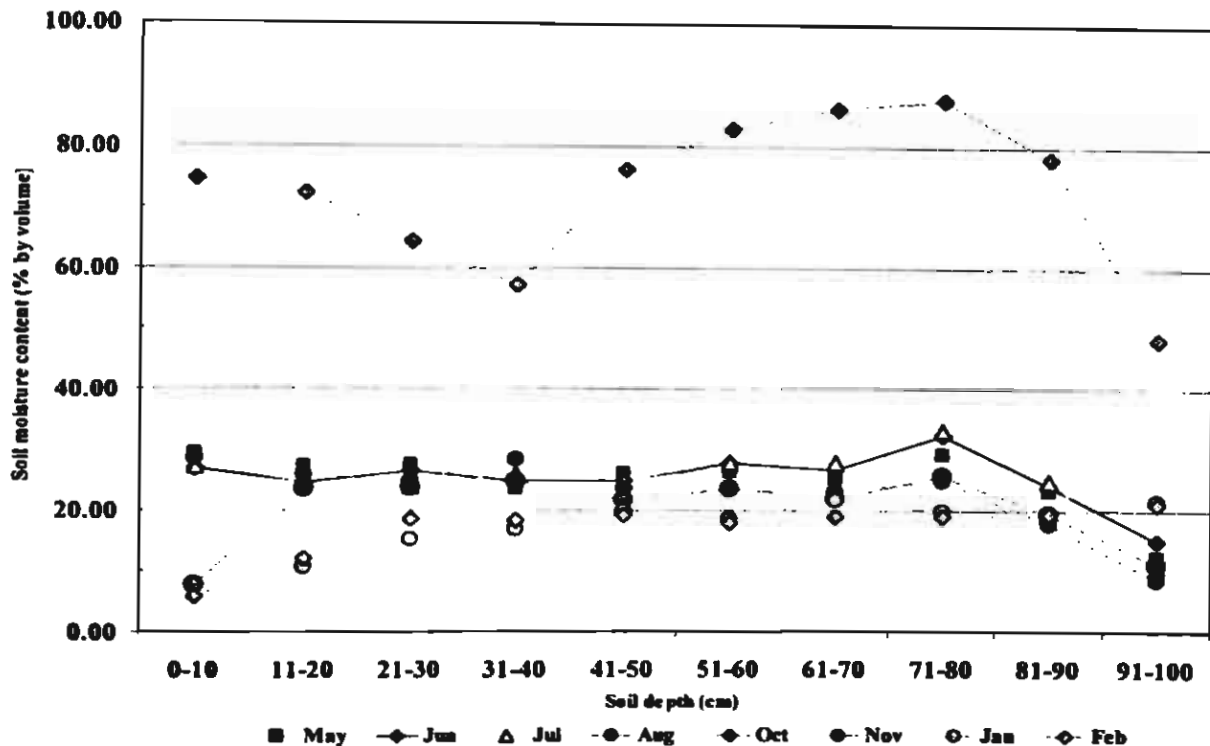


Fig. 16 Temporal and spatial variations in soil moisture along soil profile at SK site.

b) Acacia plantation (AC site). At AC site, the soil moisture profile was quite different from at SK site (Fig. 17). Soil moisture increases along with soil depth. In a given month, moisture at surface was always lowest throughout the soil profile. From all data available, moisture content in the soil profile at this site never exceeded 45%. This is in contrast with SK site where in October the soil was nearly saturated with water. Thus, the soil at AC site is well drained compared with other two sites. This may be the reason why this site acts as the net methane sink most of the time.

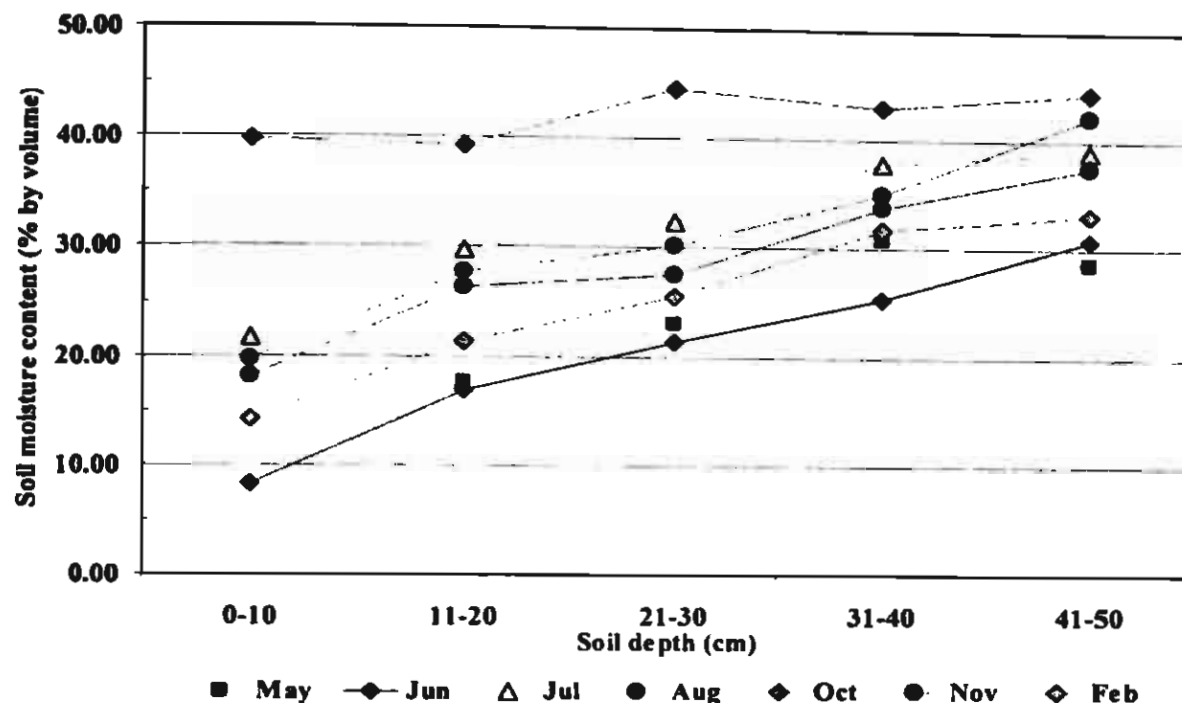


Fig. 17 Temporal and spatial variations in soil moisture along soil profile at AC site.

c) Corn cultivation (CF site). Similar to AC site, moisture at CF site increase along with soil depth. However, in the deeper layer (below 80 cm), soil moisture in every month was more than 40%. In October and November below 50 cm the moisture content was relatively high (above 60%). This high moisture content explains the net methane emission characteristic as mentioned above, possibly due through activation of methanogenesis.

3.3.3 Relationship between soil moisture and methane oxidation rate

Relationship between soil moisture at different depths and rate of methane oxidation measured on the soil surface was analyzed. At SK site, it seemed that oxidation rate decreased as water content at 0-10 cm and 20-30 cm increased from 5% to about 20%, and from 25-35%, respectively (Fig. 19A). There was no clear correlation at 10-20 and 30-40 cm soil depth in this site. On the other hand, at AC site in all soil depths, methane oxidation rate increased as water content increased from about 7% to 45% (Fig. 19B). No correlation between soil moisture and methane oxidation rate in all soil depth at CF soil

was observed. Thus, it seems that interplay between site characteristics and moisture content influences methane oxidation differently.

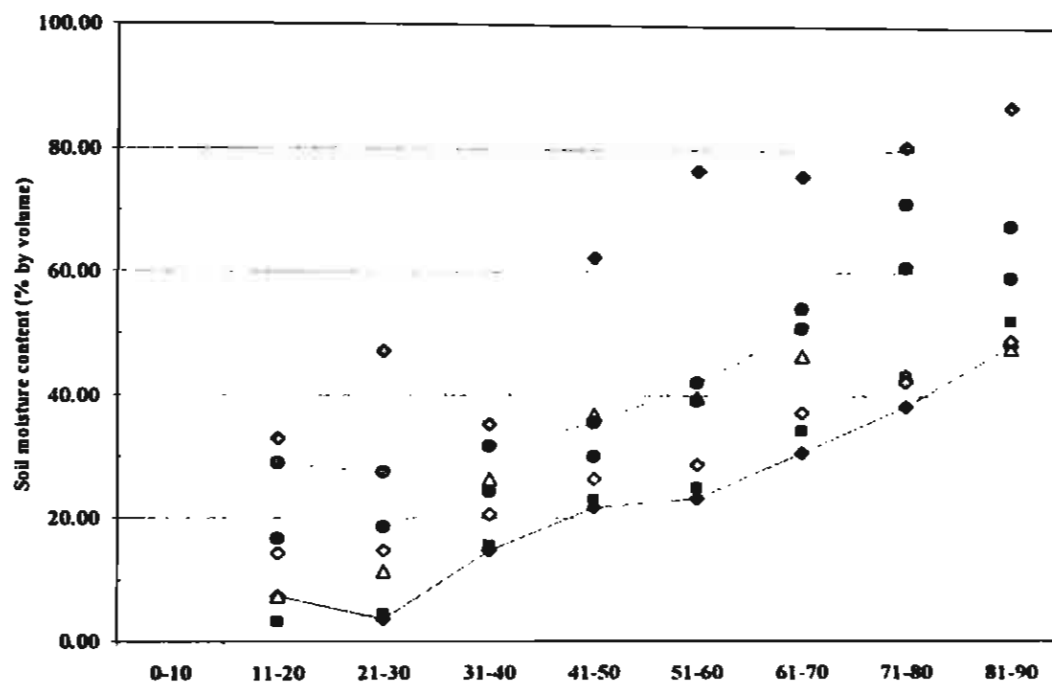


Fig. 18 Temporal and spatial variations in soil moisture along soil profile at CF site.

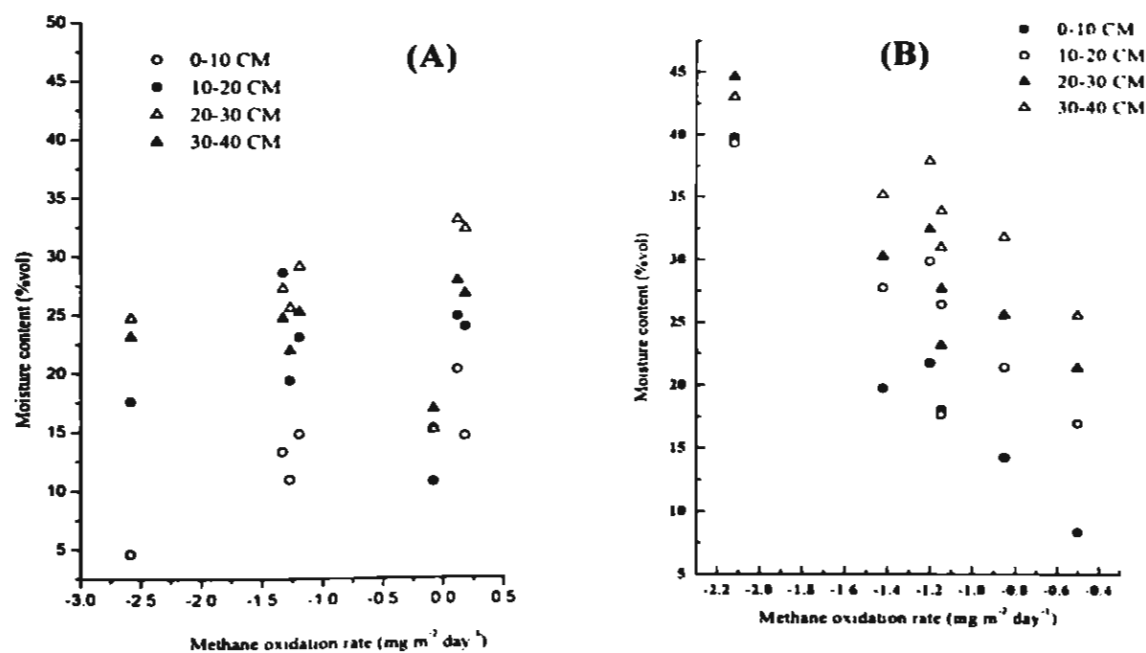


Fig. 19 Relationship between soil moisture and methane oxidation rate at SK site (A) and AC site (B).

3.4 Soil Temperature

Air and soil temperatures (at 2 cm and 5 cm) were measured during project period. The results are given in Fig. 20 below. It is clear that different land covers and land use type strongly influence temperature characteristics of soils. Highest soil and air temperatures were found at CF site. Larger seasonal variations were also observed at CF than at SK and AC sites. The average air temperature and soil temperatures at 2 and 5 cm depth during February to December are 25.4, 24.0 and 23.5 °C at SK site, 26.9, 25.6 and 25.3 °C at AC site, and 33.3, 30.7 and 28.7°C, respectively. Temperature difference between air and soil is higher at CF site than at both forest sites.

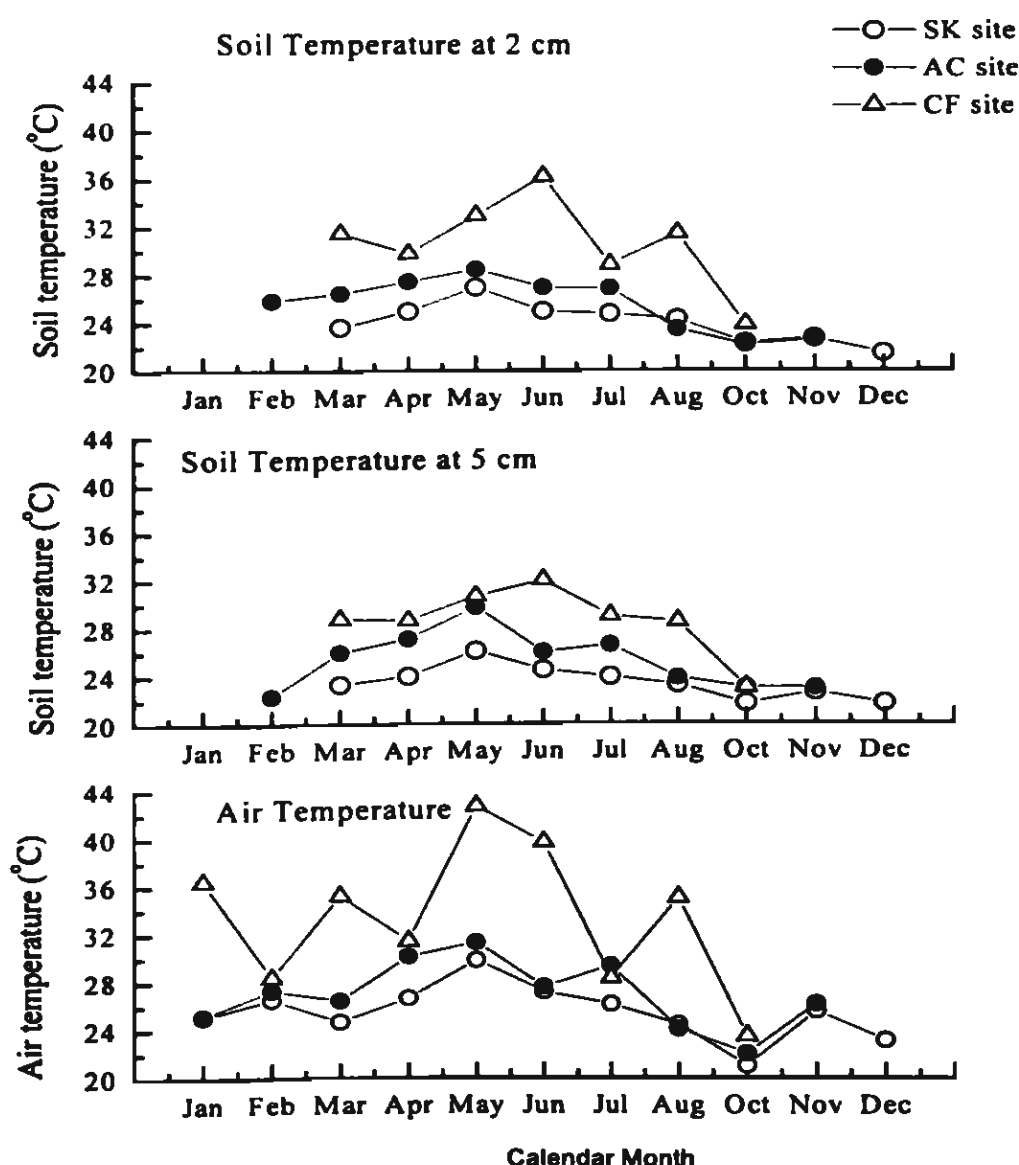


Fig. 20 Seasonal variations in soil temperature at 2 and 5 cm and air temperature in 2003

3.5 Results from Laboratory Study

3.5.1 Oxidation 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 times. Since soil from CF site shows low oxidation activity, incubation was carried out under 5 ppmv methane. Results of these incubation experiments are given in Fig. 21-24. Most of soil layers exhibit the methane oxidation activity. However, the most active methane oxidation layers for SK site is between 15 and 30 cm (Fig. 21 & 24). For AC site clear oxidation activity was found only at 10-15 and 20-30 cm (Fig. 22 & 24) 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. 23 & 24). There was no distinct active layer for in this site. However, the most active layer was still in the subsurface at 15-20 cm. From these results, it is concluded that the active layers for methane oxidation lie below the soil surface in forest soils. The reason for this is not know. 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. 10). In addition, the pH at the surface layers is very low in the present study (Fig. 9). Thus, The soil at the surface layers may be too acidic for methane oxidation.

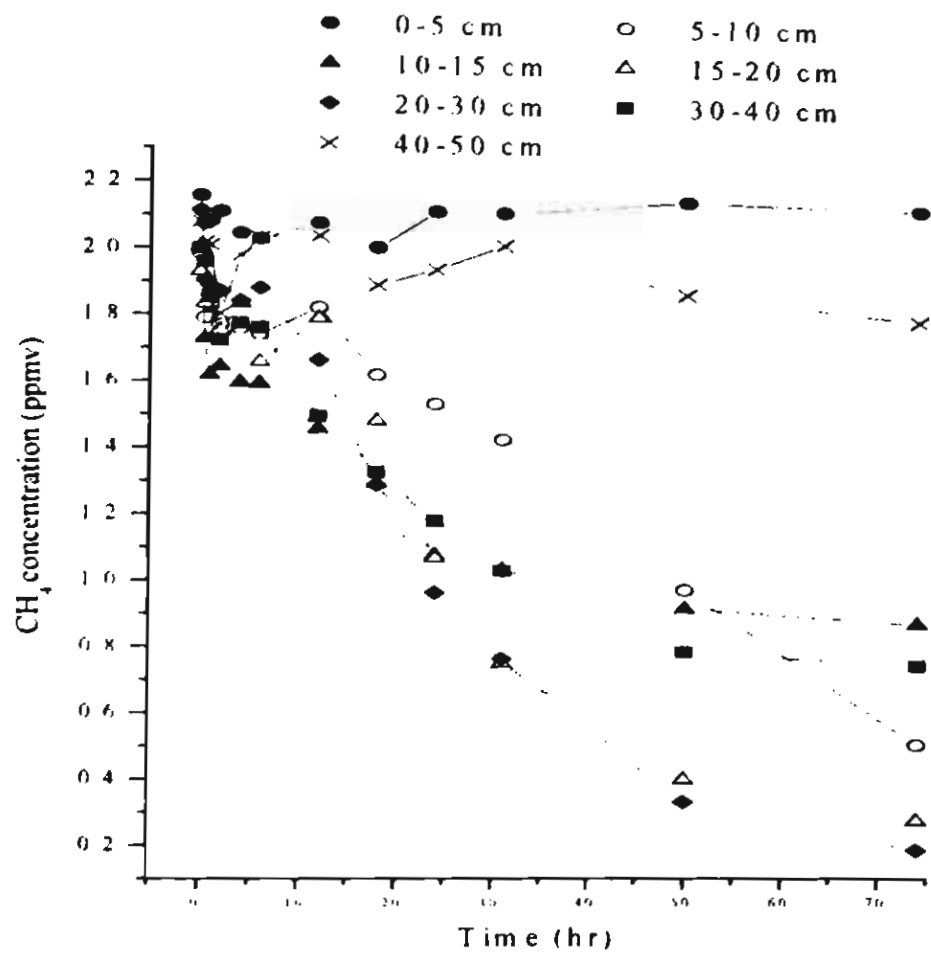


Fig. 21 Oxidation of methane by different soil layers taken from SK site and incubated under ambient methane concentration.

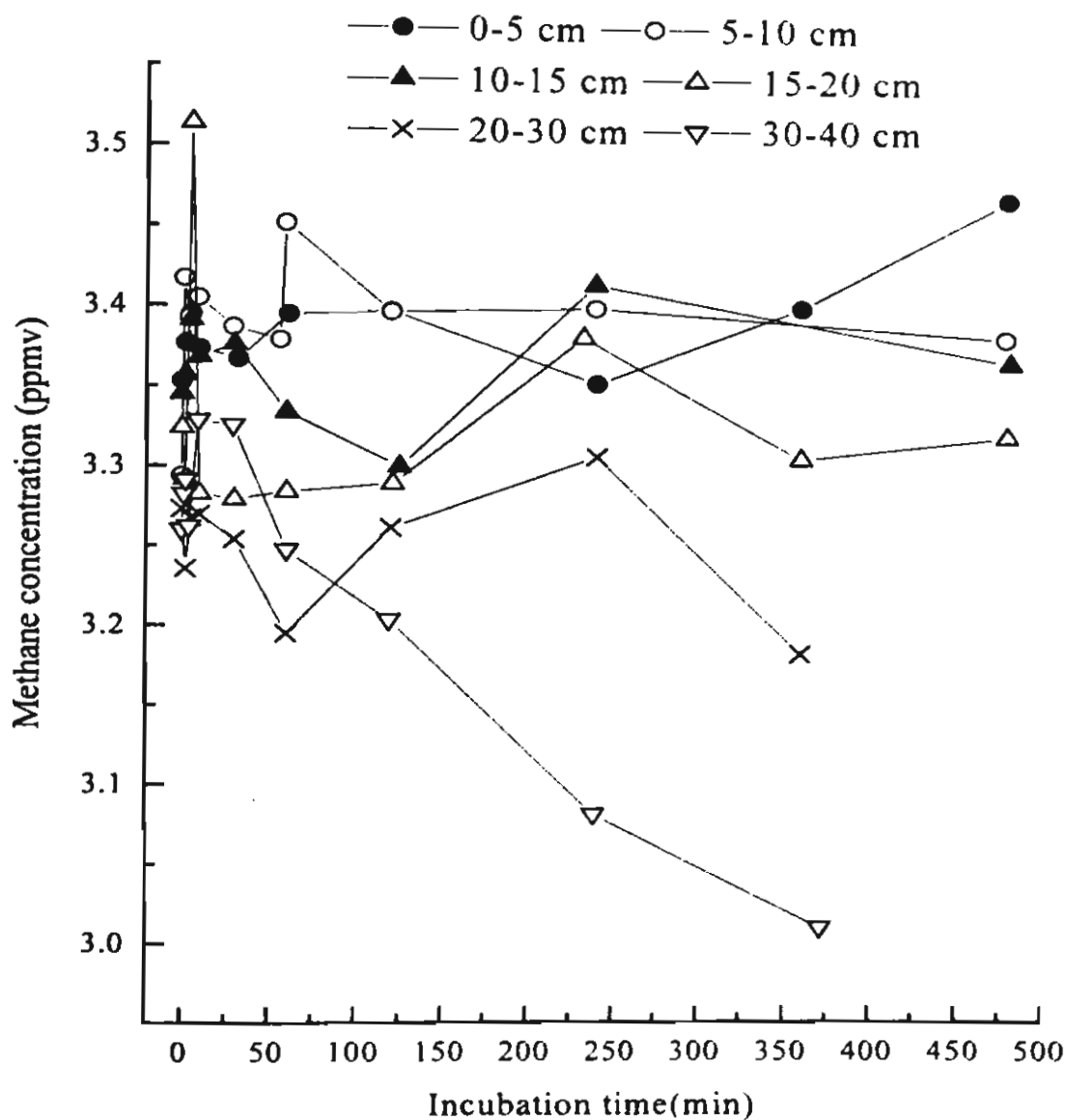


Fig. 22 Oxidation of methane by different soil layers taken from AC site and incubated under ambient methane concentration.

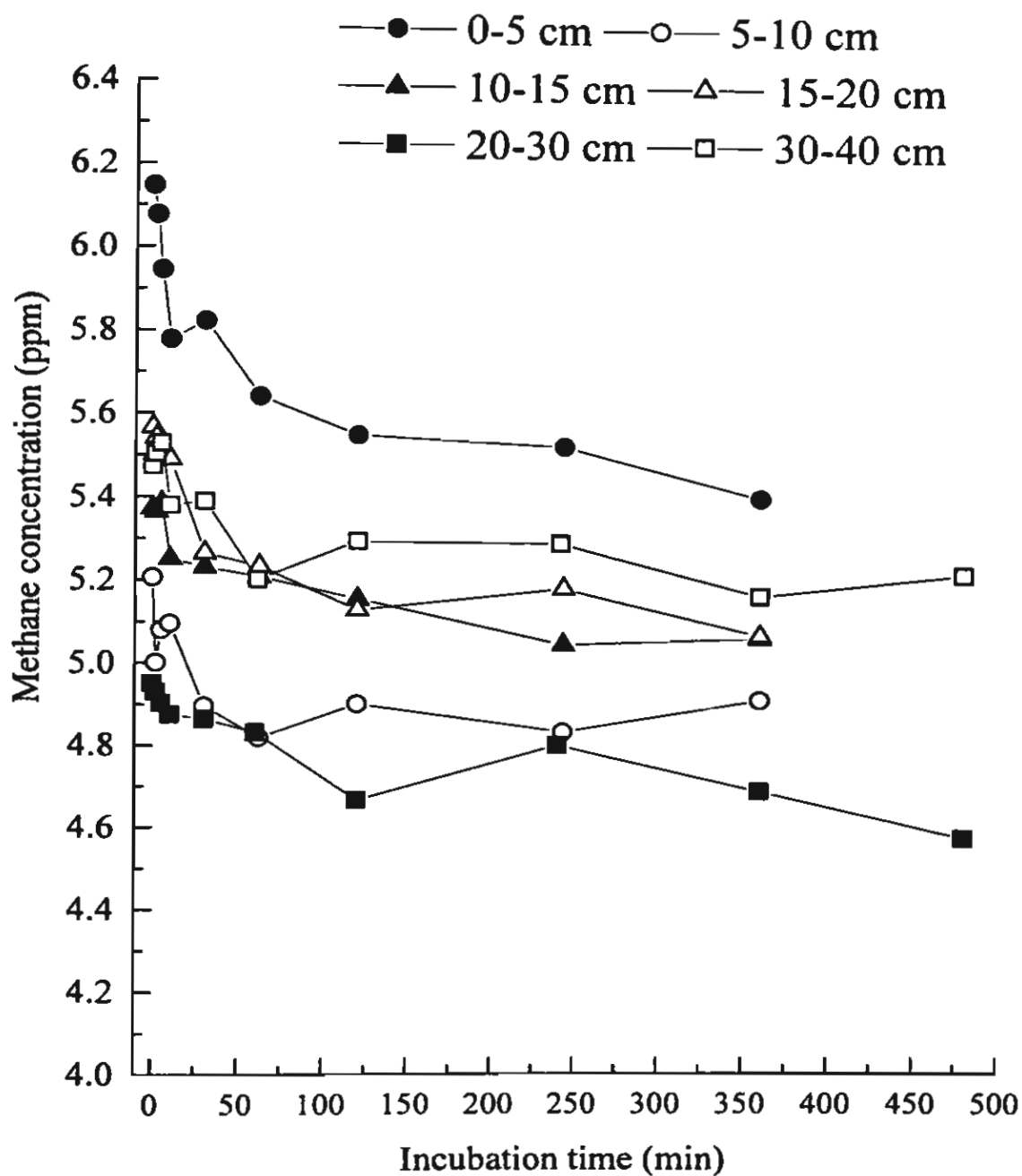


Fig. 23 Oxidation of methane by different soil layers taken from CF site, incubated under 5-6 ppmv of methane.

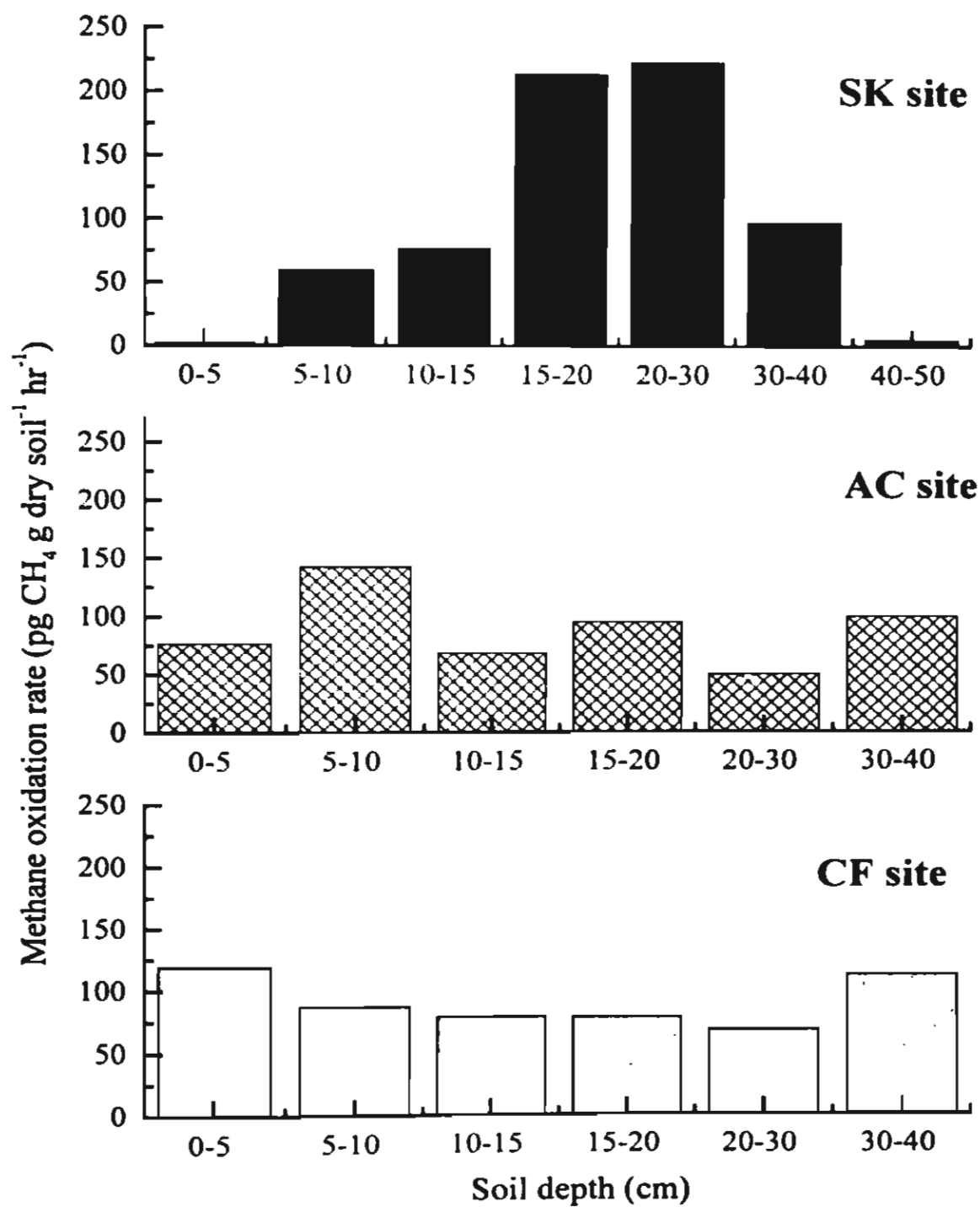


Fig. 24 Oxidation of methane along soil profile and in different land use type.

3.5.2 Kinetic study

From the results of section 3.5.1, soil from the active layer was used for kinetic study of methane oxidation. The results are described below.

Methane oxidation rate at each concentration was estimated by linear regression between CH_4 mixing ratios versus time. A plot of initial methane concentration and methane oxidation rate is given in Fig. 24. 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. 26).

From Fig. 26, 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 atmospheric concentration better than soils at AC and CF site. However, above about 200 ppmv, the oxidation rate becomes concentration independence at SK site. Thus, SK soil will not oxidize methane if its concentration is too high. In AC and CF sites oxidation rate still increased and then stabilized around 800 and 1000 ppmv, respectively. Comparing between AC and CF soils, 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 3. 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. It is interesting 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).

From these results, it is apparent that land use type affects oxidation kinetics of methane oxidizing bacteria. 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. Difference in kinetic parameters among land use types could be due to various factors associated with land use such as nitrogen fertilization (Dunfield and Knowes, 1995). In additions, availability of substrate which is limited by methane and oxygen diffusion into the oxidation site, different in methanotrophic community as shown in the Section 3.6 below, all could account for such variations. 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 $3.6 \text{ nmol g}^{-1} \text{ hr}^{-1}$.

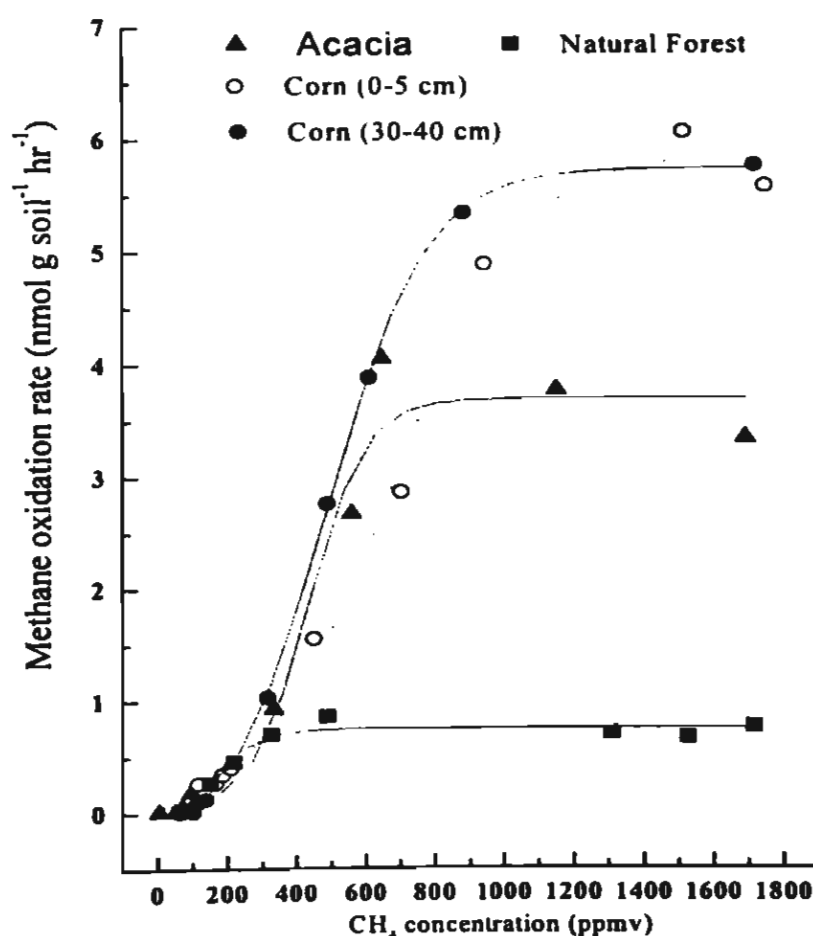


Fig. 25 Kinetic plots for initial methane concentration vs methane oxidation rate. Soil samples used in experiments at SK and AC sites were taken from 10-20, 5-10 cm depth, respectively, while at CF site two depths (0-5 and 30-40 cm) were examined.

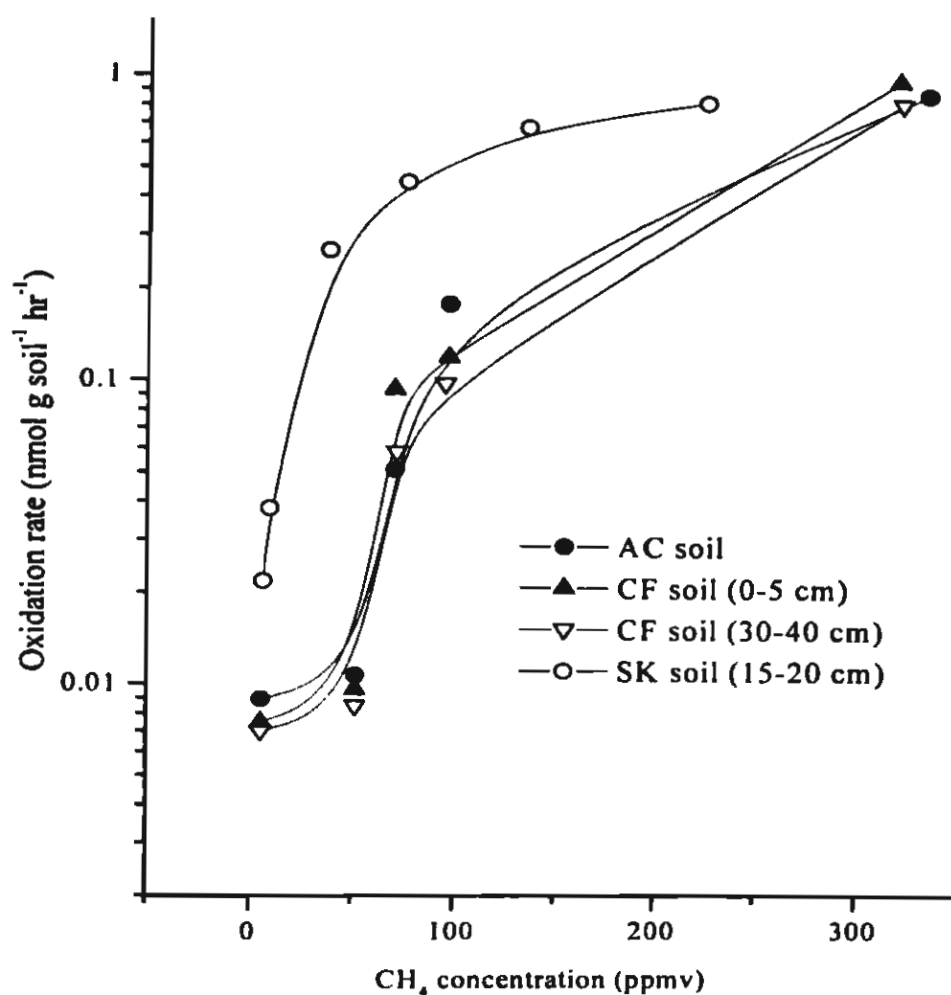


Fig. 26 Exponential plot of relationship between oxidation rate and methane concentration below approximately 350 ppmv.

Table 3 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

3.6 Molecular biology aspects of methanotrophs

3.6.1 DGGE

DGGE photographs of the A682b DGGE and the mb661 DGGE are shown in Fig. 27. Black bars indicate the excised bands. More bands were obtained with primer A682b than the primer mb661. Some of these bands (bands 1 and 4) contained neither *pmoA* (encoding a subunit of pMMO) nor *amoA* (encoding a subunit of ammonia monooxygenase). This is due to the fact that primer A682 is not as specific as mb661. For some bands (A682b DGGE) more than one PCR product was identified. This is due to the different procedures applied to identify the bands (cloning, application of different backward primers). These procedures, especially the application of different backward primers, may lead to the detection of “background smear”. Therefore, most bands were additionally cloned to identify the PCR product(s). The reverse primer mb661 is more specific for methanotrophs. Each visible band in the DGGE gel represented only one methanotrophic taxon. *Methylocystis* and *Methylobacter* sequences were the only taxa detected with both primer systems, but only in some of the soils (AC and CF, respectively, Table 2). Application of the reverse primer A682b lead to a preferential amplification of *amoA* genes, of genes with sequences which cluster in between those of *pmoA* and *amoA* (= PmoA/AmoA), and of USC α sequences.

3.6.2 Amplification of the *pmoA* gene (encoding the α subunit of pMMO)

Using cultivation-independent methods, the communities of MB were characterized in soil samples taken from 10 – 20 cm soil depth during the second half of the wet season. Duplicate soil samples were taken from each sampling site. Two different reverse primers were used for the amplification of the *pmoA* gene fragment. Primer A682 is the less specific of the two, since it was designed to amplify both *pmoA* and *amoA* gene fragments (Holmes et al., 1995). Primer mb661 was designed to amplify *pmoA* gene fragments specifically, excluding amplification of *amoA* gene fragment (Costello and Lidstrom, 1999). The *pmoA* gene fragment, used as a functional marker for the characterization of the methanotrophic community, was successfully PCR-amplified from all soil DNA extracts (Fig. 27). A gene fragment of the *mmoX* gene could not be amplified. This is in accordance with results from upland soils from Central Europe (Knief et al., 2003) 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 (Hanson and Hanson, 1996).

3.6.3 Detected taxa in soils from different land use

The PmoA sequences related to the nitrifier *Nitrosospora muliformis* was only detected in CF soil (Fig. 28, Table 4). Those closely related to the genera *Methylocystis*, *Methylobacter*, *Methylococcus*, and distantly related to *Methylocapsa*, were detected. *Methylocystis* sp. (Type II) was not detectable at site SK (Fig. 28, Table 4). However, at site AC it was detected with both primer systems. USC α was represented by band 3 in the A682b DGGE. The USC α is a novel sequence cluster usually called the “fiorest sequence cluser” and as indicated in this study and previous reported (Bourne *et al.*, 1997, Holmes *et al.*, 1999), it is present only in SK and AC soil samples (For sample AC a “clean” PCR product could not be obtained, therefore this sequence is missing in the phylogenetic tree). The most closely related pmoA sequence from a pure culture is that of *Methylocapsa acidiphila*, a type II methanotroph isolated from acidic peat. Interestingly, the soils under forest (both SK and AC) are also highly acidic (pH below 4 between 10 and 20 cm-depth, Fig. 9). This may indicate that this methanotroph prefers the acidic environment. The disappearance of USC α at site CF (a cornfield) confirms the result of Knief *et al.* (2003) and, who did not detect sequences of this cluster in farmlands (correlated with low methane oxidation activity). This also supports the hypothesis that this bacterium prefers an acidic habitat. Primer mb661 does not amplify the *pmoA* gene of the USC α (Bourne *et al.* 2001, this study). But PCR products were obtained with this primer that formed a cluster related to the sequences of USC α . The highest amino acid identity of “Thailand AC3141, band A” and “Thailand SK3141, band B” was 83% to “uncultured bacterium, DGGE band EL4-4 (Fig. 26). The corresponding DGGE band (band 2) was very intensive in soils SK and AC but not detectable at site CF. This correlates with the results for the USC α sequences, obtained by primer A682b. *Methylobacter* sp. sequences were