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Emergence and behaviors of acid-tolerant *Janthinobacterium* sp. that evolves N_2O from deforested tropical peatland

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Abstract

Using a soilless culture system mimicking tropical acidic peat soils, which contained 3 mg of gellan gum and 0.5 mg NO_3^--N per gram of medium, a greenhouse gas, N_2O emitting capability of microorganisms in acidic peat soil in the area of Palangkaraya, Central Kalimantan, Indonesia, was investigated. The soil sampling sites included a native swamp forest (NF), a burnt forest covered by ferns and shrubs (BF), three arable lands (A-1, A-2 and A-3) and a reclaimed grassland (GL) next to the arable lands. An acid-tolerant *Janthinobacterium* sp. strain A1-13 (Oxalobacteriaceae, β-proteobacteria) isolated from A-1 soil was characterized as one of the most prominent N_2O-emitting bacteria in this region. Physiological characteristics of the N_2O emitter in the soilless culture system, including responses to soil environments, substrate concentration, C-source concentration, pH, and temperature, suggest that the N_2O emitting *Janthinobacterium* sp. strain A1-13 is highly adapted to reclaimed open peatland and primarily responsible for massive N_2O emissions from the acidic peat soils. Regulation of N_2O emitters in the reclaimed peatland for

agricultural use is therefore one of the most important issues in preventing the greenhouse gas emission from acidic peat soil farmlands.

Keywords: Tropical peatland; *Janthinobacterium*; Forest fire; Nitrous oxide (N_2O) emission

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1. Introduction

Nitrous oxide (N_2O) is a greenhouse gas (GHG) contributing to about 6% of global warming (IPCC, 2001) and to the depletion of the ozone layer in the stratosphere (Crutzen, 1981). It therefore has a large impact on global systems. Approximately 20% of annual N_2O production is from agricultural soil (estimated at 3.5 Tg N yr^{-1}) and reclaimed tropical forests (3.0 Tg N yr^{-1}), which is a large fraction of total global production (16.2 Tg N yr^{-1}) (Maltby and Immirzi, 1993; IPCC, 1996) from mineralized N via nitrification and/or denitrification processes (Conrad, 1996). Therefore, arable land which is excessively fertilized with nitrogen (N) is a major source of N_2O flux from soils (Bouwman, 1990). Extensive areas of Indonesian tropical peat swamp forest that were destroyed by repeated forest fires (Page et al., 2002) and agricultural exploitation (Muhanmad and Rieley, 2002) are known to have had a large impact on the global carbon cycle by accelerating CO_2 and CH_4 emissions from peat soil decomposition (Page et al., 2002; Inubushi et al., 2003). By monitoring soil gas emission for 2 years, it was determined that peat forests which were converted for use as arable land emitted large amounts of N_2O (Hadi et al., 2000; Takakai et al., 2006). It is known that the area of

agricultural peat soil farms in Central Kalimantan, Indonesia, is one of the most active N_2O emitting sites in the world by regular monitoring of gas flux in the soil (Takakai et al., 2006).

The objectives of this study were to search for major N_2O-emitting microorganisms from the tropical peat soils that have been utilized for decades as arable land and actively been producing N_2O, and to also characterize and investigate biological properties of the N_2O emitter. This enabled us to propose and conduct strategy for controlling N_2O emission in the field. In the study site, conversion rates of applied N (as manure and chemical fertilizer, ranging from 665 to 1,278 kg N ha^{-1} yr^{-1}) to N_2O were estimated to be a maximum of 39% of annual input N emitted as N_2O from the peat soil (Takakai et al., 2006). This bioconversion rate in the local arable land varies over an unexpectedly wide range, suggesting that alternative factors promote and/or suppress N_2O emission by soil microorganisms. Under water-saturated conditions, the peat soil of the monitoring sites in Central Kalimantan is moderately or medium-strongly acidic (pH 4.2 to 3.7) at a depth of 10 to 30 cm and composes of large amounts of organic materials containing C and N. We thus report the isolation and characterization of soil bacteria primarily responsible for N_2O emission in tropical acidic peat soil employed as arable land, using an acidic peat soil-mimic soft gel medium.

2. Materials and methods

2.1 Site description.

Soil sampling sites are located in the area of Kalampangan village near Palangkaraya (2˚S, 114˚E) in Central Kalimantan, Indonesia. The experimental plots are two forest sites and four tracts of arable land. One forest site is in a natural forest (NF: 2˚21’S, 114˚02’E) and the other site is in a burnt forest (BF: 2˚19’S, 114˚01’E). The natural forest had not been affected by the forest fires in 1997 and 2002, whereas the burnt forest had been destroyed by the fires. The

forest trees and the surface peat soils up to 30 cm in depth in the BF site were burned during the fires. In the NF site, the depth of the peat soil ranges from 3.50 to 4.85 m (Tuah *et al*., 2001). This forest consists mainly of deciduous trees such as *Tetramerista glabra* Miq., *Calophyllum* sp., *Shorea* sp., *Combretocarpus rotundatus* (Miq.) Danser, *Palaquium* sp., *Buchanania sessifolia* Bl., *Syzygium* sp., *Dactylocladus stenostachys* Oliv. in Hook, *Dyera costulata* (Miq.) Hook.f., *Ilex cymosa* Bl., *Tristaniopsis obovata* R. Br., and *Dyospyros* sp. (Tuah *et al*., 2003). The burnt forests are now being dominated by ferns (*Stenochlaena palustris* and *Nephrolepis* sp.) at the time of study. Arable land sites (2˚17'S, 114˚01'E) are located in an unfertilized grassland (GL) and three fertilized arable tracts of land (A1 to A3). The GL site is dominated by dwarf grasses. The main crops of the croplands were maize, cruciferous vegetable, edible amaranth, edible cosmos, and cassava. The rate of N fertilization of the croplands was 665 to 1,278 kg N ha^{-1} yr^{-1} (545 to 1,098 and 40 to 180 kg N in the form of chemical fertilizer and manure, respectively). Ash from plants and peat was also added to the cropland soils in order to supply mineral elements to the peat soil. These four sites (A1-A3, and GL) are adjacent to each other. The major chemical properties of soil in each site are shown in Table 1. The peat soils at the study sites were all classified as Typic Tropofibrists (USDA Soil Taxonomy).

2.2 Soil sampling and its N_2O production assay.

Soil samples were taken from depths of 10 to 30 cm at each site in September 2004; gas flux measurements were also done. The soil samples were collected into sterile Falcon tubes and brought to the laboratory. A soil sample of 0.1 g was added to 5 ml of sterile water and vortexed for 30 s and stood for 10 min. As an inoculant, 100 μl of the clear, resulting supernatant was taken with a sterile micropipette and inoculated to the soft gel medium

prepared as described below under aseptic conditions. The medium was again vortexed thoroughly and subsequently incubated at 25°C in the dark. After 2, 7, and 12 days of incubation, headspace gas (100-500 μl) from each vial was sampled using a gas-tight syringe, and the gas was analyzed for both N_2O and CO_2. N_2O concentration was determined by using a gas chromatograph equipped with a micro-electron-capture detector (ECD) detector (Shimadzu GC-14B, Kyoto, Japan) maintained at 340°C, using a 1 m Porapak N column (Waters, Milford, MS, USA) maintained at 60°C, with a carrier gas of Ar with 5% CH_4. The headspace gas sampled from the soilless culture vial was injected into the gas chromatograph. CO_2 concentration was determined using an infrared CO_2 analyzer (Fuji Electric ZFP5YA3I, Tokyo, Japan).

2.3 Preparation of tropical peat soil-like gel media.

Two types of soft gel media containing NH_4^+ or NO_3^- were prepared as follows: as a source of mineral N, $(NH_4)_2SO_4$ (500 mg NH_4^+-N l^{-1}) or KNO_3 (500 mg NO_3^--N l^{-1}) was added to a Winogradsky mineral mixture-based nitrogen-free medium solidified with 0.3% gellan gum (Hashidoko et al., 2002) without any other carbon source. The pH values of NH_4^+ and NO_3^--containing media were adjusted with 2 M H_2SO_4 to 4.7 and 3.8, respectively. To this sugarless medium was further added 0.3% of gellan gum (w/w, Wako Pure Chemical Industries, Osaka, Japan), and this was then heated until the gel powders were completely dissolved. After cooling to room temperature, 10 ml of the liquefied medium was poured into a 30-ml screw cap septum vial sealed with a Teflon-coated silicon rubber pad (Pierce, USA), and was then autoclaved at 120°C for 15 min and finally cooled. The headspace volume of this vial was exactly 20 ml. This soft gel medium was used as the standard for N_2O production assays. Since this medium contained almost no C-source necessary for

auxotrophic bacteria and saprophytic fungi, incubation of the soil suspension led to effective selection for acid-tolerant, oligotrophic soil bacteria.

2.4 Screening and isolation of N_2O emitting bacteria.

Based on the N_2O analysis, the cultured media that actively produced N_2O were selected for further testing of N_2O emitting bacteria. When significant N_2O production (>200 μl l^{-1} in the headspace over 5 days) and bacterial growth were observed, 10 μl of gel medium was drawn using a sterile micropipette at a shallow level (less than 5 mm) or a deep level (10 mm or more) of the gel medium, and the resulting gel droplet was spread over a modified Winogradsky agar plate (1× Winogradsky mineral solution without an N-source, 0.5% sucrose as the sole C-source, and 0.005% w/w of yeast extract (Difco, USA), the pH was adjusted to 5.0 with 2 M H_2SO_4, and this was gelled with 2% agar). The isolation plate was incubated at 23°C in the dark for 4 days, and the bacterial colonies which covered 10% or more of the relative colony-forming frequency on the plate were each obtained as individual colonies and subjected to re-isolation on a new agar plate. Subsequently, we obtained 14 isolates from 4 different active vials of the samples from the sites A1 and BF to test them for N_2O production assay in pure-culture conditions.

2.5 Identification of three N_2O emitters.

Three isolates (strains A1-13, A1-12, and BF-4) that exhibited a higher N_2O producing activity than the background level in the N_2O production assay for pure culture were identified by means of sequence determination in the region of the 16S rRNA gene (Weisburg et al., 1991). Their target genes were amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAAGGAGGTGATCCAGCC-3')

universal primers for PCR with HotStarTaq^{TM} DNA Polymerase (Qiagen, USA) as follows: for initial activation of the DNA polymerase, kept at 95^{o}C for 15 min, and subsequent 30 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 56 ^{o}C for 1 min and extension at 72 ^{o}C for 1 min were done. The PCR reaction was finalized at 72^{o}C for 10 min, and then subjected to sequencing as described previously (Hoo et al., 2004).

2.6 Alternative culturing conditions influencing N_2O production of Janthinobacterium *sp. A1-13.*

Cell concentration in the inoculant, incubation time, alternative substrates, NO_3^- concentrations, C-source concentrations, medium pH, and culturing temperature were examined. As alternative substrates, NO_3^- was substituted by NO_2^- (pH 3.8) or NH_4^+ (pH 4.7) in the same N ratio as in the original medium containing 5 mg NO_3^--N. Substrate (anhydrous KNO_3) concentration was at 0.3, 1.0, 3.0, 10, and 30 mg g^{-1} medium, whereas the concentration of the C-source (D-glucose) was 0, 0.3, 1.0, 3, and 10 mg g^{-1}, all at pH 3.8. Furthermore, the standard medium was adjusted to several pH values ranging from 2.5 to 8.4 (n=14) using 2 M H_2SO_4 and 1 M KOH. Optimal temperature for the standard medium was determined using a growth chamber Multi-Incubator (NK System, Osaka, Japan) at 25, 30, 35, 40, 45, and 50°C in triplicate. All of the above media with alternative chemical and physical factors were inoculated with approximately 10^5 cells of strain A1-13, incubated at 25°C for 7 days, and then analyzed for N_2O concentration.

3. Results

3.1 N_2O emitting capability of soil microorganisms in soilless culture assay.

In searching for N_2O-emitting microorganisms in soil samples, supernatant of soil

suspension (100 µl each, equivalent to 2 mg of soil) was inoculated in a N_2O emission assay medium in the screw cap septum vials. The soil washings from tracts of arable land (A1 to A3) and grassland (GL) exhibited significant N_2O production (60 to 300 µl l^{-1} in vial headspace compared to background levels of 0.32 to 0.35 µl l^{-1}). In contrast, no N_2O production was observed in soil samples from natural forest (NF) (Table 1). Time course experiments for A1 and BF showed that the soilless culture assay for N_2O emission allowed measurement of N_2O productivity of the inoculants over the entire 7-day culture period (Fig. 1). In parallel with the screening using the standard medium, N_2O emission in another medium containing NH_4^+-N as the sole N-source (500 mg N l^{-1}) was also used for the screening. However, none of the soil samples exhibited substantial N_2O emitting capacity with the NH_4^+-N medium, suggesting that N_2O production in peat soils mainly occurs not in ammonia-oxidizing nitrification but in a denitrification process. Among 14 isolates of oligotrophs obtained from the N_2O emission-positive cultures, three (BF-4, A1-12, and A1-13) were found to be N_2O-emitting soil bacteria (Fig. 2).

3.2 Isolation and identification of highly active N_2O-emitting bacterium, Janthinobacterium *sp.*

The three isolates were tentatively identified as *Janthinobacterium* sp. strain A1-13, *Burkholderia cepacia* strain A1-12, and *Burkholderia tropica* strain BF-4. They were then deposited at the DNA Data Bank of Japan (DDBJ) and assigned accession numbers AB252072, AB252073, and AB252074, respectively (Table 2). The most prominent N_2O emitter A1-13 identified as *Janthinobacterium* sp. (Holt et al., 1990) showed a micro-aerobic response in the soft gel medium and was widely adaptable to both nutrient-poor and nutrient-rich conditions. On a modified Winogradsky (MW) agar plate, it formed hard and

elastic colonies strongly adhering to the agar, but without violet pigment. The bacterium also exhibited an adventurous motility on a nutrient-poor soft gel and a social motility on a nutrient-rich agar plate (Shi and Zusman, 1993).

In a preliminary soilless pure culture assay, inoculation of ca. 10^3 cells on the soft gel medium and incubation at 25°C for 7 days resulted in 1290 μl l^{-1} of N_2O emission from 5 mg NO_3^--N, which is 26.0 μl (52.1 μg) of N_2O in a 20 ml headspace with a 0.7% conversion ratio for NO_3^--N. When a higher bacterial concentration in the inoculant (ca. 10^5 cells) was used in the assay, N_2O production occurred in a range of 3000 to 5000 μl l^{-1}, which is 1.5-2.5% of bioconversion rate for NO_3^--N. N_2O productivity of strain A1-13 in the C-deficient medium is almost equivalent to that of *Paracoccus denitrificans* ATCC 17741 cultured in Giltay's medium for denitrification (Daum and Schenk, 1998), which is obviously richer in organic compounds than our N_2O assay medium.

3.3 Effects of inoculated cell population density and N- or C-source concentration on N_2O emission.

When KNO_3 concentration in the standard medium was increased, excessively high concentrations of KNO_3 suppressed cell growth, and decrease production of N_2O. In contrast, a reduced substrate (0.043 mg N g^{-1} medium) resulted in the same level of N_2O emission (3,640 μl l^{-1}), suggesting that not substrate concentration but rather cell concentration is the most important factor for N_2O production in the sugarless culture medium. As the amount of D-glucose added as a carbon source to the standard medium for N_2O assay was increased, *Janthinobacterium* sp. strain A1-13 increased the amount of N_2O emission. After the addition of supplemental 10 mg g^{-1} D-glucose and 7-day incubation, N_2O emission into the headspace with an inoculant of 10^5 cells was more than 0.38 mg g^{-1}, which was calculated to be 2.45 mg

N (49% N-conversion) (Fig. 3). In these experiments above, CO_2 emission from the media containing various concentrations of D-glucose or NO_3^- exhibited a fine linear correlation with the N_2O production (Fig. 4).

3.4 Effects of other physicochemical factors of soils on N_2O emission.

It is clearly difficult to compare bulk peat soil with the medium used in the soilless culture system because soil conditions in arable land are far dynamic. Soils irregularly supplied with lower concentrations of N and C are always a diverse mosaic of pH, temperature, moisture content, C-source and N-source contents, and microorganisms. Therefore, investigation of appropriate physical and/or chemical conditions for N_2O production of *Janthinobacterium* sp. strain A1-13 in the soilless culture system may reflect soil conditions appropriate for N_2O production in the bulk soil. We therefore investigated the influence of several physicochemical properties, including pH (2.5 to 8.2), temperature (25 to 50°C), concentrations of substrate (NO_3^-), and upstream and downstream substrates (NH_4^+ and NO_2^-), on N_2O production in a soilless culture assay.

Although *Janthinobacterium* sp. strain A1-13 exhibited good cell growth over a pH range of 3.8 to 6.8, N_2O production was greatest at pH 3.8. CO_2 production and N_2O emission also showed a certain correlation in the pH range (Fig. 5A). In soil, N_2O emission is generally suppressed in acidic areas (Daum and Schenk, 1998), nevertheless, a N_2O production pulse occurred at pH 3.8 in acidic soil-tolerant *Janthinobacterium* sp. strain A1-13. This suggests that moderately to strongly acidic soils (pH 3.5 to 4.6 in H_2O, shown in Table 1) having a high buffering capacity (cf. CEC, 76 to 159 $cmol_c$ kg dw^{-1} in the research plots) (Takakai et al., 2005) increased N_2O production. The N_2O pulse was observed when the level of emitted CO_2 was over the threshold level (Fig. 5B). When nitrite (5 mg N per vial) was used as the

alternative substrate at pH 3.8, N_2O production of *Janthinobacterium* sp. strain A1-13 was only 0.8 to 2% of that from NO_3^-. On the other hand, strain A1-13 never produced N_2O in the 5 mg of NH_4^+-N-containing medium at pH 4.7, suggesting that nitrifying bacteria are not important mediators in N_2O emission in the peat soil.

In the temperature-sensitivity test (5 mg NO_3^--N, pH 3.8), it was found that N_2O emission was stable from 25 to 40°C (Fig. 6), which encompasses topsoil temperatures in open tracts of arable land, as shown in Table 1. Although cell growth of *Janthinobacterium* sp. strain A1-13 was observed to be low at 45°C, there was only a trace amount of N_2O production at this temperature (ca. 20 times the background rate), and at 50°C, no significant cell growth or N_2O emission occurred.

3.5 Vertical distribution of N_2O reactant in the arable land soil.

To investigate differences in N_2O emission in the vertical profile of the bulk soil at the A1 site, soil samples were taken from arable tracts of land at five depths as shown in Table 3, except 30-40 cm, and were tested for N_2O production ability in the soilless culture system. Topsoil (0-10 cm depth, mainly taken at 2 cm depth) and shallow soil (10-20 cm depth) under aerobic conditions had the highest levels of N_2O production. In general, nitrate respiration occurs under anaerobic conditions (Richardson and Watmough, 1999), but some microorganisms and soils actively produce N_2O under aerobic conditions (Bouwman, 1998; Dundee and Hopkins, 2001; Khalila and Bagg, 2005). Although deeper soils (20-30 and 40 -50 cm deep) exhibited relatively lower levels of N_2O production than did the soil 0-10 cm deep, it still exhibited remarkable levels of N_2O production. In contrast, the soil at 50-60 cm deep did not exhibit any significant N_2O-producing activity. These results along with chemical properties of the sample soils (Table 3) suggest that soil microorganisms near the

relatively aerobic topsoil actively denitrify NO_3^-. Moreover, shallow soils contain excess inorganic matters provided from chemical fertilizers and ash.

Discussion

The N_2O-emitting ability of *Janthinobacterium* sp. A1-13 was unexpectedly high; almost half of nitrate N was converted into N_2O gas under appropriate conditions. Biologically, N_2O is often emitted due to incomplete denitrification reaction (Zumft, 1992). In this reaction process, nitrous oxide reductase (nos) is responsible for catalytic reduction of N_2O to release N_2 ($N_2O \rightarrow N_2$). If nos cannot function in denitrifying microorganisms, then most of the N_2O that is yielded from NO by catalytic reaction of nitric oxide reductase (nor) is released into atmosphere ($NO \rightarrow N_2O$) (Holtan-Hartwig et al., 2002). In fact, disruption of *nos* genes has been reported in some active N_2O-emitting, soil microorganisms, including *Fusarium oxysporum* MT811 (Zhou et al., 2001) and *Achromobacter cycloclastes* (McGuirl et al., 1998). In view of the approximately 50% bioconversion rate from NO_3^- to N_2O in the glucose-supplemented mineral mixture medium (Fig. 4), active N_2O production in *Janthinobacterium* sp. strain A1-13 *via* denitrifying nitrate respiration is reasonably due to inactivation of the final step by the *nos* gene product. Therefore, *nos* gene clusters will be key to understand the potent ability of *Janthinobacterium* sp. A1-13 to produce N_2O actively.

It has also been observed that high rates of soil respiration are often coupled with active N_2O production in soil because C:N balance also affects N_2O emission (Huang et al., 2004). In *Janthinobacterium* sp. A1-13 in the soilless culture system, its N_2O emission is controlled by available carbon source in the assay medium, in which the highest performance is of 5 mg N with 12 mg C (*cf.* in the standard sugarless culture medium, 5 mg N with 0.02 mg available C in calculation of CO_2 flux, as shown in Fig. 4). CO_2 in the C-source supplemented culture

medium is a reliable indicator of bacterial cell growth and respiration, whereas NO_3^- is utilized as an electron acceptor in a nitrate-respiration process during denitrification (Zumft, 1992; Khalila and Bagg, 2005). Strain A1-13 exhibited characteristics of aerobic respiration near the gel surface in the glucose-supplemented media. Since inoculated bacterial cell concentration greatly affected N_2O emission in this sugarless medium, a high N_2O production by the strain A1-13 in glucose-supplemented medium is certainly due to explosive cell growth of strain A1-13. Oxygen deficiency reasonably triggers active nitrate-respiration, leading to production of excessive N_2O by strain A1-13. As another contribution, organic acids produced from glucose by the bacterium may cause N_2O production in the C-source supplemented medium as did in sugarless medium at pH 3.8 (Fig. 5B). These theories are supported by a field observation reported by Takakai et al. (2006) that N_2O emission in the field conditions irregularly increase after the rains.

In views of vertical soil profiles and capabilities for N_2O emission (Table 3), bulk soil microorganisms down to a depth of 5-15 cm was found be the primary contributor to N_2O emissions. We therefore calculated N_2O productivity in the field and headspace vial conditions to determine whether *Janthinobacterium* sp. strain A1-13 is responsible for the remarkably high levels of N_2O emission from the peat soils in the A1 sites. N-source concentration of the standard N_2O assay medium is a reasonable level (500 mg N kg^{-1} of NO_3^-), which is calculated from the annual rate of fertilizer application on local tracts of arable land. Therefore, the calculated N_2O-emitting level in the vial assay probably reflects the N_2O-production of strain A1-13 in fertilized peat soil.

The N_2O emitting performance of the A1-13 inoculated to 10 cm^3 of the sugarless culture medium (0.73-1.7 μg g^{-1} d^{-1}) was comparable with the bulk soil in A1 site, in its calculated volume per 1 m^2 (1.0 x 10^5 cm^3, from a depth of 5 cm to 15 cm). Most of the N_2O emission in

the equivalent volume of the sugarless medium (1.9-4.5 mg N m^{-2} h^{-1}) is a similar level to the arable tract of land A1, which exhibited the highest N_2O emission in the range from 0.3 to 2.9 mg N m^{-2} h^{-1}. In consideration of the N_2O-emitting capabilities of the shallower (above 5 cm) and deeper (below 15 cm) soils (Table 3), N_2O emissions of the arable soil and strain A1-13 in the soilless culture medium are a similar level. *Janthinobacterium* sp. strain A1-13 is, therefore, likely to be one of the most prominent N_2O emitter in this region.

At a depth of 4 cm, the wet peat soil in open land throughout Central Kalimantan had a soil temperature of 27 to 35°C, 3~5°C higher than natural forest bed soils, but the temperatures of the soils in the open tracts of land were within the tolerance level of N_2O production by *Janthinobacterium* sp. strain A1-13 (Fig. 6). Therefore, increase of the soil temperatures in deforestation area rarely affect the suppression of N_2O production. In contrast, performance of N_2O emission in the soilless culture medium under alternative pH conditions indicated that N_2O emission by strain A1-13 is highly linked with acidic soil environments in this region (Fig. 5). Characteristic response of strain A1-13 in a narrow acidic region is likely to contribute to active N_2O emission of A1-13, due to a high buffering property of the peat soil (Table 1).

In water-saturated, acidic peat soil paddocks in South Kalimantan, active CH_4 and CO_2 emission and trace amounts of N_2O efflux have been recorded from the soil (Inubushi et al., 2003). Natural forest bed soil in peat swamp forest regions in Central Kalimantan also produces only trace amounts of N_2O, indicating that high levels of N_2O emission are rarely observed in acidic peat soils (De Boer and Kowalchuk, 2001; Takakai et al., 2006). Nevertheless, cleared arable tracts of land with acidic peat soils in Central Kalimantan produced extraordinarily high levels of N_2O. Although it is known well that nitrification activity in natural, acidic soils is generally low, some researchers have reported relatively

active N_2O emissions from acidic soils, in which ammonium oxidizers (e.g. *Nitrosospira* sp.) play an important role in N_2O production (Nagele and Conrad, 1990; Martikainen and De Boer, 1993; De Boer and Kowalchuk, 2001). In the monitoring site, it has also been observed that rainfall leads to active N_2O emission (Takakai et al., 2006), probably suggesting that ammonium oxidizing and nitrifying bacteria are activated along with mineralization of organic N into ammonium under moistened soil conditions, leading to N_2O efflux from NO_3^-. However, neither *Janthinobacterium* sp. strain A1-13 nor soil supernatant produced any N_2O from the NH_4^+-N containing media in the soilless culture assay. Hence, the relative contributions of ammonia oxidizing and nitrifying bacteria remain to be questioned at least under the experimental conditions of this study.

The soilless culture assay for N_2O emission using soil washings indicated a possibility that both natural and burnt forest soils contain potent N_2O-emitting microorganisms. They were, however, minor or inactive in the bulk soils. The presence of living plants, particularly those of native trees, may positively suppress N_2O efflux by the N_2O-emitters. Mineralized nitrogen loss as N_2O emission from the topsoil of the tropical forest bed would also result in loss of nitrogen for the ecosystem, particularly for perennial forest trees. In fact, large parts of nitrogen in wetland peat soils are unavailable, tannized organic forms, so the plants are speculated to be exposed to nitrogen-starvation in peat soil. We thus hypothesize that the tropical swamp forest ecosystem has an ability directly or indirectly to suppress N_2O-emitting soil microorganisms.

In these respects, the simplest and most effective way to prevent production of the greenhouse gas N_2O from tropical peat soils is to conserve and re-establish peat swamp forest ecosystems. In tropical peatland that has already been cleared and fertilized for agricultural use, however, suppression of *Janthinobacterium* sp. and other N_2O emitters is the most urgent

issue. Hence, A1-13 can be used as a model bacterium in studies of biological regulation of N_2O production in tropical peat soils. Currently, regulation of rhizosphere bacteria by plant root via plant-producing quorum sensing mimic compounds came under a spotlight (Bauer and Teplitski, 2001; Teplitski et al., 2000), and such approach is also applicable to *Janthinobacterium* sp. A1-13 in the future.

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Table 1

Sampling sites and physical and chemical properties of the bulk soils.

Physical and chemical properties (temperature, pH, and EC) of bulk soils at 6 sites: a natural forest (NF), a burnt forest (BF), three arable tracts of land (A1 to A3) and a grassland (GL), were measured in the field in 2002 and 2003. N_2O emissions were analyzed in the laboratory after the soil gases and the soils were sampled. Values of N_2O emission are of 2002. Soil temperature at a depth of 4 cm was measured approximately every 4 weeks from November 2001 to October 2004. Soil pH was measured after ca. 1 g of bulk soil was suspended in 10 ml of ion-exchanged water. EC (electrical conductivity) was also measured for soil suspended in water. C and N percentages were measured in triplicate as described previously (Takakai et al., 2006). Loss on ignition was measured after treatment at 550^{o}C for 4 h. [a] Takakai et al. (2006)

Table 2

Identification of N_2O-emitting bacteria isolated from the acidic peat soils.

Three bacterial isolates were each identified by DNA sequencing of the 16S rRNA gene regions and listed along with the determined base-pair numbers and sequence homology in percentage.

Table 3

Vertical profiles and N_2O emitting performance of site A1 soils.

Physical and chemical properties (pH, EC, C content, N content, C:N ratio, and loss of ignition) of bulk soils at different depths were analysed in triplicate. Loss on ignition was measured after treatment at 550^{o}C for 4 h. N_2O emission was analysed in the soilless culture assay at 25^{o}C for 5 d.

Fig. 1.

Time-course experiment for N_2O emission in a soilless culture system.

N_2O production in the test medium inoculated with three different soil samples (A1 from a depth of 2 cm, A1 from a depth of 10 cm, and BL from a depth of 2 cm) was measured at 17 to 34 hour intervals for 8 days in triplicate. After day 2, the soilless culture medium started to evolve N_2O as shown by the generally sigmoid curve through the incubation period, but from days 2 to 7, the N_2O emission generally increased linearly. Therefore, to evaluate N_2O production, we analyzed the concentration of N_2O in headspace gas at day 7, and when necessary, we calculated N_2O production rate per day by using the absolute amount of emitted N_2O at day 7. N_2O volume in the headspace (20 ml) shown in microliters per liter was converted to an absolute amount by dividing by a factor of 24.75 to yield an amount in

micrograms. The bars in the graph show standard deviation (SD, *n*=3). At least up to day 7, the N_2O emission was linearly related to incubation day.

Fig. 2.

N_2O producing activities of the soil samples and isolated bacteria in soilless culturing assays.

N_2O productivity of 14 isolates was tested together with a blank (Blank-1) in the NO_3^--containing assay medium (pH 3.8). Among those tested, three isolates (BF-4, A1-12, and A1-13 marked with an asterisk at the top of the columns) produced significant amounts of N_2O compared with background levels of N_2O (0.32 to 0.35 μl l^{-1}), shown by a horizontal line (arrow).

Fig. 3.

Acceleration of N_2O production at different concentrations of supplemental D-glucose.

In the standard medium, different concentrations of D-glucose were added, and N_2O produced in the headspace was analyzed after 7 d-incubation at 25°C in the dark. At 0 mg concentration of supplemental D-glucose, emission of N_2O and CO_2 was 4,480 and 16,100 μl l^{-1} (in 20 ml headspace), respectively. Conversion rates of carbon in the supplemented D-glucose (3, 10, 30 and 100 mg/10 ml) into CO_2 at 25°C were 40.5, 16.3, 12.3 and 4.0%, respectively.

Fig. 4.

Correlation between the N_2O emission and CO_2 emission by *Janthinobacterium* sp. at different concentrations of D-glucose and KNO_3 supplemental C- and N-sources.

Correlation between evolved N_2O and CO_2 were plotted among those assayed for

alternative D-glucose or KNO_3 concentrations. This correlation between N_2O and CO_2 was linear (R^2=0.98, n=10). N_2O emission begun when A1-13 emitted beyond the threshold level of CO_2 (8,500 μl l^{-1}).

Fig. 5.

Effect of pH on N_2O production in relation to CO_2 emission.

Correlation between pH and gas emission. (A) In the range of pH 8.4 to 3.8, CO_2 flux exhibited a linear correlation with pH values (R^2=0.71). The higher the pH, the less CO_2 strain A1-13 emitted, but below pH 3.4, both bacterial cell growth and CO_2 flux were drastically reduced. (B) Therefore, excluding the data below pH 3.8, correlation between CO_2 flux and N_2O emission were shown here. A N_2O pulse occurred at pH 3.8, along the highest CO_2 level beyond 25,000 μl l^{-1}.

Fig. 6.

Effect of temperature on N_2O production.

The bacterial cell growth was active in the temperature range from 20°C to 40°C which encompassed soil temperatures at a depth of 4 cm in open arable tracts of land. Although cell growth of *Janthinobacterium* sp. strain A1-13 was observed at 45°C, N_2O production at this temperature was a relatively low amount (107 ±110 μl l^{-1}, ca. 20 times higher than the background), while at 50°C, cell growth did not occur along with a background level of N_2O emission (0.58 ±0.10 μl l^{-1}). Wet peat soil in open tracts of land throughout Central Kalimantan had soil temperatures, at depths of 4 cm, of 27 to 35°C, a temperature range that was favorable for N_2O production of *Janthinobacterium* sp. strain A1-13. Although lack of shade in deforested areas allowed the ground temperature to be more than 5°C higher,

temperatures are still within the tolerance of *Janthinobacterium* sp. strain A1-13. Therefore, deforestation rarely resulted in suppression of N_2O production by increasing soil temperature.

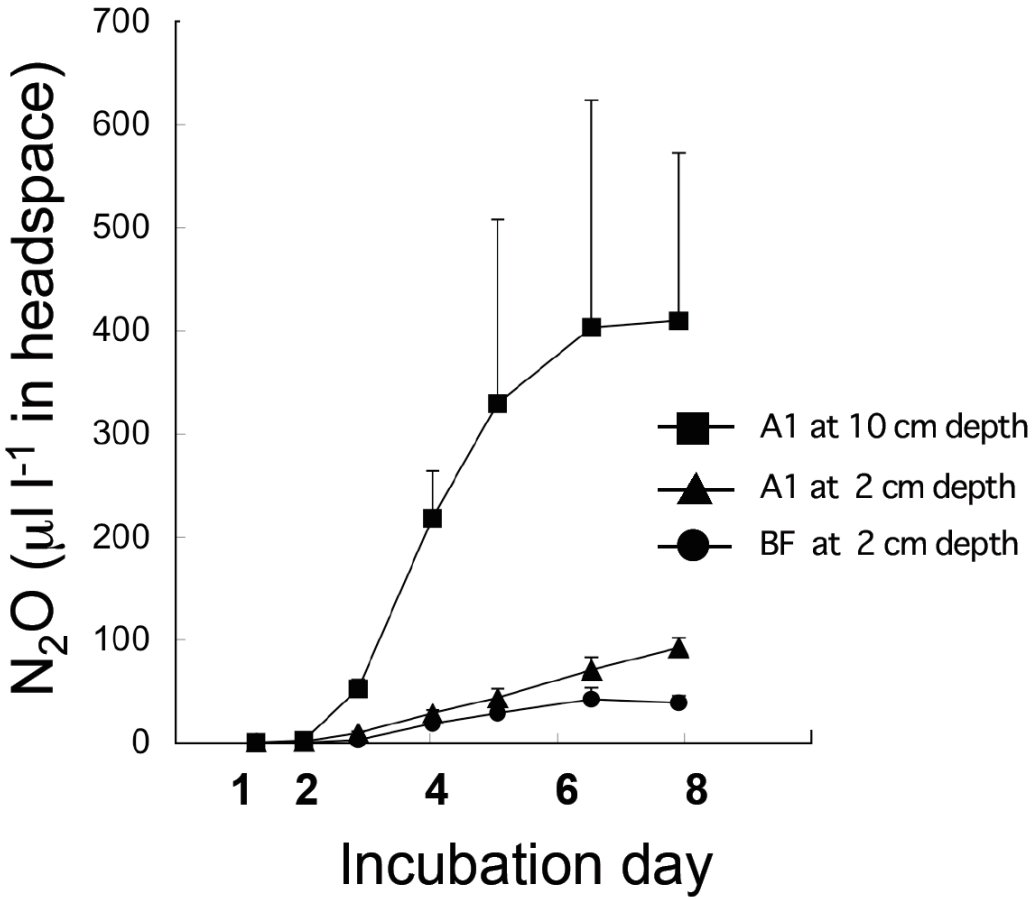

700
600
500
400
300
200
100
0
N_2O (µl l^{-1} in headspace)
1
2
4
6
8
Incubation day
A1 at 10 cm depth
A1 at 2 cm depth
BF at 2 cm depth

Fig.1

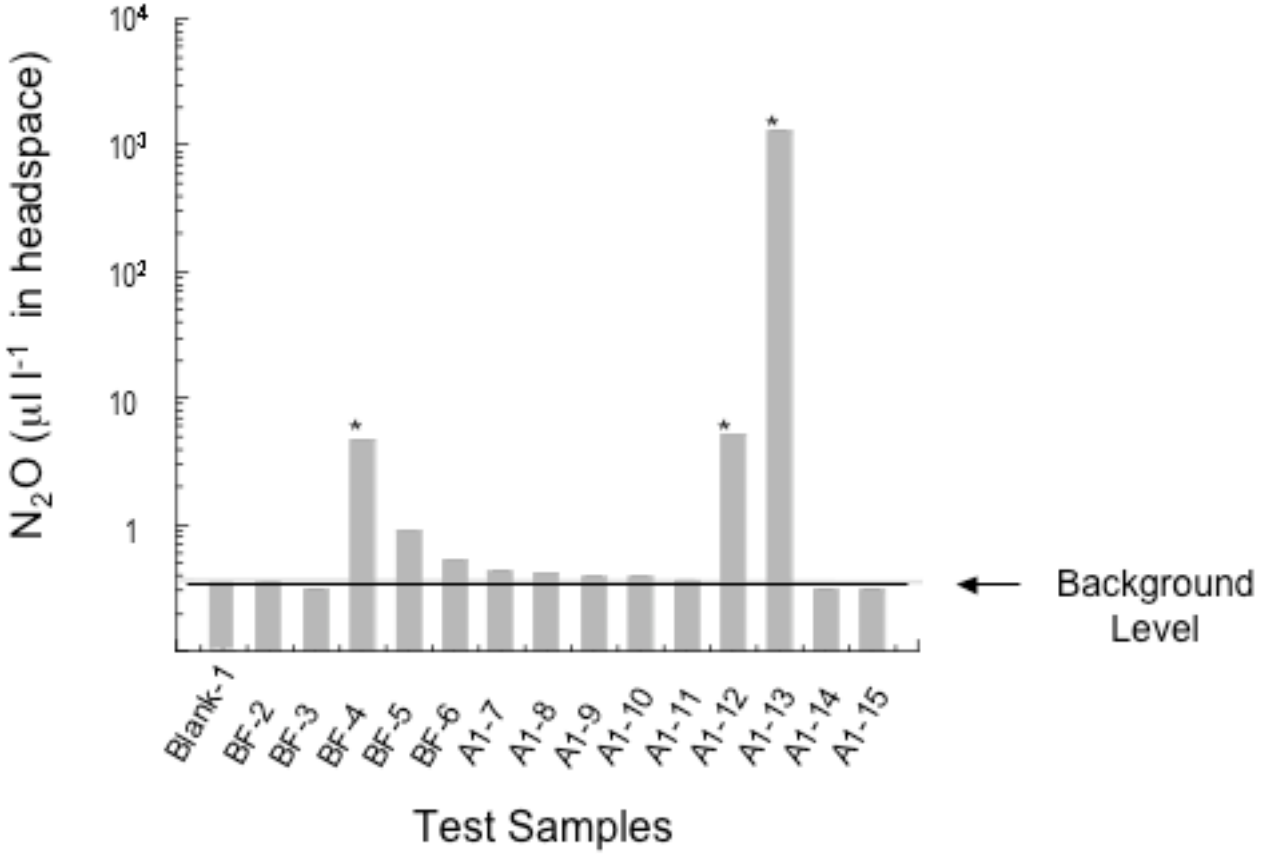

10^4
10^3
10^2
10
1
N_2O (μl l^{-1} in headspace)
*
*
*
Background Level
Blank-1
BF-2
BF-3
BF-4
BF-5
BF-6
A1-7
A1-8
A1-9
A1-10
A1-11
A1-12
A1-13
A1-14
A1-15
Test Samples

Fig.2

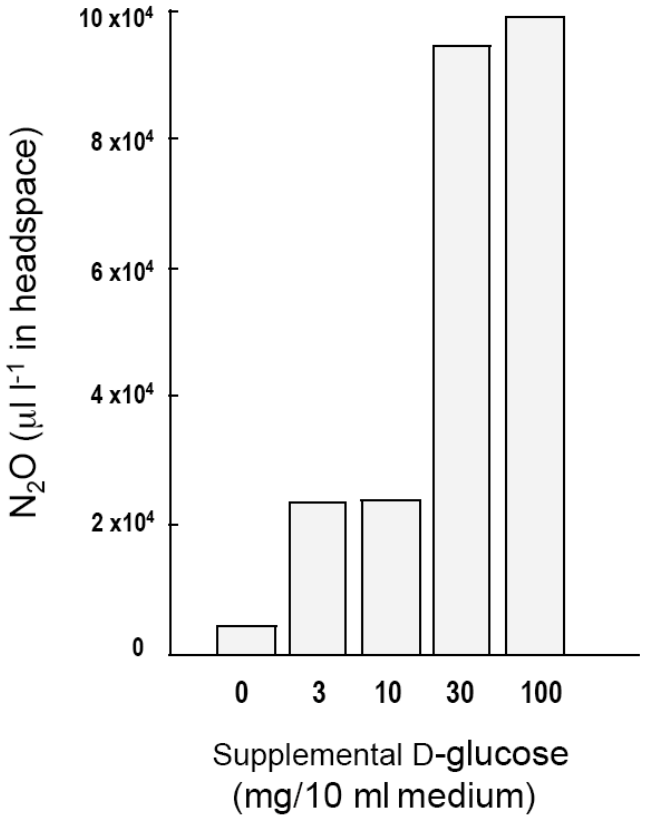

10 x10⁴
8 x10⁴
6 x10⁴
4 x10⁴
2 x10⁴
0
N2O (µl l-1 in headspace)
0
3
10
30
100
Supplemental D-glucose
(mg/10 ml medium)

Fig.3

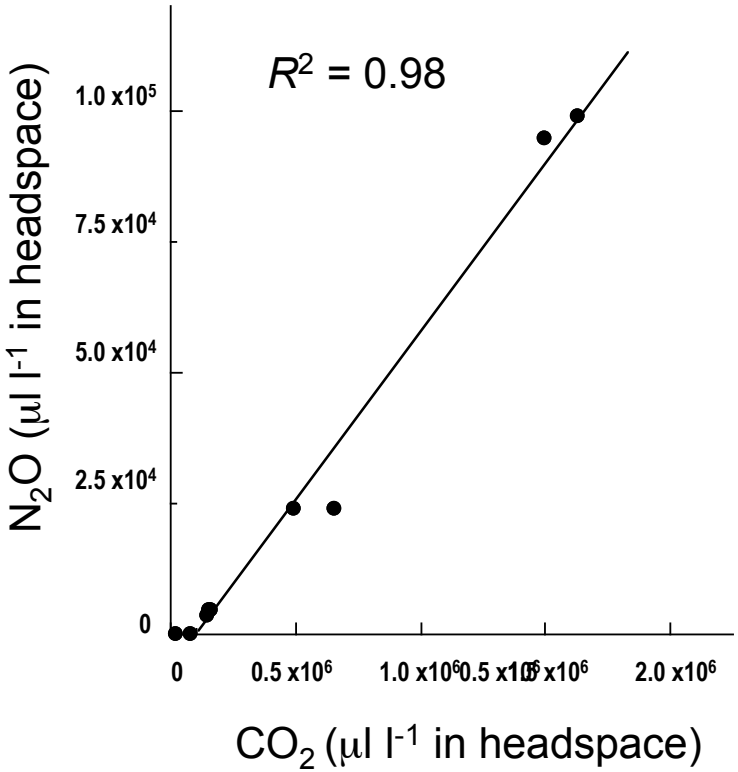
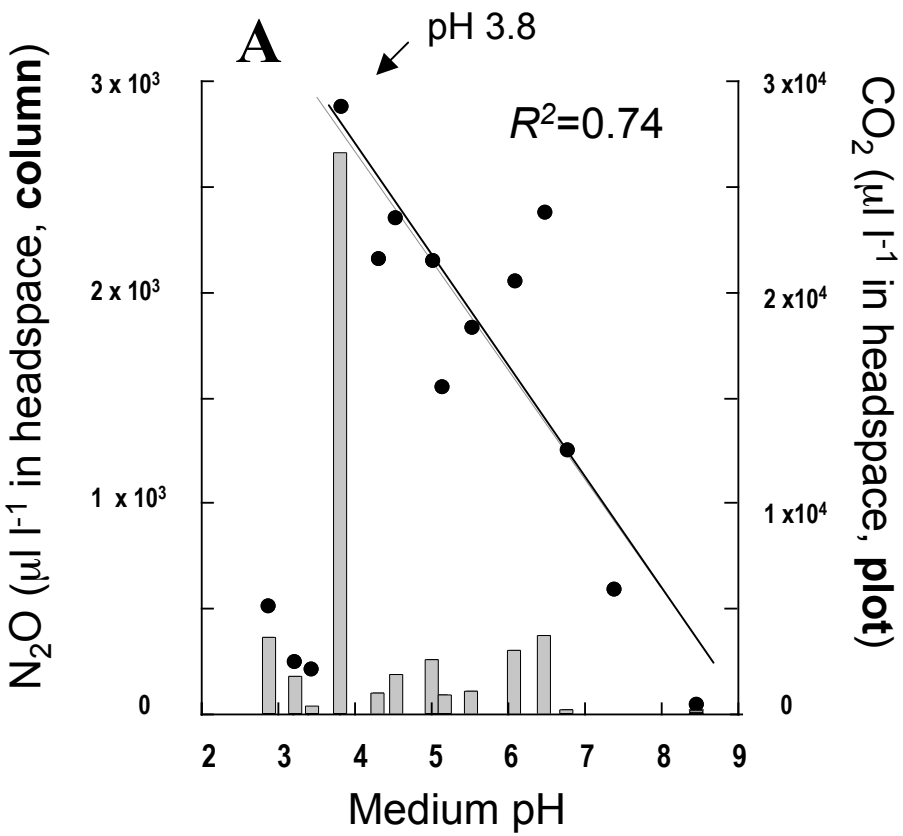

R^2 = 0.98
1.0 x10^5
7.5 x10^4
5.0 x10^4
2.5 x10^4
0
N_2O (µl l^{-1} in headspace)
0
0.5 x10^6
1.0 x10^6
2.0 x10^6
CO_2 (µl l^{-1} in headspace)

Fig.4


A
pH 3.8
R^2=0.74
N_2O (µl l^{-1} in headspace, **column**)
CO_2 (µl l^{-1} in headspace, **plot**)
3 x 10^3
2 x 10^3
1 x 10^3
0
3 x10^4
2 x10^4
1 x10^4
0
2 3 4 5 6 7 8 9
Medium pH

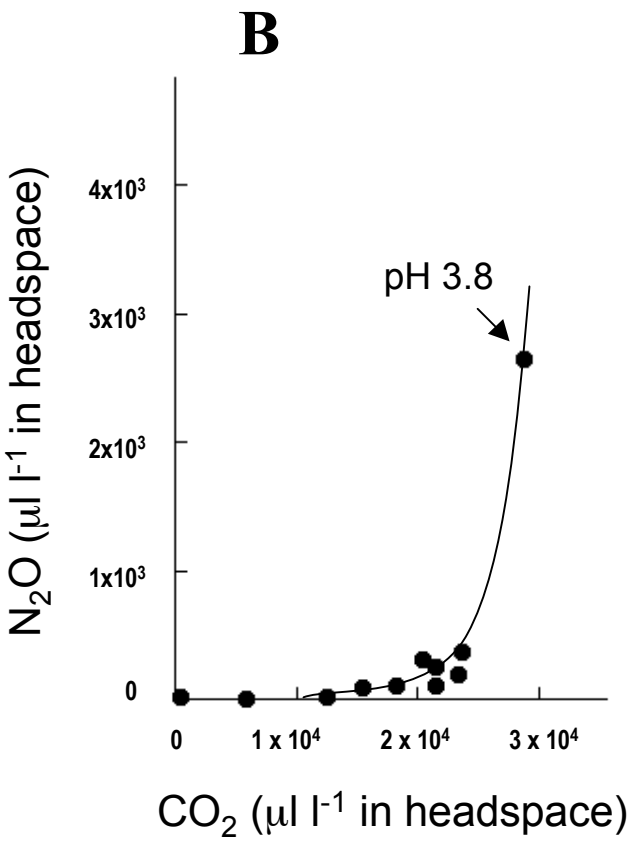

B
pH 3.8
N_2O (µl l^{-1} in headspace)
4x10^3
3x10^3
2x10^3
1x10^3
0
0 1 x 10^4 2 x 10^4 3 x 10^4
CO_2 (µl l^{-1} in headspace)

Fig.5

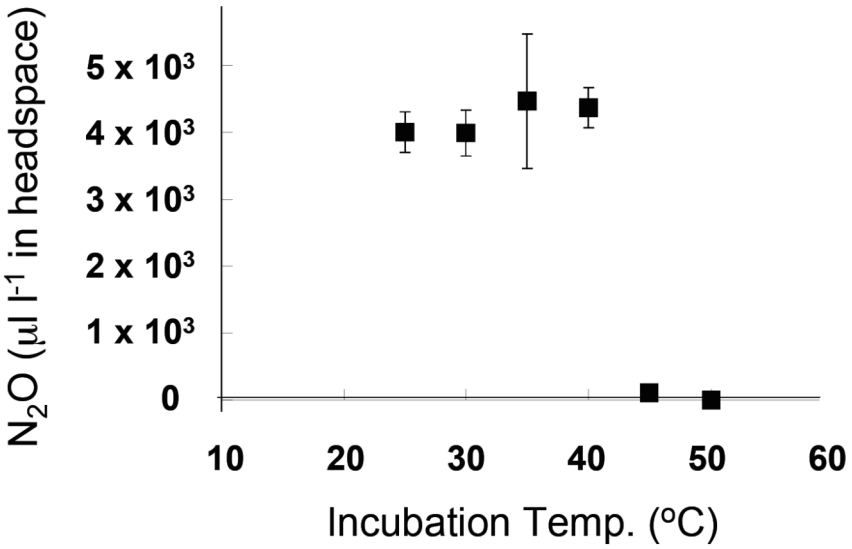

N2O (µl l-1 in headspace)
5 x 10³
4 x 10³
3 x 10³
2 x 10³
1 x 10³
0
10
20
30
40
50
60
Incubation Temp. (ºC)

Fig.6

Site	*n*	Soil temp.			*n*	pH (H_2O)		EC (mS/m)	N_2O emission[a]
		Mean ± SD	Max.	Min.		0–10 cm (± SD)	20–30 cm (± SD)	0–10 cm	(kg N ha^{-1} yr^{-1}, 2002)
NF	23	27.5 ± 1.10	30.2	25.6	3	4.06 ± 0.04	3.86 ± 0.03	11.21 ± 0.37	0.62
BF	24	30.5 ± 1.61	34.1	28.0	3	3.84 ± 0.11	3.52 ± 0.03	3.96 ± 0.32	0.97
A1	30	29.4 ± 1.48	32.4	26.8	3	5.32 ± 0.02	3.90 ± 0.02	4.19 ± 0.11	131
A2	30	30.0 ± 1.48	33.7	27.4	3	4.45 ± 0.04	3.54 ± 0.04	7.00 ± 0.24	21
A3	30	29.4 ± 2.17	35.4	26.7	3	5.61 ± 0.30	3.90 ± 0.30	2.54 ± 0.24	83
GL	21	31.3 ± 2.13	35.1	27.6	3	4.44 ± 0.05	3.63 ± 0.05	3.15 ± 0.10	7.1

Site	*n*	C (%)		N (%)		C:N ratio		Loss on ignition(%)	
		0–10cm (±SD)	20–30cm (±SD)	0–10cm (±SD)	20–30cm (±SD)	0–10cm (±SD)	20–30cm (±SD)	0–10cm (±SD)	20–30cm (±SD)
NF	3	59.7 ± 0.26	66.6 ± 0.15	2.25 ± 0.06	1.48 ± 0.03	26.5 ± 0.66	45.0 ± 0.87	99.2 ± 0.22	99.8 ± 0.11
BF	3	63.8 ± 0.18	67.3 ± 0.54	1.65 ± 0.07	1.12 ± 0.02	38.6 ± 1.59	59.6 ± 1.17	98.9 ± 0.04	99.6 ± 0.08
A1	3	48.7 ± 0.69	62.4 ± 0.73	1.40 ± 0.01	1.16 ± 0.01	34.9 ± 0.21	53.8 ± 0.50	68.5 ± 0.83	90.3 ± 0.21
A2	3	60.6 ± 1.66	66.6 ± 0.46	1.34 ± 0.07	0.97 ± 0.01	45.2 ± 1.20	68.5 ± 0.64	83.3 ± 0.19	98.8 ± 0.04
A3	3	52.9 ± 2.15	66.7 ± 0.38	1.37 ± 0.06	0.92 ± 0.02	38.7 ± 0.63	72.8 ± 1.68	72.5 ± 0.55	98.5 ± 0.40
GL	3	32.8 ± 0.82	68.4 ± 0.12	0.87 ± 0.05	1.03 ± 0.02	37.9 ± 1.12	66.5 ± 1.18	52.7 ± 2.45	98.7 ± 0.30

Table 1

Table 2
Identification of N_2O-emitting bacteria isolated from the acidic peat soils

Isolated strain	Identification	DNA base pair	Homology (%)	Accession no.
BF-4	*Burkhodleria tropica*	1526	99.7	AB252074
A1-12	*Burkholeria cepacia*	1526	99.7	AB252073
A1-13	*Janthinobacterium* sp.	1522	98.9	AB252072

Three bacterial isolates were each identified by DNA sequencing of the 16S rRNA gene regions and listed along with the determined base-pair numbers and sequence homology in percentage.

Table 3
Vertical profiles and N_2O emitting performance of site A1 soils

Depth (cm)	*n*	pH (mean ± SD)	EC (mS/m) (mean ± SD)	C (%) (mean ± SD)	N (%) (mean ± SD)	C:N ratio (mean ± SD)	Loss on ignition (%) (mean ± SD)	N_2O production ($\mu l\,l^{-1}$ ± SD, at 5 days)
0–10	3	5.32 ± 0.02	4.19 ± 0.11	48.7 ± 0.69	1.40 ± 0.01	34.9 ± 0.21	68.5 ± 0.83	90.4 ± 29.7
10–20	3	4.58 ± 0.06	5.60 ± 0.11	58.2 ± 1.27	1.27 ± 0.06	45.7 ± 1.01	83.3 ± 0.36	99.8 ± 49.9
20–30	3	3.90 ± 0.05	6.89 ± 0.26	62.4 ± 0.73	1.16 ± 0.01	53.8 ± 0.50	90.3 ± 0.21	14.5 ± 18.5
30–40	3	3.96 ± 0.06	6.04 ± 0.25	63.9 ± 0.16	1.18 ± 0.03	54.2 ± 1.29	93.0 ± 0.40	–
40–50	3	3.63 ± 0.02	7.95 ± 0.09	66.6 ± 0.20	1.02 ± 0.02	65.1 ± 1.23	98.4 ± 0.14	13.4 ± 0.9
50–60	3	3.68 ± 0.05	7.15 ± 0.32	64.7 ± 0.42	1.09 ± 0.02	59.4 ± 1.16	93.4 ± 0.63	5.5 ± 3.5

Physical and chemical properties (pH, EC, C content, N content, C:N ratio, and loss of ignition) of bulk soils at different depths were analysed in triplicate. Loss on ignition was measured after treatment at 550 °C for 4 h. N_2O emission was analyzed in the soilless culture assay at 25 °C for 5 days.