



Linking Nitrous Oxide and Nitric Oxide Fluxes to Microbial Communities in Tropical Forest Soils and Oil Palm Plantations in Malaysia in Laboratory Incubations

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Current understanding of greenhouse gas (GHG) fluxes associated with land-use change from forest to oil palm on mineral soil is not sufficient to provide reliable estimates of emission rates or advice on GHG mitigation strategies. Monocultures of oil palm have expanded in Southeast Asia, mostly replacing tropical forests. The limited data available have indicated that the land-use conversion is associated with a potentially aggravated GHG burden, including nitrous oxide (N₂O) and nitric oxide (NO) emissions, with unclear underlying biological mechanisms. In this study, we investigated N₂O and NO emission potentials of tropical soils with different land-uses from Sabah, Malaysian Borneo, under laboratory incubation. Under similar controlled conditions, logged forest and oil palm soils showed high and similar potentials of N₂O and NO emissions following increase in soil moisture, while the emissions were negligible in a riparian reserve soil irrespective of moisture conditions. Soil N₂O and NO emission rates from logged forest soils and oil palm (OP) plantations were of similar magnitude, with average fluxes over the 35 and 22 day incubation periods, respectively, of 11.5 and 1.6 ng N g⁻¹ h⁻¹ (OP) and 15.6 and 6.0 ng N g⁻¹ h⁻¹ (logged forest). Contrarily, the riparian reserve soil did not respond to rewetting and nitrogen application and fluxes were negligible. Furthermore, N₂O fluxes were on average about 10 times higher than NO fluxes. The fact that forest soils also have the potential to emit large amounts of N₂O and NO, has important implications for land-use change scenarios in the tropics, especially as some scenarios suggest atmospheric N deposition is likely to drastically increase in tropical regions due to biomass burning, increased N-fertilizer use and fossil fuel consumption. Quantification of related gene transcripts implied that Proteobacterial *nirS* and Ania-*nirK* (betaproteobacterial clade of *Neisseria*) containing denitrifiers might continuously contribute to the N₂O emissions, while the nitrifiers (ammonia oxidizing archaea in this study) are conditionally active to produce N₂O. This study therefore provides some

evidence for N₂O and NO emissions associated with phylogenetically diverse groups of microorganisms, which might be of importance in modulating the GHG emissions under different land-uses and field conditions.

Keywords: logged tropical forest, riparian areas, greenhouse gases, nitrification-denitrification, *AniA*, *nirK* and *nirS*, *amoA*, *nosZ*

INTRODUCTION

Increasing expansion of secondary forests, plantation mosaics and other human-modified habitats has changed the tropical landscape, which was once dominated by primary forests in Southeast Asia, and this might consequently jeopardize ecosystem service provision at local, regional, and global scales. For example, deforestation for oil palm (OP) agriculture, due to its high profitability, has become one of the world's most rapidly expanding cropping trend in tropical areas (Dislich et al., 2017). Asia-Pacific palm oil and forestry products each accounted for around a third of the embodied deforestation (Pendrill et al., 2019). As tropical forests represent a crucial ecosystem for global carbon (C) and nitrogen (N) cycles (Galloway et al., 2004; Bonan, 2008; Hedin et al., 2009), a better understanding of the effect of land-use conversion (from deforestation to oil palm plantation) on the key biogeochemical processes, including greenhouse gas (GHG) emissions, is required. Particularly, the fertilization regime for oil palm growth can result in low N use efficiency, resulting in common over-use of chemical fertilizers (Drewer et al., 2018). High levels of ammonium (NH₄⁺) and nitrate (NO₃⁻) fertilizers associated with oil palm agriculture will potentially increase nitrous oxide (N₂O) and nitric oxide (NO) emissions over time (Fowler et al., 2011), and modify the community structure of associated key soil microorganisms which play a central role in the N cycle, e.g., nitrifiers and denitrifiers.

N₂O has a much larger global warming potential than carbon dioxide (CO₂); 298 times larger on a 100-year time horizon (Myhre et al., 2013). Nitric oxide (NO) plays a major role in the formation of tropospheric ozone (O₃) which in itself is an important GHG (Lammel and Graßl, 1995). Tropical forest soils are known to be major sources of N₂O and NO (Werner et al., 2007; Liu et al., 2016), but relatively little is known of the impact of land-use and land-use-change on their fluxes and associated soil microbial communities in the tropics. These knowledge gaps make it difficult for tropical countries to determine their annual GHG emission inventories and develop effective mitigation strategies. The need to study GHG fluxes from forests and land converted from forests to agricultural use has been recognized (Van Lent et al., 2015). Due to growing concern of converting peatland to plantations and the associated negative environmental impacts due to soil subsidence and fires (nutrient depletion, soil erosion, destabilization of soil structure), increasingly plantations are established on mineral soil (Shanmugam et al., 2018). Much more attention has been given to studying carbon fluxes and storage from peatlands (Germer and Sauerborn, 2008; Hassler et al., 2015). Recent reviews of the non-CO₂ GHG emissions from tropical forests and

their conversion to agricultural and plantation crops, especially from Malaysia, is distinctively lacking (Van Lent et al., 2015; Shanmugam et al., 2018). There are insufficient number of studies of N₂O emissions from mineral soils to draw firm conclusions of the increase of N₂O emissions after land-use change from secondary forest to oil palm plantations (and also other plantations). The only field study including NO emissions as well as N₂O emissions was carried out in Sumatra, Indonesia (Hassler et al., 2017).

In terrestrial systems, heterotrophic denitrification and nitrification-related pathways are the primary sources of N₂O and NO production (Hu et al., 2015; Medinets et al., 2015). Soil denitrifiers, whose activity is dependent of different oxygen levels, can incompletely reduce nitrate (NO₃⁻) and nitrite (NO₂⁻) through nitrite reductase (encoded by *nirK* or *nirS* genes) to NO, which can be further reduced by nitric oxide reductase to N₂O as an end-product. Under limited oxygen conditions, N₂O can be catalyzed to N₂ by *nosZ* gene encoding the nitrous oxide reductase, responsible for mitigation of a large amount of N₂O produced from deeper soil layers (Clough et al., 2005). The abundance and expression of *nirS*, *nirK*, and *nosZ* genes are commonly used to indicate the denitrification-derived N₂O emissions in soils (Morales et al., 2010; Dandie et al., 2011; Krause et al., 2017). However, these studies usually used conventional primers targeting only Proteobacterial *nir* genes, which ignored highly diversified denitrifier communities from other bacterial phyla such as *Nitrospirae*, *Actinobacteria* and *Firmicutes* as well as archaeal and eukaryotic denitrifiers (Graf et al., 2014). Recent phylogenetic analysis also revealed novel clade II *nosZ* distinct to typical clade I *nosZ* denitrifiers, which might be responsible for an unaccounted N₂O sink in soils (Sanford et al., 2012; Jones et al., 2013). Nitrification-related N₂O and NO are mainly emitted by ammonia oxidizers in soils, including ammonia oxidizing archaea (AOA) and bacteria (AOB) (Bollmann and Conrad, 1998; Hink et al., 2017, 2018) and potentially by recently discovered complete ammonia oxidizers (comammox) (Kits et al., 2019). The relative contribution of denitrifiers and nitrifiers to N₂O and NO emissions varies in different ecosystems and largely depends on environmental conditions. Soil moisture is considered a critical factor determining the relative activity of denitrification and nitrification. It was observed that under 60% water-filled pore space (WFPS), N₂O was produced mainly from nitrification due to sufficient oxygen, while more anoxic soils with higher water content could stimulate the denitrification-derived N₂O (Bollmann and Conrad, 1998; Bateman and Baggs, 2005). However, the relative contribution of nitrification and denitrification to N₂O emissions can be quite variable under similar WFPS conditions (Liu et al., 2016), as emissions are potentially driven by different communities of nitrifiers and/or

denitrifiers in distinct land-use soils. The type of N applied and its availability are also crucial parameters influencing both the microbial activity (Hink et al., 2018) and the GHG production (Cowan et al., 2019). Indeed, high NO_3^- concentration favors denitrification pathways and results in N_2O emissions (Wang et al., 2013). There is also evidence that AOA prefer ammonium coming from mineralization of organic N, while AOB growth is preferential under high levels of inorganic ammonium supply (Di et al., 2009; Verhamme et al., 2011; Levičnik-Höfferle et al., 2012). In addition, N_2O yield (ratio of N_2O to NH_4^+ consumed) is higher in AOB than AOA (Hink et al., 2017, 2018), resulting in different amounts of N_2O emission under various fertilization strategies. It is therefore important to predict and identify the biological production of N_2O and NO emissions in tropical soils of different land uses, particularly with different soil moisture and nitrogen supply conditions. This can be achieved by targeting the activity of diverse groups of denitrifiers and nitrifiers using clade specific primers, in order to gain a comprehensive understanding of their activity and potential contributions to the GHG emissions.

To fill these knowledge gaps, we have carried out laboratory studies to investigate the soil N_2O and NO emission potentials and their underlying microbial processes in tropical forests and nearby oil palm plantations of different ages and a riparian reserve in Malaysian Borneo, Sabah within the Stability of Altered Forest Ecosystems (SAFE) project landscape (Ewers et al., 2011).

The specific objectives of the laboratory study were to investigate:

- 1) The impact of simulated rainfall events on dry soil on rates of N_2O and NO fluxes from logged tropical forest, riparian reserve, and oil palm plantation soil;
- 2) The N fertilization effects on soil N_2O and NO fluxes from oil palm and riparian soils.
- 3) The role of nitrifiers and denitrifiers in the production of N_2O and NO in the different land-use systems and under different N rates and moisture conditions.

MATERIALS AND METHODS

Site Description

This study was carried out within the Stability of Altered Forest Ecosystems (SAFE) project in Malaysian Borneo ($4^\circ 49' \text{N}$, $116^\circ 54' \text{E}$). The SAFE project was set up in Sabah in 2011 in a secondary forest area designated by the Malaysian government for conversion to plantations for palm oil production ($4^\circ 49' \text{N}$, $116^\circ 54' \text{E}$). It is a long-term landscape-scale experiment designed to study the effects of anthropogenic activity related to deforestation and oil palm agriculture on the ecosystem as a whole (Ewers et al., 2011). The soils at SAFE are classed as orthic Acrisols or Ultisols (Riutta et al., 2018). The climate in Malaysian Borneo is wet tropical with a typical wet season from November to March and a dry season from April to October. Average monthly temperatures of 32.5°C and average monthly rainfall was 164.1 mm (climate-data.org, 2019). At SAFE, the mean monthly rainfall during the 2 years of study period (2015 and 2016) was 190 mm (Walsh, unpublished data). Annual rainfall was 1,927 mm in 2015 and 2,644 mm in 2016.

For this study, we have selected logged forest soils from two different locations, two oil palm plantations of different ages, and a riparian area. All sites were within a 15 km radius. Soils were collected in November 2016, at the end of 2 years of *in situ* soil GHG flux measurements (Drewer et al., 2019a). At the start of the *in situ* measurements, we selected a young plantation, around 2 years old at the time we started the project (OP2) and another young to medium aged oil palm plantation, around 7 years old at the start of the project (OP7). So effectively, the plantations were 4 and 9 years old when the soils were collected for this study. However, for consistency with other publications in preparation, we will refer to these plantations as OP2 and OP7. The riparian area was adjacent and down slope from OP7. The general oil palm management was application of 2 kg N per palm three times per year in form of ammonium sulfate.

Experimental Setup

Soil was collected in November 2016 from the top 10 cm at randomized locations at each site and collected as a bulk sample, after brushing away any loose organic matter (leaf litter, twigs), of the logged forest, oil palm plantations, and riparian reserve. The soil was air-dried in the laboratory, then passed through a 2 mm sieve to remove large stones and twigs, and transported by air to the UK Center for Ecology and Hydrology, Edinburgh, Scotland, where the experiments were conducted following a similar protocol of previously published studies on dried, sieved and re-packed soil (Dick et al., 2001, 2006; Sanchez-Martin et al., 2010). It was not possible to conduct these experiments on fresh soil as these sites were very remote without permanent electricity and soils could not be kept cool after sampling. We carried out two experiments. Experiment 1 comprised oil palm soil from two oil palm plantations OP2 and OP7, and Experiment 2 incorporated logged forest soil from sites E and LFE. The common soil used in both experiments was from the riparian reserve (RR) adjacent to OP7. Details of the soil bulk density and pH values as well as total C and N of these sites are shown in **Table 1**. For the two experiments, 170 g of dry soil was placed into 5 cm diameter Perspex tubes (each land-use in triplicate), packed to approximately field bulk density and incubated at 25°C . An ammonium nitrate (NH_4NO_3) solution was applied to simulate rainfall ($5 \text{ kg N ha}^{-1} \text{ y}^{-1}$, J. Sentian pers.com.) to both experiments: Experiment 1: oil palm (OP2, OP7) and riparian reserve (RR) (objective 1) and Experiment 2: logged forest (LFE, E,) and riparian reserve (RR) (objective 1). In Experiment 1, 14 days after the addition of the low N dose, a second dose of NH_4NO_3 solution was added to simulate a typical N fertilization rate of $50 \text{ kg N ha}^{-1} \text{ y}^{-1}$ to OP2, OP7, and RR (objective 2). The soil was left to dry naturally over time (**Figure 1**). Cores were reweighed daily to monitor weight loss as a proxy for changes in soil moisture. The conversion from weight loss to soil moisture throughout the study period was inferred from the moisture content measured at the start and end of the incubation period. For Experiment 1 (oil palm soil), fluxes of NO, N_2O , CH_4 , CO_2 were measured on days 0, 1, 2, 4, 7, 11, 14, 15, 16, 18, 21, 24, 36 with the fertilization event on day 14. For Experiment 2 (forest soil), fluxes were measured on days 0, 1, 3, 6, 7, 8, 9, 15, 17, 22. Soil mineral N concentrations (NH_4^+ , NO_3^-) and

TABLE 1 | Soil properties from soil taken from the top 10 cm including mean pH, bulk density (g cm^{-3}), total Carbon (C) and total Nitrogen (N) in %, sd is standard deviation of $n = 3$.

Site	pH	sd	Bulk density	sd	Total C	sd	Total N	sd
OP2	4.87	0.07	0.75	0.07	0.70	0.21	0.05	0.02
OP7	5.08	0.21	0.78	0.04	0.97	0.47	0.07	0.05
LFE	7.09	0.14	0.70	0.08	3.21	2.04	0.24	0.14
E	6.31	0.23	0.81	0.07	6.40	6.72	0.38	0.26
RR	6.54	0.11	0.81	0.11	1.18	0.32	0.14	0.06

OP, oil palm plantation; LFE and E, logged forest; RR, riparian reserve.

samples for microbial analysis were taken from separate cores treated identically to the cores used for flux measurements, in order not to disturb the soil profile of the flux cores. Subsamples were taken from identical “clone” cores for pH, KCl extractable N (NH_4^+ , NO_3^-) and microbial analysis and stored at -20 and -80°C , respectively.

All experiments were carried out at 25°C reflecting average field conditions (soil temperature) in Sabah. On the first day the applications of N were staggered, in order to observe the immediate response (~ 0.5 h after application) of NO and GHG (N_2O , CH_4 , CO_2) fluxes and mineral N (NH_4^+ , NO_3^-) concentrations. On all subsequent measurement days, samples for GHG analysis were collected within 1 h from all treatments and before starting with the NO flux measurements, which took between 20 and 30 min for each core.

NO and N_2O Flux Measurements

Nitric oxide (NO) fluxes were measured from the soil cores using a gas flow-through system, as used in previous studies (Dick et al., 2006; Drewer et al., 2015). Ambient air, filtered through charcoal and aluminum/ KMnO_4 to remove O_3 and NO_x , was passed over the headspace of the core into the analyzers (flow rate 15 ml min^{-1}) to measure NO by using a chemiluminescent analyzer (42C $\text{NO-NO}_2\text{-NO}_x$, Thermo Environmental Instruments Inc., Franklin, MA, USA) and O_3 using a UV photometric O_3 analyzer (49C Thermo Environmental Instruments Inc., Franklin, MA, USA). As the NO_x and O_3 analyzer each require a flow rate of 11 min^{-1} , filtered laboratory air, to take out NO_x and O_3 , was added to make up the volume. The NO_x and O_3 analyzers were calibrated against a zero air standard before starting this study. Ozone concentrations were only measured to ensure that concentrations were sufficiently low (<4 ppb) to avoid reactions with NO_x . The flow rates through the core and into the analyzer were monitored using mass flow meters (Aera FC 7700C; Advanced Energy Industries Inc., Fort Collins, CO, USA). NO and O_3 concentrations, air temperature and flow rates were recorded at 10 s intervals using a $21\times$ data logger (Campbell Scientific, Shropshire, UK). Typically, measurements from each core lasted ~ 20 – 30 min and until the NO concentration reached equilibrium. These measurements were interspersed with NO concentration measurements from empty control cores, in order to take into account reactions with chamber walls and lids. The

NO flux ($\text{ng N g}^{-1} \text{ h}^{-1}$) was calculated as the product of the flow rate of the air stream through the repacked soil core, the increase in NO concentration above the control (empty core) and the dilution rate, by supplying additional air to the analyzer, divided by the soil dry weight (170 g).

GHG fluxes were always measured within 4 h of measuring the NO fluxes. The cores were sealed with a rubber bung for a period of 20 min, and air samples were extracted from the headspace at time 0, 10, and 20 min, using 10 ml syringes fitted with a luer lock. The extracted gas samples were analyzed within 4 h by gas chromatography (GC). The samples and three sets of four certified standard concentrations (N_2O , CH_4 in N_2 with 20% O_2) were analyzed using a GC (Agilent GC7890B; Agilent, Santa Clara, California) with micro electron capture detector (μECD) for N_2O analysis and methanizer and flame ionization detector (FID) for CH_4 and CO_2 analysis. These detectors were setup in parallel, allowing the analysis of all three GHGs at the same time. The limit of detection was 5 ppb for N_2O , 40 ppb for CH_4 and 5 ppm for CO_2 . Peak integration was carried out with OpenLab® Software Suite (Agilent, Santa Clara, California). The GHG flux was calculated as the product of the increase in concentration over time and volume of the headspace in the repacked soil core, divided by the time the core was sealed and the soil dry weight.

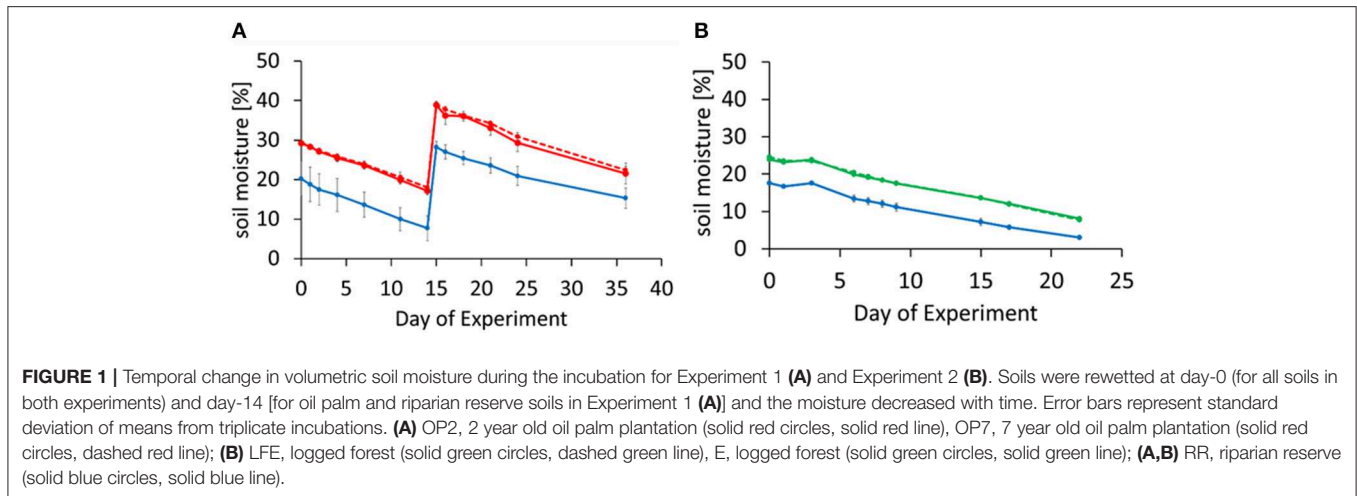
Soil Nutrient Analysis

To determine soil mineral N (NH_4^+ , NO_3^-) content, 6 g of soil was extracted with 20 ml of 1 M KCl solution for 1 h at 100 rpm on an orbital shaker (Stuart Orbital Shaker SSL1 Barloworld Scientific Ltd.). The extract was filtered through Whatman No 42 filter paper and frozen until analysis. Concentrations of NH_4^+ and NO_3^- were analyzed by colorimetric methods (Harwood and Huyser, 1970; Henriksen and Selmer-Olsen, 1970) using the SEAL AQ2 discrete analyzer. Gravimetric soil moisture contents were determined by drying approximately 10 g of soil at 105°C until constant weight, usually for 24 h. Moisture content of the individual cores was calculated from the weight difference between the wet and oven dried soils.

Bulk density was measured from samples directly taken in the field at the time of collecting the soil for the incubations. Soil samples were collected one-off using a galvanized iron ring (98.17 cm^3) with a sharp edge that was inserted in the upper soil layer with a hammer to 5 cm depth without compaction. Samples were oven-dried at 105°C until constant weight (usually 48 h) and bulk density (g cm^{-3}) was calculated based on the dry weight occupying the volume of the ring. Soil pH was measured using a MP 220 pH meter (Mettler Toledo GmbH, Schwerzenbach, Switzerland) from soil solutions of a 1:2 ratio, consisting of 10 g of soil and 20 ml of deionized water. Total C and N in soil was analyzed by high temperature combustion gas chromatography (Vario El III C/N analyzer; Elementar, Stockport, UK) using 30 mg of soil.

Soil Microbial Analysis

Even though sub-samples for microbial analysis were taken every time a flux measurement was carried out, resources were limited and we had to select a subset of samples for analysis. N_2O



fluxes were used as selection criterion in order to be able to determine potential processes leading to N_2O emissions. For each soil (including all three replicates), five time points during incubation were used for downstream transcript-based analysis, including day-0, the time point with highest N_2O emission, two intermediate time points and the incubation endpoint. Consequently, we selected samples from day-0, 1, 4, 7, and 14 for OP2 and OP7 soils, and day-0, 1, 3, 6, and 15 for RR, LFE, and E soils. Only one set of RR soils (from experiment 2) was used for microbial analysis as in both experiments fluxes from the RR soil were very low and did not show any discernible trend.

Soil RNA Extraction and cDNA Synthesis

The RNA extractions were performed using RNeasy PowerSoil Total RNA Kit (QIAGEN) from 2g of each soil sample. The nucleic acid extracts were further treated with TURBO DNA-free™ Kit (Thermo Scientific) to eliminate DNA residuals. Reverse transcription was performed using random hexamer primers and Maxima Reverse Transcriptase (Thermo Scientific) following manufacturer recommendations. RNA without reverse transcription step was used as a negative control by PCR of target genes to ensure complete removal of genomic DNA.

Quantitative Reverse Transcription PCR

The presence of ammonia monooxygenase subunit A (both archaeal and bacterial *amoA*), nitrite reductase (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*) genes from the N_2O producing and reducing microbes were tested by using specific primers targeting diverse groups of ammonia oxidizers and denitrifiers. In particular, for the nitrite reductase gene, we attempted to cover phylogenetically diverse clades (based on currently known diversity) for the quantitative analysis. To achieve this, we selected multiple clade specific primers for both *nirS* and *nirK* genes based on an *in silico* evaluation of different primer pairs (Bonilla-Rosso et al., 2016). Two *nosZ* primer pairs were used to cover both conventional clade I (Henry et al., 2006) and newly established clade II lineages. All selected primer pairs were tested using DNA extracts from soil samples,

based on the theoretical melting temperature of the primers. Consequently, seven primer pairs resulting in clear target PCR fragments visualized from agarose gel electrophoresis were used for quantitative reverse transcription PCR (RT-qPCR) assay, including bacterial *amoA*, archaeal *amoA*, *Proteobacteria nirS*, *Proteobacteria nirK*, *AniA*-affiliated *nirK*, clade I, and clade II *nosZ* genes (Table S1).

Abundance of target gene transcripts were determined on a Mastercycler RealPlex2 (Eppendorf, Germany). Each quantitative reverse transcription PCR reaction was performed in a 20 μ L mixture containing 10 μ L of QuantiFast™ qPCR master mix (Qiagen, UK), 0.2 mg mL^{-1} bovine serum albumin (BSA), 0.6 μ M of each primer and 2.5 μ L of cDNA (2.5-fold diluted). The primers and cycling-parameters for archaeal and bacterial *amoA* genes were used as previously described (Hink et al., 2017). Transcript abundance of proteobacterial *nirS* was determined by primer pair NirS1F/R3cd, with following cycling-parameters: 5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 56°C, 45 s at 72°C and a plate read after incubation for 15 s at 80°C. Proteobacterial *nirK* transcript abundance was quantified using primer pair Cunir3F/nirK5R and the cycling-parameters as following: 5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 62°C, 45 s at 72°C and a plate read after incubation for 15 s at 80°C. For *AniA*-affiliated *nirK* transcript, the primers were nirKA2_4F/nirKA2_4R and the cycling-parameters were: 5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 52°C, 45 s at 72°C and a plate read after incubation for 15 s at 80°C. The primers and cycling-parameters for quantification of clade I and clade II *nosZ* genes were described previously (Henry et al., 2006; Jones et al., 2013). Standard templates with dilution series containing 10^1 - 10^7 copies of target genes were used. For bacterial and archaeal *amoA* transcript quantification, standards were used as previously described (Hink et al., 2017). For *nirS*, *nirK*, and *nosZ* transcripts, standards were established from clone plasmids inserted with target gene fragments amplified by corresponding primers. Amplification efficiencies ranged from 82 to 98%, with R^2 values >0.99. Melting curve analysis and standard agarose gel electrophoresis were always performed after each qPCR run to assess amplification specificity.

Statistical Analysis

We used linear regression to analyze the relationship between the qPCR results and the fluxes of N₂O and NO. The model included the abundance of the five gene transcripts plus soil moisture, which varied as the cores dried, the effect of which we wanted to remove. We log-transformed the fluxes and the gene abundance data, as they varied over several orders of magnitude. All statistical analyses were conducted in R version 3.5.1 (R Core Team, 2017).

For each soil, one-way ANOVA was performed for comparison of transcript abundances at different time points for *nirS*, *AniA-nirK*, archaeal *amoA*, and clade I *nosZ* genes, respectively. Clade II *nosZ* transcript abundances were mostly lower than detection limit in soils, thus were precluded for statistical analysis.

RESULTS

Soil Properties

The two oil palm soils were more acidic, with a pH around 5, compared to the logged forest or riparian soils, which were between pH 6.5 and 7 (Table 1). Bulk density was similar at all sites. The percentages of total C and total N were much larger in the forest soils compared to the oil palm and riparian soils. Rewetting the soils with equal volumes of water on day-0 of the respective experiments resulted in the oil palm soils reaching a volumetric soil moisture content of around 30%, forest soils around 25%, and riparian soils around 20% (Figures 1A,B). All soils dried out at the same rate.

Nitrogen Fluxes (N₂O and NO)

Although we measured the three greenhouse gases nitrous oxide (N₂O), methane (CH₄) and carbon dioxide (CO₂), we will only present the N₂O results in relation to the nitric oxide (NO) fluxes and microbial analysis which links to nitrification and denitrification processes. The microbial results would not be able to explain the carbon fluxes. Methane fluxes fluctuated around zero for all land-uses with no discernible trend whilst CO₂ showed an initial pulse and then decreased over time. The results for CH₄ and CO₂ are available in Figures S1, S2.

In Experiment 1, both oil palm soils (OP2, OP7) showed peak emissions after rewetting the soil with the equivalent of 5 kg N ha⁻¹ as ammonium nitrate solution. Highest measured mean N₂O fluxes of around 130 ng N g⁻¹ h⁻¹ for OP2 were measured on day-1, whilst highest N₂O fluxes for OP7 were measured at day-3 with an average of 45 ng N g⁻¹ h⁻¹, however, the emission peak was prolonged over a few days whilst for OP2, fluxes were down to single digits after the initial pulse (Figure 2A). This was also notable in NO fluxes, where the peak for OP2 at day-3 reached a mean of 12 ng N g⁻¹ h⁻¹ and for OP7 only 2 ng N g⁻¹ h⁻¹. Generally, NO fluxes were a factor of 10 lower than N₂O fluxes (Figure 2B). After the initial peak, fluxes of N₂O and NO decreased substantially. Fluxes of N₂O and NO from the riparian soil (RR) were barely above zero. The fertilization event on day 14 resulted in only slightly elevated N₂O fluxes from the oil palm and riparian soils (15 and 20 ng N g⁻¹ h⁻¹), within the first 3 days after N application. The effect of applying 50 kg N

ha⁻¹ on N₂O was a lot smaller than the initial re-wetting with a N concentration ten times lower. The fertilization event did not increase NO emissions from the OP and RR soils.

Soil mineral N concentrations in Experiment 1 did not show a discernible trend over the duration of the experiment and the standard deviation of the three replicates was high, especially for NO₃⁻ (Figure 2C). Mean values were around 50 μg NO₃⁻-N g⁻¹ of dry soil for oil palm and riparian soil alike. NH₄⁺ concentrations increased from around 20 μg NH₄⁺-N g⁻¹ of dry soil on day-0 to about 75 μg NH₄⁺-N g⁻¹ of dry soil on day-7 and then decreased again (Figure 2D). Standard deviation of the three replicates was, however, too large to identify differences amongst the OP and RR soils.

In Experiment 2, comparing logged forest soils (LFE, E) with riparian (RR) soil, N₂O fluxes also peaked on day-1 at a mean of 95 ng N g⁻¹ h⁻¹ for LFE and 145 for E, whilst fluxes from the riparian soil were barely above zero (Figure 3A). Similar to experiment 1, NO fluxes were about a factor of 10 smaller and peaked slightly later (day-4) with 25 ng N g⁻¹ h⁻¹ for LFE and 5 ng N g⁻¹ h⁻¹ for E (Figure 3B). Again, fluxes from the riparian soil (RR) were barely above zero for the entire duration of the experiment. Mineral N concentrations were higher than from the oil palm soil, NO₃⁻ increased from around 100–300 μg N g⁻¹ of dry soil with no discernible difference between forest and riparian soil (Figure 3C), NH₄⁺ increased from about 50–200 μg N g⁻¹ of dry soil on day-9, again with high variability within the three replicates of each land-use (Figure 3D).

Abundance of the Gene Transcripts

The transcript abundances of genes related to N₂O production and reduction were quantified over time to identify the potential pathways and organisms responsible for NO and N₂O emission during the incubation. Gene transcript abundances (Table 2) are displayed as mean values of three replicates per soil and date along with their standard error, which was usually quite high. The proteobacterial *nirS* transcripts were continuously detected but displayed no significant changes throughout the incubation in most of the soils, except for the OP2 soil where the transcript abundance was highest at day-7. The expression of denitrifier *nirK* genes was not detected throughout incubation in any of the soils by conventional primers targeting mainly *Proteobacteria* clades. Surprisingly, the transcripts of *AniA-nirK* gene, which encodes copper-containing nitrite reductase from the *Neisseria* betaproteobacterial clade, were detected abundantly in all soils using the newly developed primers specific to this clade. In most soils, however, the transcript abundance did not change significantly during incubation, except for logged forest E soil. Notably, despite large variability among replicates, *AniA-nirK* transcript abundance in OP2 soil was highest during the earliest period of incubation (day 1–4), which was consistent with the pulse of N₂O emission. In addition, the transcript abundance in LFE soil peaked at day-1 and then sharply decreased, displaying a similar trend to the N₂O emissions. For ammonia oxidizers, archaeal *amoA* transcripts were below detection limit in OP7 and E soils during the earliest period of incubation (day 0–4), but highly increased after 7 days of incubation ($p < 0.05$ for OP7). Additionally, archaeal *amoA* transcripts were only detected

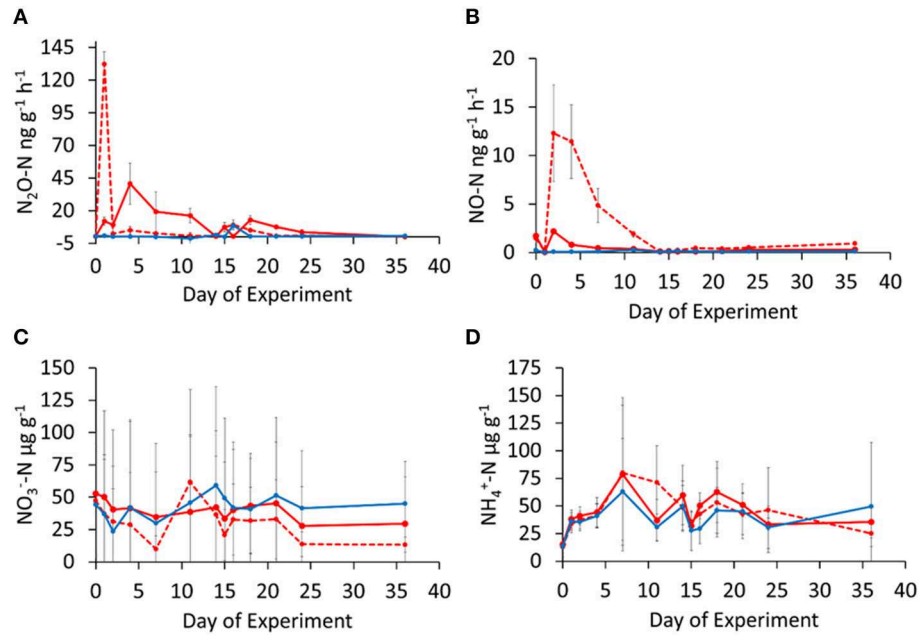


FIGURE 2 | Temporal changes in N₂O (A) and NO (B) emission rates and soil NO₃⁻ (C) and NH₄⁺ (D) concentrations in oil palm (OP2: solid red circles, solid red line and OP7: solid red circles, dashed red line) and riparian reserve (RR: solid blue circles, solid blue line) soils. Error bars represent standard deviation of means from triplicate incubations. Soils were rewetted at day-0 with N supply at 5 kg N ha⁻¹ equivalent to field N deposition condition, and at day-14 with NH₄NO₃ amendment at 50 kg N ha⁻¹ simulating a field N fertilization.

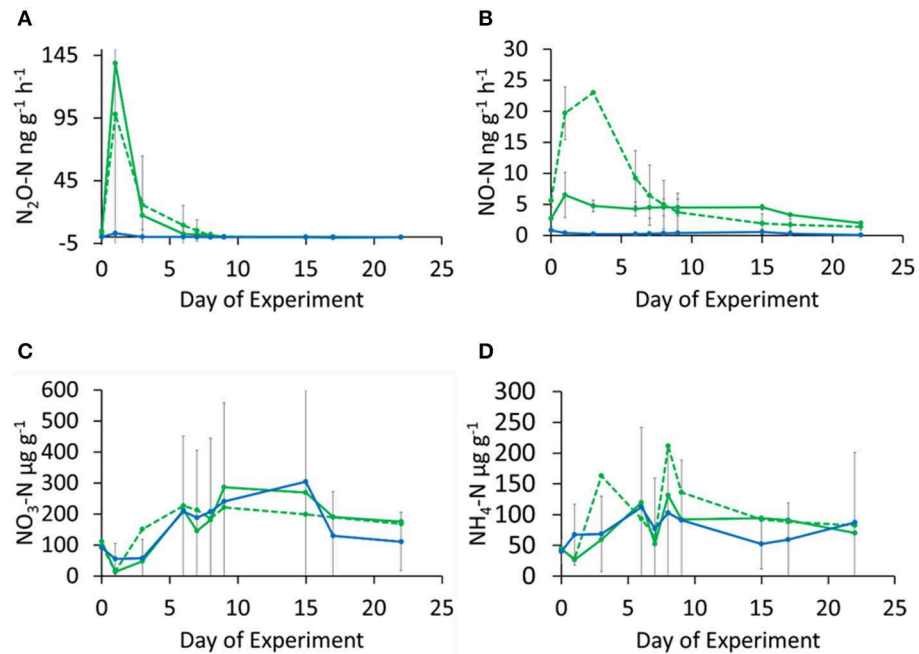


FIGURE 3 | Temporal changes in N₂O (A) and NO (B) emission rates and soil NO₃⁻ (C) and NH₄⁺ (D) concentrations in logged forest (LFE: solid green circles, dashed green line and E: solid green circles, solid green line) and riparian reserve (RR: solid blue circles, solid blue line) soils. Error bars represent standard deviation of means from triplicate incubations. Soils were rewetted at day-0 with N supply at 5 kg N ha⁻¹ equivalent to field N deposition condition.

in LFE soils between days 3 and 6, indicating stimulation of archaeal ammonia oxidation during this period. However, their transcripts were not detected in OP2 and RR soils throughout

the incubation time, implying either absence or very low AOA activity. Similarly, the bacterial *amoA* transcript abundances were below detection limit in all soils, suggesting absence of bacterial

ammonia oxidation activity in these soils under such conditions. Low abundance of clade I and II *nosZ* transcripts were detected in a few time points in several soils, although genes from clade I were more frequently transcribed than clade II. Interestingly, expression of clade I *nosZ* genes was detected in RR, LFE and OP2 soils at day-1 when the N₂O emissions were highest. This suggested that the balance between N₂O emission and reduction is toward the net production in such experimental design under aerobic conditions.

Statistical Analysis

Based on the adjusted r^2 , the linear model explained 48% of the variation in the log N₂O flux. N₂O flux increased with *AniA-nirK* and *nosZ-I* genes, as well as soil moisture ($p < 0.03$), and decreased with the *Proteobacteria-nirS* gene. The effects of *archaeal-amoA* and *nosZ-II* were inconsistent. The residual standard error was 1.7 on 18 degrees of freedom. Applying the same model to fluxes of NO showed no consistent effects of any variable. **Figures 4, 5** show the N₂O and NO data plotted, respectively, with lines fitted with separate slopes and intercepts for each gene in each land-use type. This shows some differences across land-use types, but the sample size in these combinations is too small to draw strong conclusions.

DISCUSSION

N₂O and NO Fluxes

This experiment was designed to identify the emission potential of N₂O and NO, with focus on the important drivers of microbial nitrification and denitrification rates, soil moisture and nitrogen additions. The focus of this study lies on potential emissions as a result of the pulsing effect and N fertilizer additions. The pulsing effect, also known as the Birch effect, is the burst of soil microbial activity after rewetting dry soil. The onset of a rain event re-activates dormant microorganisms and stimulates soil organic matter turnover, fueled by nutrient accumulation from the drought-induced death of microorganisms and fine roots (Borken and Matzner, 2009). In our study, the pulsing effect significantly stimulated N₂O and NO emissions for both, the forest and oil palm soil. Drying and sieving soil can cause loss of aggregate structure, which could affect the denitrification rate (i.e., can increase the capacity to denitrify nitrate) or nitrate ammonification by reducing the overall abundance of anaerobic microsites in the soil (Patten et al., 1980; Sexstone et al., 1985). Consequently, the nitrous oxide fluxes observed in our experiment may be conservative, because they represent the lower limit of what is possible. Interestingly, the magnitudes of N₂O and NO fluxes were very similar for the oil palm and forest soils. In fact, the highest fluxes were measured from the forest soil and not the oil palm as might have been expected from field measurements (Drewer et al., 2019a). As the incubations were carried out in a controlled environment, we conclude that soils of both land-uses (logged forest and oil palm plantation) have the same potential for N₂O and NO emissions. The forest soils had much larger total C and N concentrations compared to the oil palm soils (**Table 1**). The wet and warm climate of tropical forests is conducive to fast mineralization rates of organic matter, and

consequently large sources of N₂O emissions. Not surprisingly, tropical forests are the largest natural source of N₂O (Zhang et al., 2019). The riparian soil did not respond to wetting and added nitrogen with increased fluxes of either N₂O or NO. In both sets of experiments, which included the riparian soil as the common soil, the riparian soil did not respond to wetting or nitrogen addition through increased N₂O or NO fluxes. Only a small N₂O peak was measured after addition of 50 kg N ha⁻¹ and such low N₂O fluxes may be due to complete denitrification to N₂, but this could not be tested in our study. The oil palm plantations sampled for this study were still quite immature. In this area in Sabah, the soil was terraced before planting which is adding another level of disturbance to the soil after logging. Especially in the very young plantation (OP2) the soil was still very disturbed at the time of sampling and had no visible layer of organic matter yet. The slightly older OP7 plantation had a small visible layer of organic matter in the top soil but a lot less than the logged forest soil.

Field measurements of soil N₂O emissions, using static chambers at the same locations where the soil for the incubations was taken from, also showed occasional high N₂O emissions from the logged forest, however, usually fluxes were higher from oil palm and riparian reserve than logged forests (Drewer et al., 2019a). However, it is difficult to compare actual magnitudes of fluxes due to the nature of artificial conditions in the laboratory incubation with small amounts of soil only. The only other study in southeast Asia that reported both N₂O and NO fluxes from oil palm plantations in Indonesia, also reported higher N₂O than NO fluxes, but with varying ratios from 3 to 10 (Hassler et al., 2017), of which our study would be at the high end. Their reported study was carried out in the field whilst ours was carried out under controlled conditions in the laboratory, preventing direct comparison. Spatial variability in the field was also quite high as typical for this kind of studies. Our measured N₂O fluxes from the logged forest soil after the relatively short-lived rewetting pulse (<1 week) were in the range of 2–200 $\mu\text{g m}^{-2} \text{h}^{-1}$ (when converting units using the diameter of the packed soil cores as surface area) and therefore not dissimilar to other reported field studies from tropical forests summarized by Castaldi et al. (2013) with 10–85 $\mu\text{g m}^{-2} \text{h}^{-1}$. This strengthens the fact that the forest soils from Sabah used in our study have the potential to emit substantial amounts of N₂O under certain conditions. Forest soil and oil palm soil showed similar trends under the same conditions. In contrast, the riparian soil did not show elevated emissions (of NO or N₂O) after rewetting and N application. This could not be explained by any other measured parameters such as soil properties, mineral N availability and microbial activity (see below). Currently there is a distinct lack of N₂O measurements from riparian areas (Luke et al., 2019) and more research is needed to understand these processes.

Diversity and Activity of Denitrifiers

Our study tested multiple primer pairs covering phylogenetically diverse denitrifier clades to provide a comprehensive evaluation of potential activities of denitrifiers, including archaea and eukaryotes, which were overlooked from most previous studies using conventional *Proteobacteria nirS* and *nirK* primers.

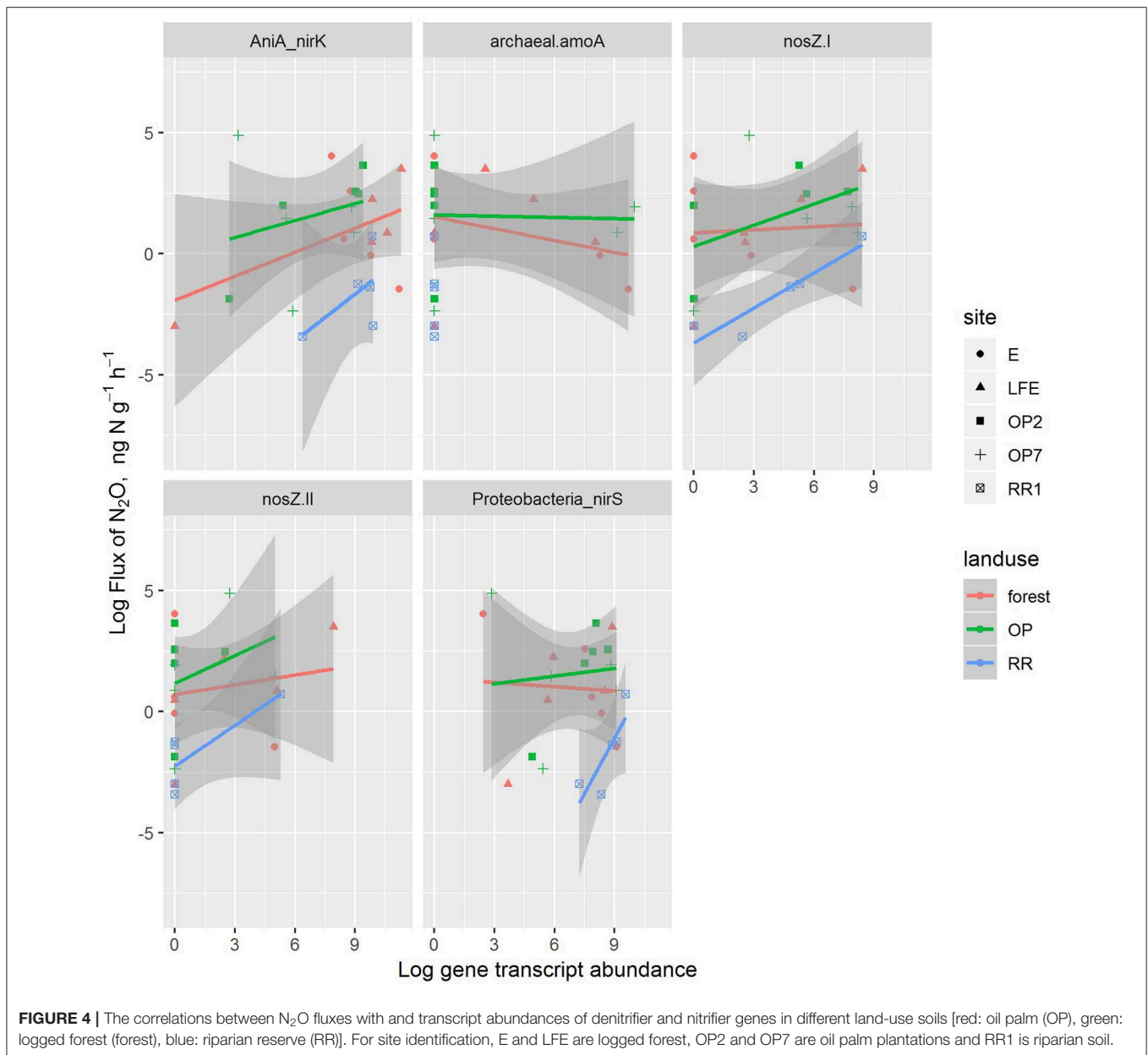
TABLE 2 | Mean gene transcript abundance from selected days of the incubation experiments with oil palm and forest soil from Sabah.

Day of experiment	Nos Z I				nos Z II							
Oil palm	OP2	se	OP7	se	OP2	se	OP7	se	OP2	se	OP7	se
0	0	0	0	0	0	0	0	0	0	0	0	0
1	3,629	2,433	1,296	1,296	617	617	1,229	1,229				
4	1,858	1,022	4,638	3,717	0	0	1,271	680				
7	2,242	429	3,696	416	0	0	0	0				
15	0	0	2,796	506	0	0	0	0				
Forest	LFE	se	E	se	RR	se	LFE	se	E	se	RR	se
0	608	608	0	0	1,896	968	1,408	708	0	0	0	0
1	4,604	893	0	0	4,908	1,566	2,883	607	0	0	1,913	1,051
3	2,296	1,467	0	0	946	475	571	571	0	0	0	0
6	717	717	1,733	1,733	488	488	0	0	0	0	0	0
15	0	0	2,833	191	0	0	0	0	1,213	653	0	0
	AniA_nirK				nirS							
Oil palm	OP2	se	OP7	se	OP2	se	OP7	se				
0	1,163	1,163	4,671	2,500	1,038	525	2404	1386				
1	10,488	2,714	4,292	4,292	2,896	598	1,792	1,792				
4	13,029	3,613	2,796	1,409	3,304	373	5,225	3,720				
7	8,638	1,469	7,763	608	6,233	1,577	8,983	1,286				
15	3,171	2,509	7,042	844	1,858	230	6,883	859				
Forest	LFE	se	E	se	RR	se	LFE	se	E	se	RR	se
0	54,292	26,649	4,779	609	9,546	1,122	6,613	3,499	2,692	368	9,229	1,056
1	84,750	17,518	2,671	671	21,708	7,833	7,775	2,378	508	508	17,617	7,945
3	49,117	32,842	6,667	1,140	17,417	723	7,354	5,946	1,945	363	7,738	1,806
6	26,204	11,146	29,946	20,851	10,396	6,101	3,408	1,892	7,133	4,998	5,313	1,981
15	688	688	75,833	8,877	7,883	6,065	988	494	9,375	1,868	2,158	523
	archaeal amoA											
Oil palm	OP2	se	OP7	se								
0	0	0	0	0								
1	0	0	0	0								
4	0	0	0	0								
7	0	0	9,379	790								
15	0	0	22,042	1,292								
Forest	LFE	se	E	se	RR	se						
0	0	0	0	0	0	0						
1	663	663	0	0	0	0						
3	1,200	686	0	0	0	0						
6	3,242	593	4,692	2,121	0	0						
15	0	0	26,692	16,510	0	0						

Mean values of three cores with standard error (n = 3). OP, oil palm plantation; LFE and E, logged forest; RR, riparian reserve.

After testing different primers DNA samples from our soils, we detected the presence of *Proteobacteria* and AniA-related denitrifiers, which potentially contributed to the NO and N₂O emission in the soils. The proteobacterial *nirS* genes were transcribed throughout the incubation, while conventional proteobacterial *nirK* transcripts were undetected, although a DNA-based approach demonstrated presence of *nirK* genes in 4 of the soils (based on standard PCR with gel electrophoresis, data not shown). Therefore, despite being present, the *nirK*-containing denitrifiers were not active under these incubation

conditions. This is in accordance with previous studies, where stimulation of *nirK*-containing microbes only occurred following amendment of carbon substrates such as leaf residues (Liu et al., 2010; Wertz et al., 2016), while *nirS* transcripts were always detected irrespective of nutrient conditions. This suggests that the activity of *nirK*-containing denitrifiers is conditional and likely limited by carbon sources while *nirS*-containing denitrifiers may play a ubiquitous role in the N₂O production under different environments and conditions. The transcripts of *Neisseria*-affiliated *nirK* gene (AniA-*nirK* gene) were abundantly detected

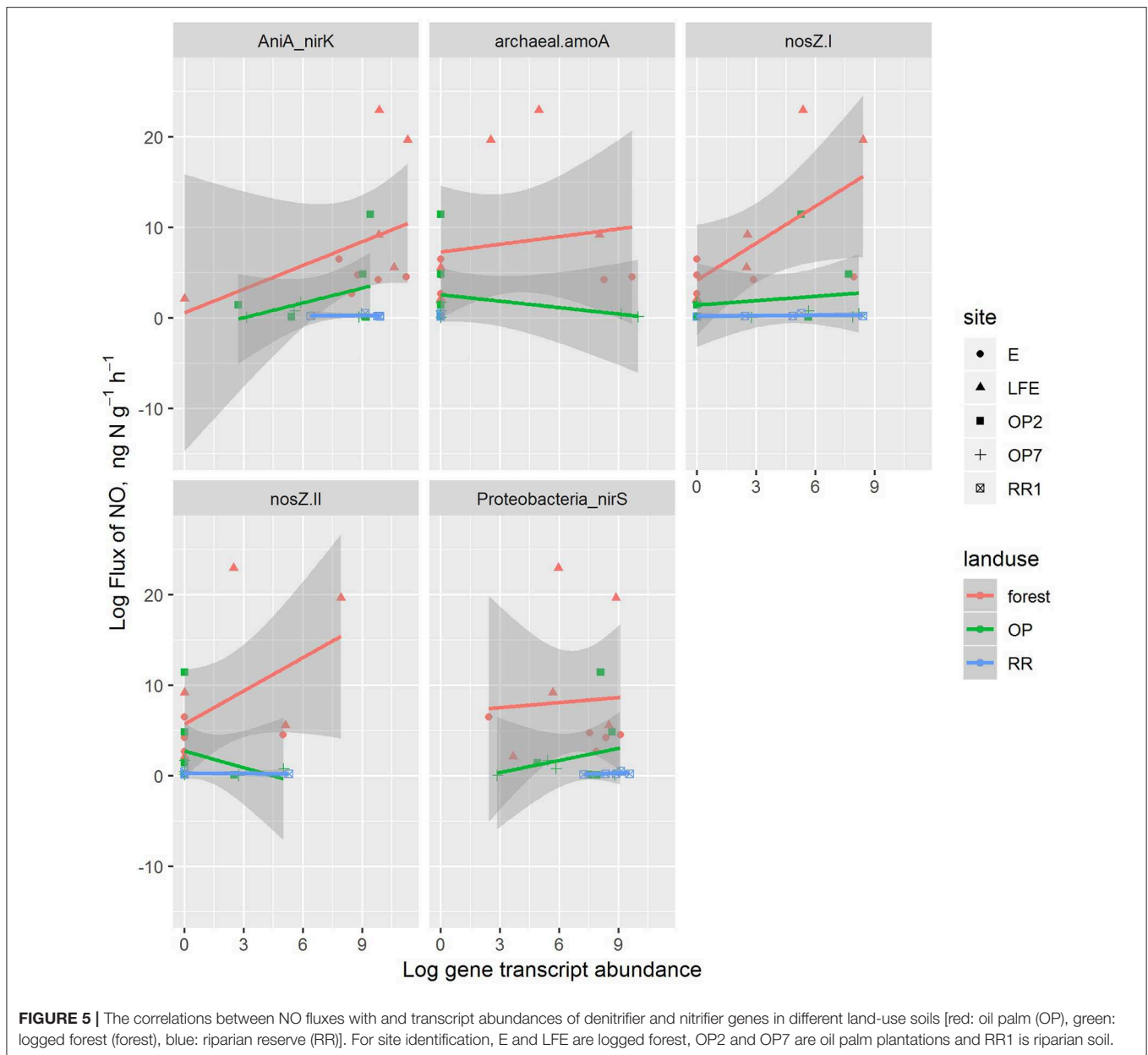


in all soils, with highest level of transcription in OP2 and LFE soils when N₂O emission reached a peak (**Figure 4**), resulting in a positive correlation between *AniA-nirK* transcription and soil N₂O emissions (**Figure 5**). *AniA-nirK* gene encodes an outer membrane lipoprotein with significant homology to the copper-containing nitrite reductases (Mellies et al., 1997). Despite being classified as *Proteobacteria*, phylogeny of *AniA-nirK* genes placed *Neisseria* as a distant outgroup from conventional clades of *nirK*-containing *proteobacteria* (Sharma et al., 2005; Graf et al., 2014; Wertz et al., 2016), suggesting that the physiology of *Neisseria* as denitrifiers might be distinct from other *nirK*-containing *proteobacteria*. The role of *Neisseria* to N₂O production has not been identified in many ecosystems (including soils), but their denitrification activities were confirmed in pure isolate,

with aerobic denitrification being an alternative pathway possibly enhancing the growth of pathogen *Neisseria meningitidis* (Anjum et al., 2002; Rock et al., 2005). In addition, detection of *AniA-nirK* transcripts in our soils suggest that this clade might be responsible for some N₂O emission in soils, which has been neglected in previous studies.

Nitrification vs. Denitrification Implication in N₂O Production

The rewetting of soils led to an immediate pulse of the N₂O emission on day-1 of the incubation (except for OP2 for which the emission peaked at day-4). The transcriptional activity of genes related to N₂O production indicated denitrification rather than ammonia oxidation as the main contributor to the N₂O



pulse emission. The nitrite reductase genes (*AniA-nirK* and *nirS*) were constantly transcribed throughout the incubation in all soils, indicating that the denitrification enzymes might be constitutionally expressed and rapidly activated in response to favorable condition, such as increased water content, which allows for more available substrates. Additionally, the N_2O emissions after the N fertilization were lower than those after the first rewetting event (with low N supply) in oil palm soils (Figure 2), suggesting that the higher emissions were mainly attributed to sufficient water availability rather than soil inorganic N amendment after the first rewetting event. The constitutional expression of nitrite reductase genes may lead to continuous presence of denitrifiers in soils, as suggested by previous study (Liu et al., 2010), which might be activated

immediately after enhanced water availability, contributing to the immediate pulse emission of NO and N_2O during the incubation.

At the later period of the incubation, the *amoA* abundance either increased or was detected, indicating that ammonia oxidation activity might also have contributed to soil N_2O emissions. As these transcripts were only stimulated at the later period of the rewetted incubation in three of the soils, a longer adaptation of these organisms to environmental condition might occur before their enzymatic activation. The relative contribution of denitrification vs. ammonia oxidation was difficult to estimate based on gene transcription only, especially as different soil core replicates were used for gas fluxes and transcription microbial analysis (as the flux cores could not be destructively sampled) and

due to high measurement variability both for gas fluxes and gene transcript abundances.

NH_4NO_3 fertilization to oil palm soils on day-14 stimulated a second pulse of N_2O emission, however, smaller than the first one. The NH_4NO_3 fertilizer serves as both NH_4^+ and NO_3^- sources that can be converted to N_2O by both nitrification and denitrification pathways. Ammonia oxidizers use carbon dioxide for biomass synthesis and previous studies demonstrated enhanced nitrification-based N_2O production upon ammonium-based fertilization (Hink et al., 2017, 2018). Therefore, ammonia oxidation could have contributed to the second N_2O emission pulse after N fertilization in the oil palm soils. However, the second N_2O pulse was lower compared to the first one both for OP2 (at day-4) and OP7 (at day-1), and this was likely caused by the restricted denitrification activity. In addition, there was no increase in the riparian soil that received the same treatment. Since the nitrogen source was sufficiently applied, we suspect that the depleted organic carbon might be the limiting factor affecting the denitrification-related N_2O production in our soils during this period.

CONCLUSIONS

Our study established that under controlled laboratory incubations logged forest and oil palm soils have equally high potential for N_2O and NO emissions following an increase in soil moisture, while riparian reserve soil releases constantly lower rates of N_2O and NO independently of soil moisture condition. The nitrogen based mineral fertilization induced the N_2O emission in soils, suggesting enhanced GHG emission potential after conversion of forest land for agriculture use. Proteobacterial *nirS* and *AniA-nirK* containing denitrifiers and archaeal ammonia oxidizers appeared to be the main contributors to N_2O emissions, with their activity differed under different conditions. Although it is difficult to make precise estimations on the GHG emissions and the relative contributions of different microbial communities to the emissions due to high variability among replicates, the present study suggests intensified N_2O and NO

emissions and impaired ecosystem functioning stability against environmental perturbation following deliberate anthropogenic exploitation of tropical forests.

DATA AVAILABILITY STATEMENT

The datasets generated/analyzed for this study can be found in <https://doi.org/10.5281/zenodo.3614876> (Drewer et al., 2019b).

AUTHOR CONTRIBUTIONS

JD and US have designed the experiment, in collaboration with ML and JS. ML carried out the laboratory experiment. JD analyzed the flux and soil data. CG-R designed the microbial analysis. JZ and CG-R analyzed the soil microbial transcript activity. PL advised on statistical analysis. JD, JZ, CG-R, and US wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ffgc.2020.00004/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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