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Functional structures of soil microbial community relate to contrasting N_2O emission patterns from a highly acidified forest

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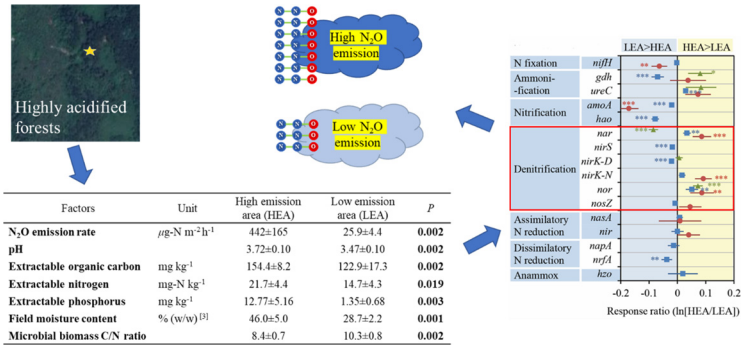
HIGHLIGHTS

- N_2O emission rates were high in the TSP forest but also diverse in different sites.
- Nitrogen cycling function genes were diverse in acidified forest soils.
- Functional genes in denitrifiers were positively correlated to N_2O emission.
- Phosphorus showed significant influence on microbial gene communities.

GRAPHICAL ABSTRACT



Factors	Unit	High emission area (HEA)	Low emission area (LEA)	P
N_2O emission rate	$\mu g\text{-}N\ m^{-2}\ h^{-1}$	442±165	25.9±4.4	**0.002**
pH		3.72±0.10	3.47±0.10	**0.002**
Extractable organic carbon	$mg\ kg^{-1}$	154.4±8.2	122.9±17.3	**0.002**
Extractable nitrogen	$mg\text{-}N\ kg^{-1}$	21.7±4.4	14.7±4.3	**0.019**
Extractable phosphorus	$mg\ kg^{-1}$	12.77±5.16	1.35±0.68	**0.003**
Field moisture content	% (w/w) [3]	46.0±5.0	28.7±2.2	**0.001**
Microbial biomass C/N ratio		8.4±0.7	10.3±0.8	**0.002**

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ABSTRACT

Nitrous oxide (N_2O) is an important greenhouse gas contributing to global climate change. Emissions of N_2O from acidic forests are increasing rapidly; however, little is known about the mechanisms driving these emissions. We analyzed soil samples from a high N_2O emission area (HEA, 224–601 μg N m^{-2} h^{-1}) and an adjacent low emission area (LEA, 20–30 μg N m^{-2} h^{-1}) of a highly acidified forest. HEA showed similar carbon and nitrogen (N) pools and microbial biomass to LEA, but significantly higher moisture and extractable nutrients than LEA did. GeoChip 4 detected 298 gene families (unadjusted $P < 0.05$; 94, adjusted $P < 0.05$) showing significantly different structures between HEA and LEA. Both areas had highly diverse N cycling functional genes. However, HEA had higher relative abundances of *nor*, *P450nor*, and archaeal nitrifier *nirK*, which provided evidence for the importance of denitrifiers in N_2O emission. HEA also showed significantly higher relative abundances of lignin- and cellulose-degrading genes, oxygen-limitation-response genes and denitrifier *ppk*, but lower abundances of N- and phosphorus (P) -limitation-response genes especially denitrifier *pstS*, corresponding to the higher moisture and extractable nutrients conducive to denitrification. The moisture, extractable nutrients and pH explained over 50% variation in microbial communities, and extractable P appeared as the key factor driving community variation and consequently regulated N_2O production.

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Capsule abstract: N_2O emission in highly acidified forest soils was related to the diverse N functional genes, especially denitrification genes, and was affected by soil properties.

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1. Introduction

Nitrous oxide (N_2O) is the third-largest well-mixed greenhouse gas, contributing to radiative forcing, with a global warming potential 298-fold higher than CO_2 (Dou et al., 2016; IPCC, 2013). In addition, N_2O is considered as the most important ozone-depleting substance emitted in the 21st century (Ravishankara et al., 2009). The atmospheric concentration of N_2O has increased by 19% from 1750 to 2011, mostly in recent 50 years (Syakila and Kroeze, 2011). Therefore, determining the causes of any abnormal increase in atmospheric N_2O is of great importance. The emission of N_2O from agriculture is the primary source of its increase (Qin et al., 2019; Syakila and Kroeze, 2011). In contrast, forest soils typically have a low or negative N_2O flux which has been either ignored or considered to be N_2O sinks in global models (Davidson, 2009; Syakila and Kroeze, 2011). However, acidic forest soils, which have a surface zone pH < 5.5, often serves as N_2O sources (Eickenscheidt and Brumme, 2012; Sitaula et al., 1995; Zhang et al., 2018; Zhu et al., 2013a). Acidification of forest soils has been significantly enhanced by increased nitrogen (N) and sulfur (S) deposition over the past several decades, and acidic forest soils are now distributed globally and occupy approximately 20% of the total ice-free lands on Earth (Dentener et al., 2006; He et al., 2012; Huang et al., 2019).

Several studies have revealed a relationship between geochemical properties and N_2O emission in acidic forest soils (Gundersen et al., 2012; Jumadi et al., 2005; Weslien et al., 2009). N deposition and acidification have been shown to significantly increase the N_2O emission in forest soils (Gundersen et al., 2012; Weslien et al., 2009; Xie et al., 2018). For example, some highly acidic (pH < 4), N-rich forest soils showed high annual N_2O emission rates ranging from 0.23 to 4.85 g-N m^{-2} yr^{-1}, comparable to or even higher than common agricultural soils (e.g., 0.39–0.87 g-N m^{-2} yr^{-1} in maize fields, 0.034–0.46 g-N m^{-2} yr^{-1} in legume fields) (Moir, 2011; Gundersen et al., 2012; Weslien et al., 2009; Zhu et al., 2013b). By 2030, worldwide N deposition is predicted to increase by between 50% and 100% compared to levels in 2000; S deposition is expected to increase substantially in Asia and South America as well (Dentener et al., 2006; Liu et al., 2013; Reay et al., 2008). As N deposition and acidification are expected to increase dramatically over the next decades, the corresponding increases in N_2O emission from acidified forests could make an obvious contribution to overall global N_2O increases. In addition, greater numbers of highly acidified forests could emerge as N_2O emission hotspots. Therefore, it is important to understand the mechanism of N_2O emission in acidic forest soils.

N_2O production is primarily mediated by microorganisms and can be dominated by either bacteria or fungi in different acidified forests (Butterbach-Bahl et al., 2013; Rütting et al., 2013; Zhang et al., 2018). Either denitrification or nitrification can be the primary pathway for N_2O production (Li et al., 2019; Moir, 2011). N_2O emissions can also be indirectly affected by microbial processes other than N cycling, e.g. carbon (C) degradation and phosphorus (P) utilization (Butterbach-Bahl et al., 2013; Zhang et al., 2015; Mori et al., 2013). Studies have demonstrated that high abundances and high diversity of functional genes related to N and C recycling were detected in acidic forests (Isobe et al., 2012; Cong et al., 2015). The microbial composition would change with the N content variation in acidic forest soils (Nie et al., 2018). Although it is known that microorganisms play a very important role in N_2O emission, their relationships with the diversity and structure of microbial communities in highly acidic forest soils and environmental factors shaping these communities are still poorly understood.

South China has large areas of acidic subtropical forest soils suffering from long-term high N deposition, often exceeding 40 kg ha^{-1} yr^{-1}, which is similar to the highest levels found in Europe and North America (Liu et al., 2013; Reay et al., 2008). Among the five sites of the Integrated Monitoring Program on Acidification of Chinese Terrestrial Systems, the Tieshanping (TSP) forest, a subtropical pine forest in southwestern China, has the highest N deposition, lowest soil pH (<4.2), and most severe defoliation (Larssen et al., 2006). An annual N_2O emission rate of 4.3–5.4 kg-N ha^{-2} yr^{-1}, which amounted to be 8% to 10% of the annual atmogenic N deposition, was reported for a hill slope area during a two-year biweekly monitoring program in TSP (Zhu et al., 2013b). Meanwhile, N_2O emission in the TSP forest was reported to exhibit high spatial variations, with a peak rate of up to 1800 μg-N m^{-2} h^{-1} in the high emission areas during monsoonal summer (Zhu et al., 2013b). These observations together make the TSP forest an excellent site to study microbial mechanisms of N cycling and N_2O emission in the highly acidic forest soils with high N deposition.

In this study, soil samples were collected from the high and low N_2O emission areas in the TSP forest, along with measurements of N_2O emission rates and geochemical properties. Functional gene microarray (Geochip 4) was used to determine the microbial functional gene composition of the forest soils in the TSP areas, with a particular focus on the composition of N cycling functional genes, and the linkage between functional gene compositions, soil geochemical properties and spatial variation of N_2O emission.

2. Materials and methods

2.1. Site description

The study site (106°41.24′ E, 29°37.42′ N) is located at TSP, a natural secondary pine forest that is 25 km northeast of the center of Chongqing City in southwest China (Fig. S1a). TSP has an altitude of 510–580 m. In 2010, the annual temperature and precipitation at the nearest metrological station were 18.6 °C and 1044.7 mm, respectively, and the annual deposition of N and S at the site was as much as 6.3 g-N m^{-2} and 15.8 g-S m^{-2} (Larssen et al., 2006; Liu, 2010). The canopy is dominated by *Pinus massoniana* (tree-age 40a), the shrub layer contains *Camellia oleifera* and *Randia cochinchinensis*, and the herbaceous layer is mainly pteridophytes. The soil is locally called yellow earth, corresponding to Orthic Acrisols in the Food and Agriculture Organization system (IUSS, 2015), with an O/A horizon of 2–4 cm and a B horizon of 40–50 cm followed by a gradual transition to the C horizon. The soil has very low pH values of 3.7–4.1 ranging from the O horizon to the lower B horizon.

2.2. Sampling and chemical analyses

In this study, two adjacent areas with distinct N_2O emission rates were selected in order to explore the influence of microbial community differences on N_2O emission rather than the distance effect. The low emission area (LEA) was 6–7 m higher and 15–20 m distant from the high emission area (HEA, Fig. S1b). In our two-year study of this hill slope, N_2O emission rates (measured in Jul and Sep 2009, May, Jun, Sep, and Oct 2010) were as high as 100–800 μg-N m^{-2} h^{-1} in most quadrats including the HEA, but <30 μg-N m^{-2} h^{-1} in other quadrats including the LEA. LEA and HEA showed typical ground characteristics and N_2O emission rates. Samples were collected from 6 locations of each area in October 2010 (Fig. S1c) after an entire season of high-emission. Litter was removed before measurements and sampling. N_2O emission rates at each location were measured within 24 h before sampling using the static chamber technique as described previously

(Repo et al., 2009). The size of cubic chamber used in this study was 50 × 50 × 60 cm. Gas samples were collected every 15 min for 3 h, and these samples were analyzed for N_2O concentration. Each location sample was a homogenized mixture of 7 sub-samples (Fig. S1d). Each sub-sample was comprised of surface soil (0–15 cm depth) collected by using a steel soil core sampler (inner diameter 50 mm). Soils were sealed in sterile sampling bags and transported on ice to the lab within 8 h. Subsamples for DNA extraction were stored at −80 °C. Microbial biomass C and N contents were measured as described previously (Berthrong and Finzi, 2006). The geochemical properties were measured according to the recommended soil testing procedures (Lu, 1999): pH was measured by the potentiometry, field moisture content (FMC) by the oven drying method, extractable organic carbon (EOC) by a total organic carbon analyzer, extractable phosphorus (EP) by the acid dissolution/Mo-Sb colorimetric method, and extractable nitrogen (EN) by the alkaline hydrolysis diffusion method.

2.3. DNA extraction

Microbial genomic DNA was extracted by a freeze-grinding method (Zhou et al., 1996) and purified by agarose gel electrophoresis. DNA was analyzed by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Purified DNA had A_{260}/A_{280} of 1.75–1.85 and A_{260}/A_{230} of >1.7. The DNA samples were stored at −80 °C until ready for use.

2.4. GeoChip hybridization and data pre-processing

The microbial communities were analyzed by a new version of GeoChip 4 which covered >140,000 protein-coding sequences (Tu et al., 2014). Comparing to the qPCR and metagenomics, which are always used to analyze the functional genes, the GeoChip has the combined advantages of these two methods, and it can quantify multiple function genes simultaneously (Zhou, 2009; Ma et al., 2017). For each sample, 1 μg DNA was labeled with the fluorescent dye Cy3 and hybridized on GeoChip 4 (Tu et al., 2014). The arrays were scanned with a NimbleGen MS 200 Scanner (Roche NimbleGen, Madison, WI, USA). The raw data were preprocessed using a data analysis pipeline (http://ieg.ou.edu/microarray/). Across all samples, spot signals were normalized by the average signal intensity of control spots and then by the sum of all sample spot signals. Then, the spots with (i) a signal-to-noise ratio <2.0, (ii) or a coefficient of variation > 0.8, (iii) or a normalized signal <1000 were removed. The microarray data presented are available at http://www.ou.edu/ieg/publications/datasets.

2.5. Statistical analysis

Pre-processed microarray and geochemical data were further analyzed: (i) hierarchical clustering for geochemical data, and before the application of geochemical data, they are subjected to dimensionless processing by *Z*-transformation; (ii) microbial diversity indices, the two-tailed *t*-test and response ratios. For the *t*-test, the Benjamini & Hochberg method was used to adjust *P* values within each gene category (Benjamini and Hochberg, 1995). But due to the defects of *P* value adjustment (Althouse, 2016) and the controversy over significance test (Amrhein et al., 2019), the unadjusted and adjusted *P* values were both showed in this study ($P < 0.05$ was considered statistically significant, unless otherwise stated). The equation of the response ratio for gene i is: response ratio$_{(i)} = \ln(\bar{x}_i/\bar{y}_i)$, where $\bar{x}_i$ is the mean of normalized signal intensity in HEA samples and $\bar{y}_i$ is the mean of normalized signal intensity in LEA samples; (iii) detrended correspondence analysis (DCA) for community structure (He et al., 2010); (iv) dissimilarity test of microbial communities by ANOSIM, adonis, and MRPP analysis (He et al., 2010); (v) canonical correspondence analysis (CCA) for linking microbial communities to geochemical properties (Ter Braak, 1986); (vi) Mantel test and Pearson correlation test for correlation analysis between functional genes, geochemical properties and N_2O emission rates ($P < 0.05$ was considered statistically signifiant, unless otherwise stated) (Mantel, 1967). At the level of gene families or gene groups, Mantel tests used the relative abundances of each gene while the Pearson correlation test used the sum of the relative abundances of all genes within each gene family or gene group. A relatively abundant gene was defined as a gene with an intensity of $Z > 1.28$ (corresponding to percentile of 90% in a normal distribution) in at least one sample, where $Z = (X\text{-}X_{ave})/S$, X is intensity of the gene, X_{ave} and S are average intensity and the standard deviation of all detected genes in the sample, respectively. For the Pearson correlation analysis, the adjusted *P* values were adopted when the multiple tests were performed. Statistical analysis was performed by R v.2.15.2 with the psych v.1.9 package and vegan v.2.0–5 package ($P < 0.05$ was considered statistically signifiant, unless otherwise stated). Gene names and the full names of corresponding enzymes were listed in Table S1.

3. Results and discussion

3.1. Microbial N functional gene diversity

About 45,896–57,323 functional genes in 689 gene families were detected in each sample from the TSP forest. The genes with higher intensities than most other detected genes ($Z > 1.28$) were considered to be relatively abundant genes. A total of 587 gene families (84% of all covered families), 106 archaeal gene groups (57%), 446 bacterial gene groups (86%) and 173 fungal gene groups (76%) contained relatively abundant genes (Table 1). These relatively abundant genes were derived from 42 different phyla, 862 different genera, and many unclassified clusters, with diverse metabolic potentials for C, N, P and S cycling, energy metabolism, metal resistance, organic pollutant degradation, stress response and antibiotic resistance (Table S2).

Geochip 4.2 contained 17 N functional gene families involved in ammonification, nitrification, denitrification, N fixation, dissimilatory N reduction, assimilatory N reduction, or anammox in bacteria, archaea, and fungi (Tu et al., 2014). From the TSP forest soils, a total of 5051 N functional genes were detected, which accounted for >70% of all covered N functional genes. The average percentages of detected N functional genes in each sample from the TSP forest varied considerably for different N cycling processes (Table S3). Briefly, the percentages of detected genes involved in the denitrification process were the highest (79.9 ± 4.2%); in contrast, the percentages of detected genes involved in nitrification (56.1 ± 3.1%) and anammox (56.5 ± 6.6%) processes were much lower.

The potential functions of microorganisms can be very diverse in acidified forests with soil pH > 4.0, based on metagenomic analyses of several forests from distinct geographic locations (Cong et al., 2015; Paula et al., 2014). However, only a few functional gene families, e.g. *pmoA* and some N cycling genes, have been analyzed in highly acidified forests with soil pH < 4 (Isobe et al., 2012; Jung et al., 2012; Nguyen et al., 2018). As an example, according to Isobe et al. (2012), only the archaeal *amoA* gene was detected in Dinghushan Biosphere Reserve, but in the case of Bukhan Mountain, both bacterial *amoA* and archaeal *amoA* were detected abundantly (Jung et al., 2012). In highly acidic environments, the denitrification guild can be very diverse or dominated by only a few bacteria (Green et al., 2012; Palmer and Horn, 2012). High acidity (pH < 4) has been proposed as selective pressure and is a feature of environment where acidophilic organisms thrive (Sharma et al., 2012). To further verify whether high acidity restricts the N functional gene diversity in TSP forest soils, we compared the diversity of N functional genes (in terms of the percentages of detected N functional genes) detected in TSP forest soils with other eight forest ecosystems via accessible Geochip 4 datasets (Table S3) (Cong et al., 2015; Ding et al., 2015; Paula et al., 2014; van Straalen et al., 2014). It was found that the diversity of soil microbial N functional genes detected in these nine forests varied drastically. For most N cycling processes, N

Table 1
The number and percentage of detected, highly detectable and overlapping gene families and gene groups and those with significantly different relative abundances between the high (HEA) and low (LEA) N_2O emission areas.

Items	Total detected (HEA or LEA)	Highly detectable[1] (HEA or LEA)	Overlapping (HEA and LEA)	HEA > LEA Significantly[2]	HEA < LEA Significantly[2]
Gene families	689 (98.3%[3])	587 (83.7%)	682 (97.3%)	178[a] (25.4%) 63[b] (9.0%)	120[a] (17.1%) 44[b] (6.3%)
Archaeal groups	171 (91.9%)	106 (57.0%)	166 (89.2%)	29[a] (15.6%) 6[b] (3.2%)	30[a] (16.1%) 13[b] (7.0%)
Bacterial groups	511 (98.5%)	446 (85.9%)	507 (97.7%)	124[a] (21.4%) 30[b] (5.8%)	54[a] (10.4%) 31[b] (6.0%)
Fungal groups	220 (96.5%)	173 (75.9%)	218 (95.6%)	21[a] (9.2%) 6[b] (2.6%)	34[a] (14.9%) 18[b] (7.9%)

[a] Unadjusted *P* value.
[b] Adjusted *P* value.
[1] A gene with *Z* value >1.28 in any sample was counted as a highly detectable gene. A gene family or gene group with at least one highly detectable gene was counted as a highly detectable gene family or group.
[2] *P* value of *t*-test < 0.05. The comparison used the relative abundance sum of a certain gene family or gene group in each sample.
[3] Percentage in total number of gene families or gene groups targeted by this GeoChip.

functional genes were the most diverse in two amazon rain forests; in contrast, they were much less diverse in the alpine coniferous forest in Shennongjia. Although the TSP forest had the most acidified soils (pH 3.3–3.8), the diversity of N functional genes in the TSP forest soils was the highest among the nine forests, demonstrating that the high acidity didn't restrict the N functional gene diversity in TSP forest soils. In summary, our results with a comprehensive snapshot of microbial metabolic potentials demonstrated that highly acidic forest soils can also possess a high versatility of microbial functional potentials.

3.2. High N_2O emission and the microbial sources

The N_2O emission rates were significantly ($P < 0.005$) higher in the HEA (224–601 μg-N m^{-2} h^{-1}) than in the LEA (20–30 μg-N m^{-2} h^{-1}, Table 2). Such high rates in HEA are similar to short-term N_2O emission rates observed in another hill slope area in TSP (Zhu et al., 2013a, 2013b). And also, these short-term emission rates were similar to or lower than those from highly acidic soils in forested drained peatlands in Sweden (up to 5700 μg-N m^{-2} h^{-1}) and Finland (400 μg-N m^{-2} h^{-1}), but higher than many reported forest soils (30–50 μg-N m^{-2} h^{-1}) (Cheng et al., 2016; Klemedtsson et al., 2010; Maljanen et al., 2013; Vanitchung et al., 2011; Weslien et al., 2009; Xie et al., 2018).

Table 2
Geochemical properties and soil biomass in the areas with different N_2O emission rates in a highly acidified forest in southwestern China.

Factors	Unit	High emission area[1]	Low emission area	*P*[2]
N_2O emission rate	μg-N m^{-2} h^{-1}	442 ± 165	25.9 ± 4.4	**0.002**
pH		3.72 ± 0.10	3.47 ± 0.10	**0.002**
Total organic carbon	g kg^{-1}	16.8 ± 2.6	15.4 ± 2.5	0.375
Extractable organic carbon	mg kg^{-1}	154.4 ± 8.2	122.9 ± 17.3	**0.002**
Total nitrogen	g-N kg^{-1}	1.22 ± 0.31	0.99 ± 0.50	0.351
Ammonium nitrogen	mg-N kg^{-1}	2.09 ± 1.52	2.83 ± 0.63	0.293
Nitrate nitrogen	mg-N kg^{-1}	2.51 ± 2.1	2.67 ± 1.5	0.883
Extractable nitrogen	mg-N kg^{-1}	21.7 ± 4.4	14.7 ± 4.3	**0.019**
Extractable C/N ratio		8.5 ± 1.6	10.3 ± 3	0.237
Extractable phosphorus	mg kg^{-1}	12.77 ± 5.16	1.35 ± 0.68	**0.003**
Field moisture content	% (w/w)[3]	46.0 ± 5.0	28.7 ± 2.2	**<0.001**
Saturation moisture capacity	% (w/w)[3]	51.4 ± 5.6	47.4 ± 2.8	0.151
Water-filled pore space	%	76.4 ± 7.9	47.8 ± 3.6	0.132
Microbial biomass carbon	mg kg^{-1}	67.3 ± 17.9	65.2 ± 16.7	0.832
Microbial biomass nitrogen	mg kg^{-1}	9.43 ± 2.95	7.36 ± 1.56	0.158
Microbial biomass C/N ratio		8.4 ± 0.7	10.3 ± 0.8	**0.002**

[1] Mean ± standard deviation.
[2] *P* value of two-tailed *t*-test between high N_2O emission and low N_2O emission areas. Factors with significant differences are in bold.
[3] Weight of water divided by the weight of soil and water.

The major microbial sources of N_2O reported in different acidic forest soils are nitrification or denitrification (Nakajima et al., 2005; Zhang et al., 2011). In the previous study of TSP, an in situ ^{15}N-NO_3 labeling experiment demonstrated that denitrification was the dominant process of N_2O production (Zhu et al., 2013a). Our results support the observations with metagenomics details. Denitrifiers produce N_2O by denitrification mediated by *nor*-encoded enzymes (Fig. 1) (Moir, 2011). In this study, the *nor* gene groups of bacterial, archaeal, and fungal denitrifiers were all more abundant in HEA than in LEA (Fig. 2a), with response ratios being 0.053 (unadjusted $P = 0.042$, adjusted $P = 0.110$), 0.091 (unadjusted $P = 0.049$, adjusted $P = 0.127$), and 0.075 (unadjusted $P = 0.002$, adjusted $P = 0.018$), respectively. Although for bacterial and archaeal *nor*, the differences were not significant after *P* values were

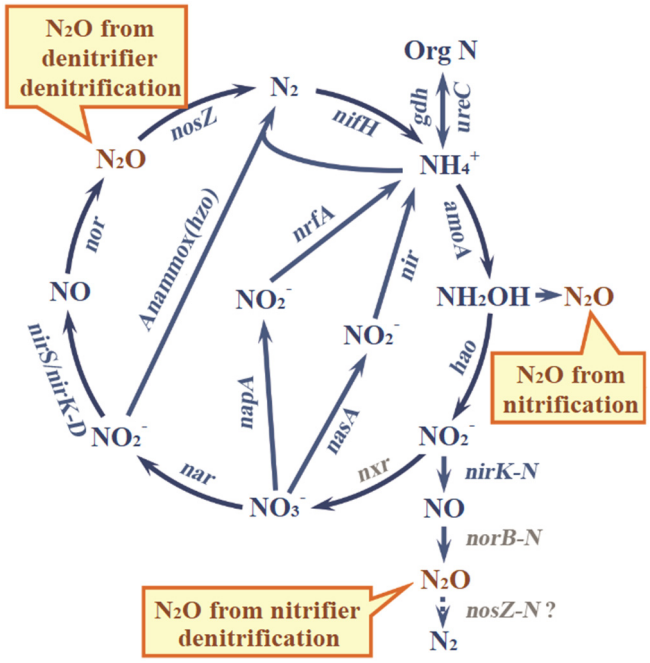

Fig. 1. Nitrogen cycling processes and functional genes. The figure was modified from He et al. (2010). Grey-colored genes were not targeted by this GeoChip. It remains unknown if *nosZ* homologs (*nosZ-N*) exist in nitrifiers. *nirK-N* and *nirK-D* are *nirK* in nitrifier and denitrifier respectively. *ppk-D* and *pstS-D* are *ppk* and *pstS* in putative denitrifiers. The description of the genes is in Table S1.

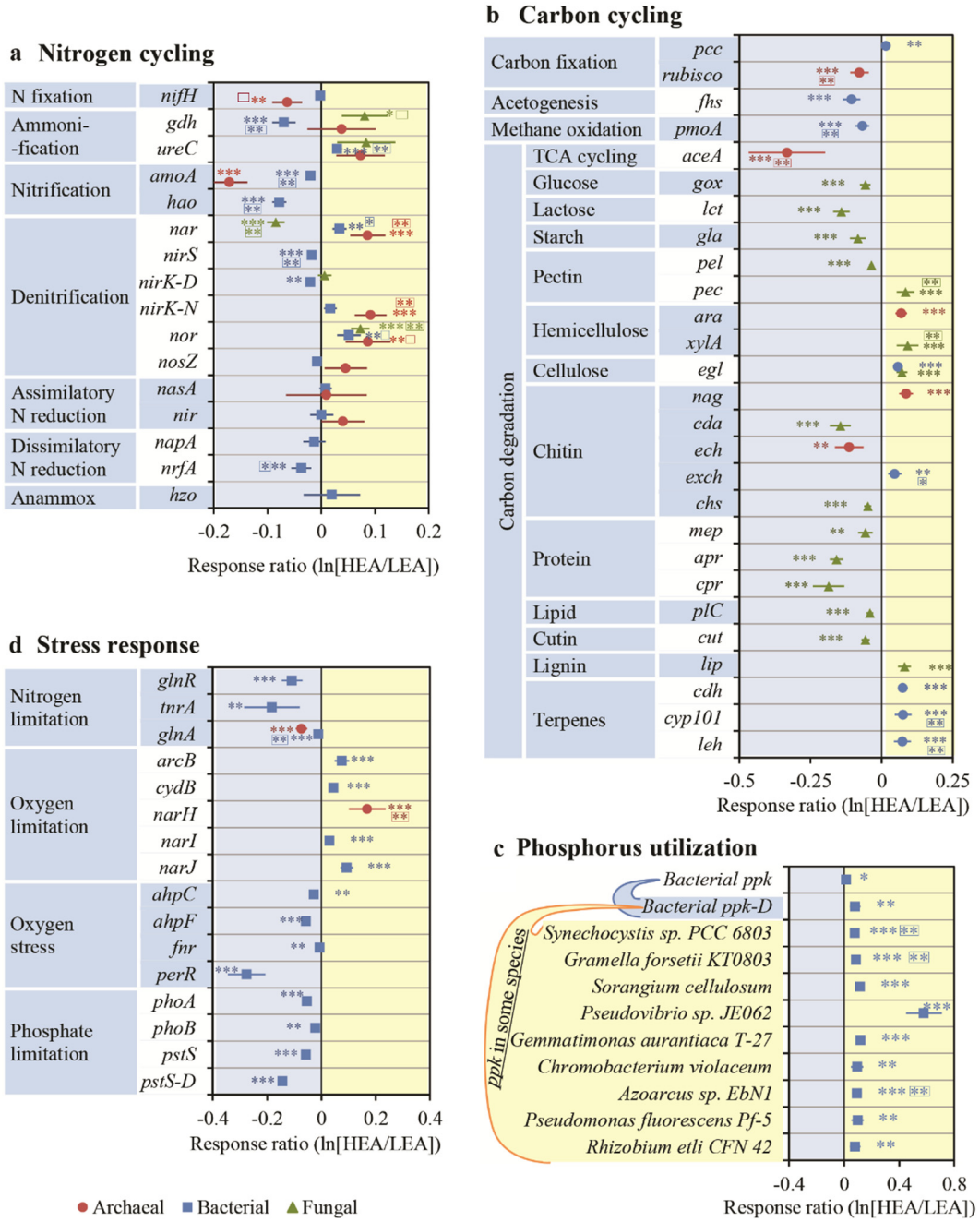

Fig. 2. Differences in functional gene abundances between high (HEA) and low (LEA) N_2O emission areas: reflected by the abundance response ratios of different gene groups. Error bars represent standard errors. *, significant at $P < 0.1$; **, significant at $P < 0.05$; ***, significant at $P < 0.01$. The significance based on the adjusted P value was provided in box if it was different from the unadjusted P value. The gene groups with insignificant ($P > 0.1$) differences between HEA and LEA are not included in Fig. 2b, c and d.

adjusted, the response ratios were close to the average response ratio (0.082) of all significantly different genes associated with N cycling. In addition, they showed positive correlation with N_2O emission rates (Fig. S2), indicating their potential contribution to N_2O emission. Nitrifiers can produce N_2O during nitrification when ammonia is oxidized by *amoA* and *hao* encoding proteins (Fig. 1). However, in this study, the relative abundances of *amoA* and *hao* gene groups were all significantly lower in HEA than in LEA (response ratios were − 0.157, −0.020 and − 0.075 for archaeal *amoA*, bacterial *amoA* and bacterial *hao*, respectively. Fig. 2a). Some nitrifiers can also produce N_2O through denitrification under oxygen limiting conditions (Fig. 1), which has been demonstrated by many studies on ammonia-oxidizing bacteria (AOB), but has not yet been proved in ammonia-oxidizing archaea (AOA) (Kozlowski et al., 2016). GeoChip 4 contains *nirK* genes from both bacterial and archaeal nitrifiers (*nirK-N*), representing AOB and AOA with denitrification potential, respectively. In our work, the archaeal *nirK-N* gene group was detected and showed a significantly positive correlation with N_2O emission rates while the detected bacterial *nirK-N* did not (Fig. S2), suggesting that some AOA might exhibit denitrification activity and contribute to N_2O emission. N_2O emission could also occur during the assimilatory and dissimilatory N process, yet the abundances of the *nasA*, *nir*, *napA*, and *nrfA* gene families were all lower or at a similar level in HEA compared to LEA. In summary, among all 17 N functional gene families that might be related to N_2O emission, only genes involved in denitrification were significantly higher in HEA. In our study, totally 1975 denitrification genes (1898 bacterial and 17 fungal) from 6 gene families were found, among which 1443 genes showed higher relative abundances in HEA (489

genes with *t*-test unadjusted $P < 0.05$, 127 genes with *t*-test adjusted $P < 0.05$) than in LEA. N_2O emissions have their optimum with >70–80% water-filled pore space (WFPS) depending on soil type (Bateman and Baggs, 2005; Butterbach-Bahl et al., 2013; Moir, 2011). In the current study, WFPS values were > 75% in HEA, which could further boost the denitrification activities of the more abundant denitrifiers in HEA, compared with the lower WFPS (<50%) in LEA.

Recent studies in TSP and other subtropical acidic subtropical forests in southern China also unveiled heterotrophic nitrification (fungal nitrification in particular) could make a significant contribution in N_2O production (Yu et al., 2017; Zhang et al., 2011; Zhang et al., 2015), although a lesser extent compared to denitrification in these ecosystems. The functional genes directly related to fungal nitrification remained obscure. However, it has been demonstrated that fungal nitrification was often linked with degradation of lignin and other recalcitrant organic N (De Boer and Kowalchuk, 2001; Kuyper and Bokeloh, 1994; Zhang et al., 2014). In this study, soil samples of HEA exhibited significant higher abundances of *lip* genes that code for lignin peroxidase in fungi than those of LEA (response ratio = 0.081, Fig. 2b), which was consistent with the higher EOC contents in HEA.

In contrast to the increased abundances of N_2O production genes, the relative abundance of *nosZ* associated with N_2O consumption was similar in both HEA and LEA. Several studies have reported that N_2O reductase is more sensitive to low pH than other denitrification reductases (Čuhel et al., 2010; Dannenmann et al., 2008; Moir, 2011). Thus, in highly acidic forest soils, the very low pH could suppress enzymes and microorganisms consuming N_2O by a greater degree than those producing N_2O through denitrification. Altogether, these findings suggest that high acidity suppressed N_2O consumption in both areas and the significantly higher N_2O emission in the HEA could likely be resulted from stimulation of N_2O production via denitrification.

3.3. Microbial biogeochemical mechanisms underlying N_2O emission

To further understand the underlying biogeochemical mechanism, the soil geochemical properties, the whole structure and various relevant gene families of functional microbial communities of HEA and LEA were compared. N_2O emission rates have been shown to increase due to higher available N (Wolf et al., 2011), higher available C (Weslien et al., 2009), higher soil moisture (Gundersen and Rasmussen, 1990), and lower pH (Weslien et al., 2009) in different forests. FMC, EP, EOC, EN, and pH were correlated with one another ($P < 0.1$) and clustered together (Fig. S3), corresponding to the correlation of extractable nutrients and acidification with water transport in acidic soils. These five properties were all significantly ($P < 0.02$) higher in HEA than in LEA (Table 2) and also significantly ($P < 0.03$) correlated with N_2O emission rates. Specifically, soil pH was closer to 4 in HEA (3.6–3.8) than in LEA (3.4–3.6), showing slight but statistically significant ($P < 0.002$) differences. EP and FMC showed more obvious differences between HEA and LEA than other properties (Table 2).

The functional gene structures were significantly different between HEA and LEA according to DCA (Fig. 3a) and dissimilarity tests ($P < 0.02$, Table S4). However, the proportion of overlapped genes between any two samples was higher than 74%, while the unique genes in every sample were lower than 1% (Table S5). The detected gene numbers and Simpson alpha diversity indices were slightly lower in HEA than in LEA (relative differences were 8.6% and 9.7%, respectively, $P < 0.03$, Table S2 and S6), suggesting most species were well spread across both areas and the community differences mainly referred to variation of abundances rather than richness.

The relative abundances of many functional genes of HEA's samples were significantly different from LEA's (Fig. 2, Table 1 and S7). A total of 120 gene families showed significantly higher relative abundances in LEA (unadjusted $P < 0.05$; 44, adjusted $P < 0.05$), while 178 gene families had higher relative abundances in HEA (unadjusted $P < 0.05$; 63, adjusted $P < 0.05$. Table 1). HEA had significantly higher relative abundances of

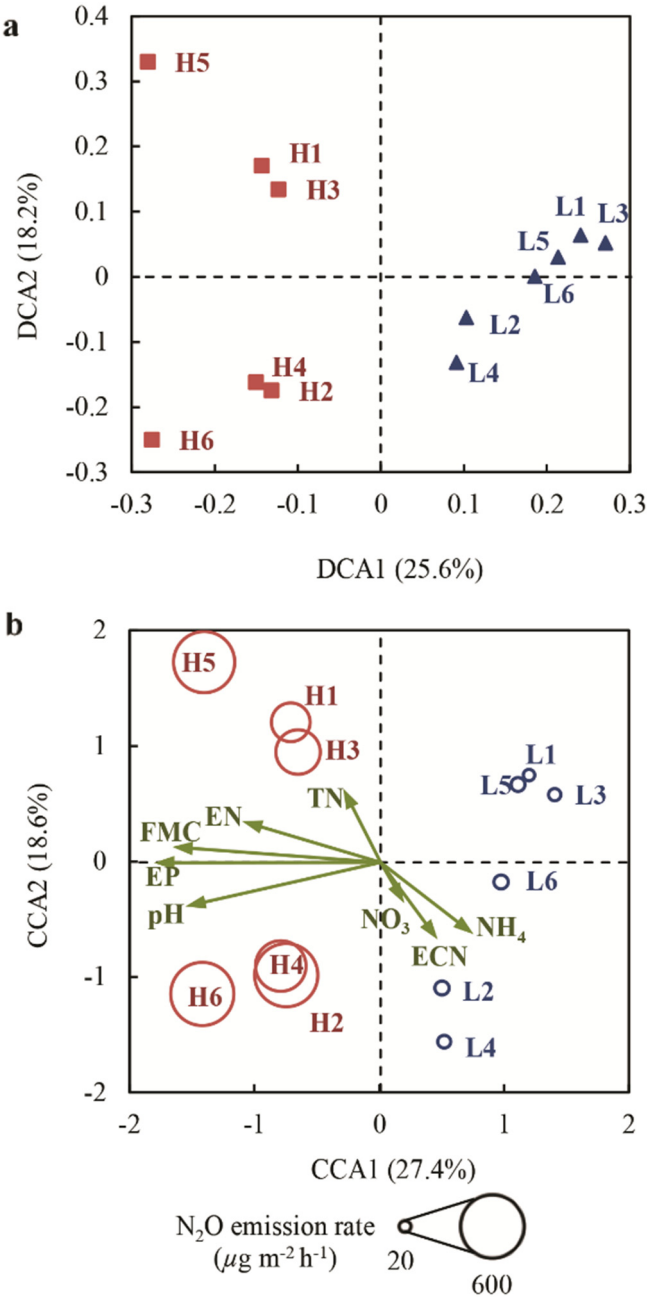

Fig. 3. (a) detrended correspondence analysis (DCA) of functional genes detected by GeoChip 4, (b) canonical correspondence analysis (CCA) of functional microbial community structures and geochemical properties ($P < 0.01$). Bubble sizes represent N_2O emission rates which were 21.9 and 597 μg-N m^{-2} h^{-1} in L3 and H6, respectively. H1~H6 (red), samples from high N_2O emission area (HEA). L1~L6 (blue), samples from low N_2O emission area (LEA). FMC, field moisture content. EP, extractable phosphorus. EN, extractable nitrogen. TN, total nitrogen. NH_4, ammonium nitrogen. NO_3, nitrate nitrogen. ECN, extractable carbon/nitrogen ratio. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gene groups involved in the degradation of lignin, cellulose, hemicellulose, terpenes, pectine and chitine while lower relative abundances of gene groups involved in the degradation of cutin, lipid, protein, chitine, pectin and labile C (glucose, lactose and starch, Fig. 2b). Since lignin and cellulose are major C sources in the forests, the higher potential for their degradation in the HEA could promote C availability and provide more electron donors for denitrification, corresponding to higher EOC in HEA. Furthermore, HEA had lower relative abundances of N- and P-limitation-response genes and higher relative abundances of oxygen-limitation-response genes (Fig. 2d), in accordance with higher nutrient

availability and moisture in HEA. All the above geochemical differences and the corresponding changes in functional genes, in addition to N cycling genes, can stimulate microbial denitrification and promote N_2O production in HEA.

To explore the drivers of high N_2O emission in HEA, the relationship between geochemical properties and microbial community structures was also analyzed. In CCA analysis, a combination of geochemical properties was qualified if the model was significant ($P < 0.05$) and individual variance inflation factors were <10. The qualified combination that explained the highest proportion of total variation of microbial communities was pH, FMC, EP, EN, total N, ammonium N (NH_4), nitrate N (NO_3) and extractable C/N ratio (81.7%, $P < 0.01$, Fig. 3b). The first axis explained 27.4% of the variation, on which the HEA samples were well separated from the LEA samples. The first axis was negatively correlated with EP, FMC, pH, and EN. Previously, microbial communities in forest soils were shown to be influenced by pH (Lladó et al., 2018), moisture (Gelsomino and Azzellino, 2011), organic C (Merilä et al., 2010), N (Cong et al., 2015), and P (DeForest and Scott, 2010). Partial CCA analysis showed that EP, FMC, pH, and EN were able to explain 51.4% ($P < 0.02$) of the community structure variation. Moreover, EP, pH, and FMC correlated with 75%, 31% and 16% detected gene families (Mantel test $P < 0.05$, Fig. S4), respectively, while other geochemical properties correlated with much fewer gene families (<6%), although some other properties also had significant variation across areas (e.g., NO_3 ranged from 0.4 to 6 mg-N kg^{-1}).

EP had the most obvious variation across sampling areas (from 0.6 to 17.9 mg kg^{-1}). Acidic environments can decrease the mobilization of soil inorganic P, resulting in P limitation (DeForest et al., 2012). In this highly acidified forest, LEA had much lower EP (1.35 ± 0.68 mg kg^{-1}, Table 2) than many other acidic forest soils (16.6–128.4 mg kg^{-1}) including P limited forests (DeForest et al., 2012; Kunito et al., 2012). LEA also showed higher relative abundances of P-limitation-response gene groups than HEA (average response ratio = −0.068, Fig. 2d). These results suggested a clear P limitation in LEA and a sharp EP gradient in this forest. However, the effect of P limitation on N_2O emission has rarely been documented in forest soils, although P addition has been shown to stimulate N_2O emission in water-saturated soil from a plantation (Mori et al., 2013). In this study, the analysis of putative denitrifiers, *pstS-D,* a P-limitation-response gene family, showed significantly higher relative abundances in LEA (response ratio = −0.142, Fig. 2d), while the whole family and many members of *ppk-D*, which related to polyphosphate production from ATP in denitrifiers, had significantly lower relative abundances in LEA (response ratio = 0.097, Fig. 2c), suggesting the obvious P shortage affected denitrifiers. Denitrification is coupled to ATP synthesis, which could also be suppressed by a P shortage (Moir, 2011). Accordingly, the significant variation of P availability could contribute to denitrification regulation and thus affect N_2O emission.

As an essential nutrient for both components and energy of all organisms, P can be critical to microbial communities when it is limited. In some acidic forest soils, available P was shown to strongly correlate with the composition of phospholipid fatty acids (PLFA) (DeForest and Scott, 2010; DeForest et al., 2012), and the effect of pH on microbial communities was proposed to be an indirect response caused by the influence of pH on available P (DeForest and Scott, 2010). In this study, we found that EP was correlated with many more functional genes than other properties (Fig. S4), and was the only measured property correlated significantly with the whole community structure of functional genes ($r = 0.557$, $P < 0.01$, Mantel test). Moreover, EP was also the only property which was significantly ($P < 0.01$) correlated with the whole and each gene group of P utilization gene category. Altogether, a sharp gradient of EP can be a key factor driving the change in functional microbial communities in highly acidic forest soils.

While not directly related to N_2O emission, we did find some interesting relationships between N cycling functional microorganisms and geochemical properties. Previous studies have shown bacteria, fungi and archaea were differentially affected by some geochemical properties in acidic soils, e.g., pH (Nicol et al., 2008; Pennanen et al., 1998) and N (Gilliam et al., 2011). In this study, the bacterial, archaeal and fungal groups within the same gene family usually showed different trends under the geochemical property gradients in this forest (Fig. S2 and S4). For instance, pH and EOC correlated with archaeal ($P < 0.05$) but not bacterial *amoA*, while NH_4 correlated with bacterial ($P < 0.05$) but not archaeal *amoA* (Fig. S2). EP and pH correlated with archaeal but not bacterial *nirK-N*, as well as bacterial but not archaeal ammonification gene groups (*gdh* and *ureC*, Fig. S2). FMC significantly ($P < 0.05$) correlated with 26% of the fungal C cycling gene groups but with a much lower percentage (8%) of the bacterial C cycling groups. In all, bacteria had higher percentages of gene groups positively correlated with EP, pH, and FMC, while fungi had higher percentages of gene groups negatively correlated with these properties ($P < 0.05$, Fig. S4).

In acidic soils, AOA were found to be the dominant ammonia oxidizers (He et al., 2012; Nicol et al., 2008). It has been proposed that ammonia is a limiting factor suppressing AOB, because the theoretically predicted ammonia N (NH_3) concentrations in acidic soils are below the substrate threshold of AOB (He et al., 2012). Correspondingly, using PCR, AOB *amoA* and 16S rRNA genes have not been detected in many acidic forest soils (Isobe et al., 2012; Schmidt et al., 2007; Wu et al., 2017). In our current study of highly acidic forest soils, AOB *amoA* genes were detected; however, they showed less variation across samples and correlated to fewer gene groups than AOA *amoA* (Fig. S5). Furthermore, AOB *amoA* showed a significant correlation with NH_4 ($r = 0.626$, $P = 0.03$, Fig. S2) and an even higher correlation with theoretically predicted NH_3 ($r = 0.839$, $P < 0.001$), while AOA *amoA* did not, supporting the supposition that NH_3 is a limiting factor for AOB growth. An anaerobic ammonium oxidation (anammox) gene family, *hzo*, was detected in this forest and showed distinctive relationships with geochemical properties (Francis et al., 2007). Mantel test results indicated that *hzo* was the only N cycling gene family significantly correlated with the combination of all measured properties ($P = 0.002$), as well as pH, FMC, EP, EOC and EN individually ($P < 0.05$, Fig. S6), suggesting the sensitivity of *hzo* family structures to environmental change. Interestingly, the total abundance of *hzo* did not significantly correlate with any measured geochemical properties ($P > 0.1$) except NO_3 ($P = 0.054$, Fig. S2), implying the different *hzo* genes responded similarly to their substrate NO_3 variation, but quite different to other geochemical gradients.

Overall, this highly acidified forest showed highly diverse functional genes and significant variation of microbial community functional structures driven by moisture and nutrient gradients, particularly the sharp EP gradient. The abnormally high N_2O emission could be due to stimulated denitrification by higher moisture and available nutrients in the HEA. However, it should be noted that the conclusion was limited to the current study site, and no consideration was made to generalize this without enough evidence. Further studies should be carried out in highly acidified forests in different regions as well as those under different restoration strategies, to reveal the effect of the worldwide development of acidified forests on ecosystem functioning and feedbacks to global change.

4. Conclusions

The geochemical properties and the microbial gene diversity of soils were analyzed, which were collected from two adjacent sites but with significantly different N_2O emission in the highly acidified TSP forest. Denitrification was believed as the main N_2O emission pathway, indicated by the evidence that genes involved in denitrifiers were positively correlated with the N_2O emission rates. In addition, the P concentration gradient was identified as a crucial factor that might affect the N_2O emission in the TSP forest. Overall, this study revealed the driving factors in N_2O emission in the TSP forest and further calls for the comparison from the future investigation in other acidic forests worldwide.

CRediT authorship contribution statement

Yina Zou: Formal analysis, Writing - original draft. **Daliang Ning:** Investigation, Formal analysis, Writing - original draft. **Yong Huang:** Investigation, Writing - review & editing. **Yuting Liang:** Writing - review & editing. **Hui Wang:** Funding acquisition, Supervision, Writing - original draft. **Li Duan:** Investigation, Writing - review & editing. **Tong Yuan:** Investigation, Writing - review & editing. **Zhili He:** Writing - review & editing. **Yunfeng Yang:** Writing - review & editing. **Kai Xue:** Writing - review & editing. **Joy D. Van Nostrand:** Writing - review & editing. **Jizhong Zhou:** Writing - review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138504.

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