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Abstract

To safeguard food security and preserve precious water resources, the technology of water-saving ground cover rice production system (GCRPS) is increasingly used to substitute traditional paddy rice cultivation. However, reduced soil water, increased aeration and temperature under GCRPS could promote soil nitrogen (N) mineralizing and nitrifying microbes and thus enhance soil nitrogen turnover and environmental N losses e.g., through emission of the potent greenhouse gas nitrous oxide (N2O). At two sites with paired GCRPS/Paddy fields in Central China, we followed the abundance and activity of N-mineralizers, nitrifiers, denitrifiers and N2-fixing microbes based on qPCR from DNA and RNA directly extracted from soil. GCRPS exerted pronounced negative effects on nifH transcripts (encoding for nitrogenase) but positive effects on qnorB and archaeal amoA transcripts (encoding for NO reductase and ammonia monooxygenase). This indicated a higher potential for N losses due to decreased biological N2 fixation and increased N2O emission in GCRPS. The latter was confirmed by increased in situ N2O emissions. In addition, the N2-fixing and denitrifying microbial community composition as measured by a community fingerprinting approach was strongly influenced by GCRPS cultivation. In contrast to previous work at other sites in the study region, we found that following 10 years of GCRPS cultivation and in fields not receiving N fertilization soil organic carbon (SOC) and total nitrogen (TN) contents decreased significantly, while this was not observed for unfertilized paddy fields. I.e., if N fertilization is not supplied GCRPS C and N losses are increased due to enhanced SOM mineralization, gaseous losses and probably also leaching. However, under standard urea fertilization, no changes in soil organic C and N were observed as GCRPS promotes root development and C and N return via residues. Overall, our study reveals the microbial mechanisms underlying the risks for increased mineralization and N2O emissions and decreased biological N fixation in GCRPS. Improved fertilizer N use efficiency and plant residue return however appear to prevent a net N loss in GCRPS.

Keywords	N cycle, microbial activity, N2O, ground cover rice production, paddy rice soil
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Submission of manuscript "Enhanced nitrogen cycling and N_2O loss in water-saving ground cover rice production systems" as original research paper in *Soil Biology & Biochemistry*.

Dear Editor,

We take pleasure in enclosing our recent work, which provides novel insights into the response of the soil microbial community as well as associated C and N cycling and N_2O emissions to GCRPS (water-saving ground cover rice production systems) cultivation.

To safeguard food security and preserve water resources, the technology of GCRPS is increasingly used to substitute traditional paddy rice cultivation. However, GCRPS might stimulate soil organic matter mineralization and increase gaseous losses such as N_2O emissions. Still a detailed investigation of the response of key soil processes such as mineralization, nitrification, denitrification and biological N_2 fixation to GCRPS cultivation is missing.

To characterize such changes, we monitored the dynamics of abundance and activity of microbial communities involved in the soil N cycle at paired Paddy/GCRPS fields in Central China. We also explored consequences of GCRPS cultivation on associated soil N_2O emissions, and on soil organic C and total N concentrations as well as on soil dissolved C and N. We show that GCRPS altered the composition of both the N_2-fixing and denitrifying microbial communities. Furthermore, GCRPS decreased nitrogenase (*nifH*) transcript levels but increased archael ammonia monooxygenase (*amoA*) and NO reductase (*qnorB*) transcript levels, thereby promoting N_2O emissions. However, soil organic C and total N concentrations may decrease in GCRPS only in absence of fertilization, likely due to plant residue return to soil and increased fertilizer N use efficiency.

We would be happy to share these novel and highly relevant findings with the audience of *Soil Biology & Biochemistry* and look forward to hearing your response to this submission.

Yours sincerely,

Prof. Dr. Shan Lin

Corresponding author on behalf of all co-authors

Enhanced nitrogen cycling and N_2O loss in water-saving ground cover rice production systems (GCRPS)

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Abstract

To safeguard food security and preserve precious water resources, the technology of water-saving ground cover rice production system (GCRPS) is increasingly used to substitute traditional paddy rice cultivation. However, reduced soil water, increased aeration and temperature under GCRPS could promote soil nitrogen (N) mineralizing and nitrifying microbes and thus enhance soil nitrogen turnover and environmental N losses e.g., through emission of the potent greenhouse gas nitrous oxide (N_2O). At two sites with paired GCRPS/Paddy fields in Central China, we followed the abundance and activity of N-mineralizers, nitrifiers, denitrifiers and N_2-fixing microbes based on qPCR from DNA and RNA directly extracted from soil. GCRPS exerted pronounced negative effects on *nifH* transcripts (encoding for nitrogenase) but positive effects on *qnorB* and archaeal *amoA* transcripts (encoding for NO reductase and ammonia monooxygenase). This indicated a higher potential for N losses due to decreased biological N_2 fixation and increased N_2O emission in GCRPS. The latter was confirmed by increased in situ N_2O emissions. In addition, the N_2-fixing and denitrifying microbial community composition as measured by a community fingerprinting approach was strongly influenced by GCRPS cultivation. In contrast to previous work at other sites in the study region, we found that following 10 years of GCRPS cultivation and in fields not receiving N fertilization soil organic carbon (SOC) and total nitrogen (TN) contents decreased significantly, while this was not observed for unfertilized paddy fields. I.e., if N fertilization is not supplied GCRPS C and N losses are increased due to enhanced SOM mineralization, gaseous losses and probably also leaching. However, under standard urea fertilization, no changes in soil organic C and N were observed as GCRPS promotes root development and C and N return via residues. Overall, our study reveals the microbial mechanisms underlying the risks for increased mineralization and N_2O

emissions and decreased biological N fixation in GCRPS. Improved fertilizer N use efficiency and plant residue return however appear to prevent a net N loss in GCRPS.

***Keywords*:** N cycle, microbial activity, N_2O, ground cover rice production, paddy rice soil

1. Introduction

Rice is the major staple food for almost half the global population, and about 90% of rice in the world is produced in Asia (FAO, 2011). To meet the food demand of a growing population, an annual increase in rice production in the range of 8 - 10 million tons is needed over the next 20 years, equaling to a global annual increase in rice production of 1-2% (IRRI, 2011; Ray et al., 2013). Due to the need for irrigation, conventional Paddy-rice systems require about 2.5 m^3 water per kg of grain (Bouman and Tuong, 2001; Tuong et al., 2005). Consequently, an increase in rice production currently goes along with increased demand for irrigation water. This is in contrast to declining water availability due to climate-induced water scarcity and increased domestic and industrial water demands, which challenges overall food security in the world. Approx. 15-20 million hectares of irrigated rice fields suffer from water scarcity (Bouman, 2007). Thus, reducing the irrigation water demand of rice production systems have been a key research area (Belder et al., 2004; Bouman and Tuong, 2001; Li et al., 2007; Qu et al., 2012; Yuan et al., 2014). One of the most promising technologies is the ground cover rice production system (GCRPS), which was introduced and promoted in China roughly two decades ago and now has been already widely adopted across China (Bouman, 2007; Lin et al., 2002).

In GCRPS the soil surface is covered with a 5- to 7-μm thick plastic film and traditional lowland rice cultivars are used and grown at soil water content nearby 80-90% of water holding capacity with no standing water layer during the entire growth period (Qu et al., 2012). This reduces evaporation by 32 - 54% and alleviates temperature limitation as the soil temperature is increased by 3 - 5 ^oC during early stages of rice cultivation

GCRPS cultivation requires an initial flooding of the field only, while during later crop growing stages the soil water filled pore space is kept at 80-90%. Using the GCRPS technique significantly increases rice yields where the water and temperature are limiting factors compared to the traditional Paddy rice cultivation (Liu et al., 2013; Qu et al., 2012). The combination of increased temperatures and aerobicity in GCRPS soils compared to soils being permanently flooded for traditional Paddy cultivation could stimulate mineralization of soil organic matter with losses of soil organic carbon (SOC) and total nitrogen (TN). However this view was recently challenged by a regional study pointing to the opposite by revealing a net increase in SOC and TN stocks due to GCRPS cultivation (Liu et al., 2015). While these authors could provide some hints on the underlying mechanisms such as increased C and N input by roots and decreased fertilizer NH_3 losses, a detailed investigation of the response of key soil N turnover processes such as mineralization, nitrification, denitrification and biological N_2 fixation is still missing. This still impedes a functional understanding of the response of the soil N cycle and associated N loss processes to GCRPS.

The N cycling processes N mineralization, nitrification, denitrification, microbial immobilization and biological N_2-fixation as well as the single enzymatic steps involved are conducted by specific soil microbial communities. Thus, microbial processes and communities regulate plant nutrient availability, ecosystem N retention, ecosystem N losses including emissions of the potent greenhouse gas N_2O and leaching of nitrate to ground and stream water, as well as the removal of reactive N from the biosphere via denitrifier N_2 production and the closing of the global N cycle (Butterbach-Bahl et al. 2013). In a recent study it was shown that compared to conventional paddy systems rice production via GCRPS leads to decreased CH_4

emissions but increased N_2O emissions (Xu et al., 2004; Yao et al., 2014). Nitrous oxide can be produced by both aerobic nitrification and anaerobic denitrification pathways (Butterbach-Bahl et al. 2013). While the underlying microbial mechanism has not yet been addressed in detail, it is commonly hypothesized that GCRPS promotes soil nitrification activity, as increased soil nitrate availability has been observed. Higher nitrate availability promotes the microbial denitrification process as a whole, i. e., the stepwise reduction of nitrate to the gaseous N forms nitric oxide (NO), N_2O and dinitrogen (N_2) under anoxic conditions (Butterbach-Bahl et al. 2013). Moreover, at increased nitrate availability, the terminal step of denitrification, i. e. the reduction of N_2O to N_2 is impaired as nitrate is preferably used as electron acceptor over N_2O so that increased soil N_2O losses are likely to occur (Butterbach-Bahl et al., 2013; Kornaros et al., 1996; Ruser et al., 2006; Scholefield et al., 1997). However, N_2O release is increasingly restricted at anaerobic soil conditions and high availability of easily degradable C as under such conditions denitrification nearly exclusively produces N_2 (Zumft, 1997). Hence several, potentially interacting microbial mechanisms may account for increased N_2O emissions observed under GCRPS cultivation. Thus in the frame of this study we analyzed the effects of GCRPS systems on microbes driving N turnover.

The soil microflora responsible for processes of N turnover can be characterized by analyzing genes encoding for specific enzymes catalyzing specific N conversions within e.g. the nitrification or denitrification processes (Braker and Conrad, 2011; Crouzet et al., 2016; Gschwendtner et al., 2014; Leininger et al., 2006; Philippot and Hallin, 2005; Philippot et al., 2013). However, the abundance of genes is not necessarily related to the biogeochemical activity of the related microorganisms, as DNA levels

129 only show the presence of such microorganisms in soil, but do not allow to conclude
130 on activity levels (Sessitsch et al., 2002). Recently, it has even been shown that the
131 abundance of some genes catalyzing selected steps of the N cycle were inversely
132 related to the biogeochemical activity patterns as indicated by the analysis of the
133 corresponding transcripts or associated gross N turnover rates (Chen et al., 2015; Di et
134 al., 2009; Liu et al., 2014).
135
136 Here we aimed to provide a holistic insight into changes in abundance and activity of
137 microbial communities involved in soil N cycle processes if rice management is
138 changed from Paddy to GCRPS. We also explored consequences for soil N_2O
139 emissions, dissolved C and N availability in soil as well as multi-year effects on SOC
140 and TN concentrations. Hence, both the abundance of marker genes encoding for
141 enzymes catalyzing key steps in N turnover as well as their transcripts were quantified
142 in this study using quantitative real-time PCR (qPCR) based on DNA and mRNA
143 extracted from soil. In contrast to most of earlier studies, our study was targeted to
144 achieve a complete understanding how key processes of the N cycle change with
145 management, so that markers for N_2 fixation (*nifH* gene), nitrification (*amoA* gene) and
146 denitrification (*nirK/S, norB* and *nosZ* for nitrite, nitric oxide and nitrous oxide
147 reduction) were simultaneously investigated. Chitinase (*chiA*) was chosen as marker
148 gene from the large pool of genes encoding for N mineralization. Since *nifH*, archaeal
149 *amoA*, and *nosZ* genes as key enzymes of nitrogen fixation, nitrification and
150 denitrification turned out to be highly abundant, these genes were chosen for tracking
151 community composition using terminal restriction fragment length polymorphism
152 analysis (T-RFLP) (Bannert et al., 2011). Furthermore, we quantified $\delta^{15}N$ as an
153 integrator of more long-term changes of the N cycle. We sampled soil in a full factorial

154 design, including effects of rice cultivation technique (GCRPS vs. Paddy cultivation)
155 and fertilization (0 and 150 kg N).
156
157 ## 2. Material and methods
158 *2.1 Sites and soil sampling*
159 The experiment was conducted in a typical mountainous rice growing region of Central
160 China - Shiyan County in Hubei province. We investigated two sites with paired
161 Paddy/GCRPS experimental fields with GCRPS established from Paddy conversion in
162 2003 and 2012. The sites were sampled in the years 2012 and 2014, i.e., 10 and 3 years
163 after Paddy conversion to GCRPS. Both field sites are located on the floor of small
164 valleys in the upper Han River basin. However, the sites differ in soil texture and SOC
165 content (Table 1) so that they were not regarded as a chronosequence. The region is
166 mountainous and has a humid subtropical climate with low temperatures and severe
167 seasonal and regional water scarcity, both limiting rice yields (Liu et al., 2013). The
168 location, soil physical and chemical properties of the two sites are provided in Table 1.
169 The weather data during the growth period were collected at a nearby meteorological
170 station. Soil temperature at the 5-cm depth was recorded every 2 h by data loggers (EBI-
171 20T, Ebro Instruments, Germany). Over 7 monitored years, the mean daily air
172 temperature during the growing season ranged from 19 ± 1.3 to 28 ± 0.7°C (Qu et al.,
173 2012).
174
175 In the present study, we investigated two fertilizer treatments under the two rice
176 production systems in a full factorial design: P0 (Paddy with no nitrogen fertilization),
177 P150 (Paddy with 150 kg urea-N ha^{-1}), G0 (GCRPS with no nitrogen fertilization) and

G150 (GCRPS with 150 kg urea-N ha^{-1}). The treatments were arranged in a completely randomized block design with three replicated plots of ca. 40 m^2 (10 years GCRPS) and 90 m^2 (3 years GCRPS) (Qu et al., 2012; Tao et al., 2015). All plots were completely isolated by levees with plastic coverings. As nitrogen fertilizer urea was applied once at a rate of 150 kg ha^{-1} y^{-1}, all treatments received 45 kg P_2O_5 ha^{-1} y^{-1} and 45 kg K_2O ha^{-1} y^{-1} as basal fertilization just before transplantation of rice plants. More details on the experimental setup are provided by (Qu et al., 2012; Tao et al., 2015). In agreement with the local water management, the experimental plots of conventional Paddy underwent a typical cycle of subsequent flooding/midseason drainage/frequent waterlogging with intermittent irrigation (Yao et al., 2014). In the present study, the mid-season drainage started on 16 July and ended on 23 July, and the period of final drainage was from 23 August to the end of rice-growing season (Fig. 1). For GCRPS, the soil water content was kept at saturated water holding capacity (WHC) until mid-tillering (May 25) and then kept at 70-80% WHC until the end of growing season (Jin et al., 2016). After fertilization, each GCRPS plot was covered with a 5μm thick polyethylene plastic film, followed by hole- punching for the transplantation of rice seedlings. After harvest, the rice straw was completely removed and all field plots were kept fallow over the winter period.

Two different times of Paddy to GCRPS conversion were investigated. The fields which were converted from traditional Paddy to GCRPS cultivation 10 years ago and the respective Paddy controls were sampled once during the middle tillering period (May 8, 2012). The fields which underwent GCRPS conversion 3 years ago were sampled – together with the adjacent Paddy controls - four times during different plant growing stages and soil moisture conditions in 2014: at middle tillering (May 25), maximum

tillering (June 22), panicle initiation (July 25), and maturity (September 5). From each plot, four soil cores were taken at every sampling date from the upper soil layer (0-20 cm), mixed to a composite sample, and immediately frozen (-20°C) for subsequent transport to the laboratory in Beijing. After that, soil samples were separated into two subsamples. The first subsample of ca 20 g was stored at -70°C for further molecular analysis. The remainder was stored at -20°C for soil physical and chemical determinations including soil inorganic N concentrations (ammonium and nitrate) as well as dissolved organic carbon (DOC) and nitrogen (DON).

2.2 Soil physical and chemical properties

Approximately 20 g of soil were extracted with 40 or 50 ml 0.5 M K_2SO_4 for measuring mineral N (NH_4^+-N and NO_3^--N), dissolved organic carbon (DOC) and dissolved total nitrogen each sampling time as described by Dannenmann et al. (2009). Extractable organic C was determined by UV-enhanced persulfate oxidation of organic C to CO_2 and analyzed using a nondispersive infrared detector (Multi N/C 3100 TOC/TNb-Analysator, Analytik Jena, Jena, Germany) (Dannenmann et al. 2009). The concentrations of NH_4^+ and NO_3^- were determined using continuous flow injection colorimetry (Skalar San plus system, Skalar Analytical B.V., Breda, Netherlands) in a commercial laboratory (Landwirtschaftliches Labor Dr. Janssen, Gillersheim, Germany). Soil dissolved organic N (DON) was calculated by subtracting NH_4^+-N and NO_3^--N from dissolved total nitrogen.

Soil pH was measured in distilled water with a soil-to-solution ratio of 1:2.5. Soil samples for total organic carbon and nitrogen and $\delta^{15}N$ analysis were powdered in a ball bill (MM200, Retsch, Hann, Germany) with the soil carbonates removed prior to

C analyses (Harris et al., 2001). Analyses were conducted using a Costech elemental analyzer (Costech International S.p.A., Milan, Italy) fitted with a Zero Blank autosampler coupled via a ConFloIII to a Thermo Finnigan Delta V Plus istotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA) as described in detail by Liu et al. (2015).

2.3 In-situ soil nitrous oxide flux measurements

The N_2O fluxes were measured in situ at the soil sampling day or maximal 2 days before or after the day of soil sampling by using the manual static chamber technique, as described in detail by Yao et al. (2014) using chambers (with a bottom area of 0.65 m × 0.90 m and a height of 0.50 m or 1.0 m depending on crop growth) per plot at a distance of approx. 3 m to the sampling spots of soil cores. During chamber closure, five headspace gas samples were taken with a 60 mL polypropylene syringe at 0, 10, 20, 30 and 40 minutes after covering. Samples were analyzed for N_2O concentrations within 6 hours following sampling by using a gas chromatograph (GC) (Agilent 7890A, Agilent Technologies, CA, USA) equipped with an electron capture detector (ECD). N_2O fluxes were calculated from linear concentration increase/decrease in the chamber headspace and mole volume was corrected using air temperature and air pressure (Yao et al., 2010). Besides these snapshot N_2O flux measurements, N_2O emissions were also followed across the entire growing season. For details see Yao et al. (2017).

2.4 DNA and RNA extraction

DNA and RNA were co-extracted from 0.4 g homogenized dry soil using the method described by Lueders *et al*. (Lueders et al., 2004) and the Precellys 24 Instrument (Peqlab, Erlangen, Germany). In view of the limitations of the chloroform fumigation

253 extraction method in Paddy soils (Marstorp *et al.*, 2000)., the extracted DNA was used
254 to estimate microbial biomass of microorganisms The quality and quantity of the
255 nucleic acids were assessed using a spectrophotometer (Nanodrop; PeqLab, Germany)
256 and agarose (1.5 % (w/v)) gel electrophoresis. Afterwards, the extract was divided into
257 two subsamples. One was used for DNA analysis and the other subsample was used to
258 prepare RNA by digestion and purification with DNase Max kit (MO BIO Laboratories,
259 Carlsbad, CA, USA). The absence of DNA in the RNA samples was confirmed by
260 performing a 16S PCR reaction, using the universal eubacterial primers 968f (5'-aac
261 gcg aag aac ctt ac-3') and 1401r (5'-cgg tgt gta caa gac cc-3'). The cDNA was
262 synthesized with the "High capacity cDNA reverse transcription kit" (Life
263 Technologies, Darmstadt, Germany) according to the instructions. The success of
264 cDNA synthesis was confirmed by performing PCR targeting the 16S rRNA gene as
265 described above. Both DNA and cDNA extracts were stored at -20°C until use.
266
267 *2.5 Real time PCR assay*
268 Quantitative real-time PCR (qPCR) was carried out on a 7300 real-time PCR system
269 (Life Technologies, Darmstadt, Germany) using SYBR green as a fluorescent dye,
270 plasmids containing the targeted gene fragments and PCR reaction mixtures as shown
271 in supplementary table S1. Dilution series of the different DNA extracts were tested in
272 a pre-experiment with randomly picked soils to avoid inhibition of PCR. DNA extract
273 dilutions of 1: 50 turned out to be most suitable and were used for the qPCR. The
274 standard curves for all the detected genes were created using 10-fold dilution series
275 (10^1~10^7 copies) of the respective plasmids containing the targeted gene fragments
276 (sources of standards are shown in Table S1). All PCR runs started with an initial
277 enzyme activation step performed at 95°C for 10 min followed by gene specific thermal

278 profiles (Table S1). The thermal profile was different for each gene, as shown in Table
279 S1, followed by a melting curve, consisting of 95°C for 15 s, 60°C for 30 s, and a
280 subsequent temperature increase until 95°C with a ramp rate of 0.03°C s^{-1}. Specificity
281 of the amplified products was checked by the observation of a single melting peak and
282 the presence of a unique band of the expected size in a 2% agarose gel. PCR efficiencies
283 (Eff) were calculated from the standard curve by the formula $Eff = [10^{(-1/slope)} - 1] \times$
284 100% and accounted for 95 to 97% for *chiA* gene, 88 to 91% for *nifH* gene, 98% for
285 *nirK* gene, 90% for *nirS* gene, 87% for *qnorB* gene, 87 to 94% for *nosZ* gene, 91% for
286 *amoA* AOA gene and 93% for *amoA* AOB gene.
287
288 *2.6 Microbial community composition*
289 Community composition and diversity analysis by terminal restriction fragment length
290 polymorphism analysis (T-RFLP) was performed with one gene of each
291 examined process, N_2-fixation (*nifH*), nitrification (*amoA* from ammonia
292 oxidizing archaea [AOA]) and denitrification (*nosZ*). Primer pairs of *nifH*, *amoA* AOA
293 and *nosZ* as well as thermal profiles were the same as described for quantitative real-
294 time PCR (Table S1) except for a reduction to 30 PCR cycles and an additional final
295 extension step of 10 min at 72°C. The forward primer was labeled with 5'-
296 carboxyfluorescein. PCR amplifications were carried out with one of the selected
297 temporal DNA samples in triplicate. The restriction enzymes AatII (*nifH*), MwoI (*amoA*
298 AOA) and HpyCH4V (*nosZ*) were selected based on *in silico* T-RFLPs using the
299 program REPK (restriction endonuclease picker) (Bannert et al., 2011; Collins and
300 Rocap, 2007). For T-RFLP migration, 1-5 ng of digested amplicons were mixed with
301 MapMarker 1000 (Eurogentec, Köln, Germany) as internal standard and separated on
302 an ABI3730 sequencer (Life Technologies, Darmstadt, Germany). Size and relative

303 abundances of terminal restriction fragments (TRFs) were analyzed using PeakScanner
304 v1.0 software (Life Technologies, Darmstadt, Germany) and T-REX
305 (http://trex.biohpc.org/) with a binning range of 1 bp. The T-RFLP profiles were
306 evaluated by calculating the relative abundance of TRFs normalized by the total signal
307 height of the respective TRF patterns. Fragments smaller than 50 bases and TRFs
308 contributing < 1% to the total peak height were excluded from the analysis (Schreiner
309 *et al.*, 2010).
310
311 *2.7 Statistical analysis*
312 The effects of GCRPS on the analyzed parameters were analyzed by using multi-factor
313 variance analysis with cultivation (GCRPS, Paddy), fertilization (N0 and N150) and
314 time (sampling date) as factors (n=3 replicated measurements per sampling day and
315 treatment). We also tested for significant differences Paddy and GCRPS cultivation at
316 each sampling time by use of One-Way ANOVA and Fishers Least Significant
317 Difference (LSD). Statistical analyses were conducted using SPSS 21 (SPSS Inc.,
318 Chicago, USA). To analyze the terminal restriction fragment data, ordination
319 techniques of correspondence analysis (CA) and canonical correspondence analysis
320 (CCA) (CANOCO version 4.53) were used for the community analysis (Bremer et al.,
321 2009; Bremer et al., 2007; Chen et al., 2010). CCA was used to assess the relationships
322 between microbial community profiles and environmental variable.
323
324 # 3. Results
325 *3.1 Soil meteorological, physical and biogeochemical parameters*

At the sites with 3 years old GCRPS plots, we observed pronounced temperature effects of GCRPS cultivation: in the first two months after the soil was covered with plastic films, soil temperature at 5 cm depth was 24.9 ^{o}C on average in GCRPS and 21.3 ^{o}C on average in the Paddy fields (Fig 1). This GCRPS-induced warming effect disappeared after the maximum tillering stage, i.e., 2 months after transplanting (Fig. 1) due to shading by the growing rice crop. Due to the increased aeration of the topsoil a significantly higher soil E_h in GCRPS than Paddy soil was observed (Table 2).

In May, i.e. at the onset of the growing season, soil NH_4^+-N concentrations were significantly higher in the fertilized treatments than in the unfertilized treatments, but not affected by the cultivation system (Table 2). However, the NH_4^+ concentrations decreased much faster in GCRPS than in Paddy soil until June. Generally, the soil NO_3^- concentrations were more than an order of magnitude lower compared to the NH_4^+ concentrations (Table 2) and neither influenced by fertilization nor by the cultivation system during the entire growing season (Table 2). Soil DOC concentrations were overall significantly lower in GCRPS compared to Paddy, and significantly lower in the fertilized treatments than in the unfertilized treatments (Table 2). Soil DON concentrations were in the same order of magnitude than soil NH_4^+ concentrations, but did not decline over time. At the first sampling date, DON concentrations were higher in GCRPS than in Paddy soils but then tended to be higher in Paddy. The SOC and TN concentrations as well as soil δ ^{15}N signatures were not significantly affected by the cultivation technique three years after conversion to GCRPS (Fig. 2). Soil microbial biomass as measured by the extracted DNA amounts was not influenced by fertilization or cultivation technique 3 years after conversion to GCRPS (Table 2).

At the site with 10 years old GCRPS plots, effects on soil DOC concentrations were similar to those observed at the other site with a significant reduction of approx. 21 to 24% in GCRPS compared to Paddy (Fig S1). Similarly, DON concentrations were smaller in GCRPS soils than in Paddy soils (Fig. S1). Soil NH_4^+ and NO_3^- concentrations were not significantly affected by GCRPS except for the NO_3^- content in the N-fertilizer treatments (G150 was significantly higher than P150, Fig S1). However, a significant reduction of microbial biomass was found during the single sampling date at the 10 years old GCRPS compared to Paddy fields (Fig. S1). The unfertilized plots affected by 10 years of GCRPS showed significantly lower SOC and TN concentrations but higher $\delta^{15}N$ signature. However, these trends were less pronounced and not statistically significant for the plots receiving fertilizer (Fig. 2).

3.2 Abundance and expression of functional genes involved in nitrogen cycling

In general, the genetic potential (based on the abundance of the investigated genes) was not or not consistently affected by 3-years of GCRPS cultivation except for *nifH,* which showed a decline, and *nirS* and *nosZ*, which increased in soils of GCRPS (Fig S2, Table 3). The increase of *chiA* copy numbers in soils of GCRPS was marginally significant ($p=0.052$, Fig S2, Table 3). *AmoA* abundance which was expected to increase under GCRPS cultivation even were significantly lower under GCRPS for some sampling dates (Fig S2). In contrast to DNA levels, transcripts showed pronounced effects in response to 3 years of GCRPS cultivation with partly diverging patterns as observed for DNA levels. Transcript levels for *nifH* were generally much higher in unfertilized fields, and persistently reduced in soils of GCRPS throughout the entire growing season (Fig 3 and 4). In contrast, both GCPRS cultivation and N fertilization increased the transcript levels of the *chiA, amoA* (AOA and AOB) *nirS* and *qnorB* after the flooding

period, i.e., when the soils were dryer in GCRPS compared to Paddy (Fig 3 and 4, Table 3). Interestingly transcripts of *amoA* AOB were not detectable after the mid-season (Fig 3). Transcripts levels related to genes catalyzing other steps of denitrification were not persistently affected by GCRPS, but followed the general trend for denitrification transcripts to decrease over the growing season (Fig. 3, Table 3). The ratio of *qnorB* : *nosZ* and *nirS* : *nosZ* was higher in soils of GCRPS, suggesting increased net N_2O production under GCRPS cultivation (Fig S3).

For the single sampling date in May at 10-year old GCRPS field sites, we found a very clear and persistent pattern of reduced abundance of all examined genes in soils of GCRPS compared to Paddy soils (Fig S4). The observed patterns of gene expression (transcripts) at the 10-year old GCRPS field resembled those observed at the 3 year old sites especially with regard to strongly reduced *nifH* and increased *nirS* gene transcript levels (Fig. S4).

3.3 Nitrous oxide fluxes and relationships to microbial community data

Nitrous oxide fluxes as obtained from the three year old GCRPS site at sampling ranged from -10 to 1200 μg N m^{-2} h^{-1}. Average N_2O fluxes at unfertilized fields were -0.09 ± 0.9 and -0.58 ± 1.4 μg N m^{-2} h^{-1} at Paddy and GCRPS sites, respectively. Fertilization drastically increased N_2O fluxes with average fluxes of 85.2 ± 79.8 for Paddy and 316.0 ± 152.3 μg N m^{-2} h^{-1} for GCRPS. Over the whole rice-growing season, GCRPS significantly increased N_2O emissions at the fertilized fields (Table 2).

To identify the importance of functional groups of microbes for soil N_2O emissions we performed regression analysis between *in-situ* N_2O emissions and the expression of

401 genes coding for enzymes driving nitrification and denitrification at the respective
402 sampling dates. Transcripts of *nirS* and *qnorB* showed significant explanatory power
403 for N_2O emissions, but only for the fertilized treatments of both Paddy- and GCRPS
404 cultivations (Fig 5). For *amoA* (AOA and AOB), neither gene abundance (DNA level)
405 nor expression (RNA level) showed significant correlations with N_2O emissions.
406
407 *3.4 Shifts of microbial community composition*
408 N_2-fixing, nitrifying and denitrifying community composition was analyzed based on
409 community fingerprinting both for soil sampled at the 3- and 10-years old
410 GCRPS/Paddy sites. The community composition of both, N_2O reducing denitrifiers
411 (*nosZ)* and N-fixing microbes (*nifH),* was clearly separated in the ordination plot,
412 indicating significant shifts of the community composition triggered by GCRPS (Fig
413 6). In contrast, the respective results for the ammonia oxidizing archaea (a*moA* AOA
414 genes) scattered around the centre of the ordination plot (Fig. 6), indicating no
415 significant change in community composition after 3 or 10 years of GCRPS cultivation.
416 Furthermore, the ordination plot also showed a well pronounced effect of fertilization
417 on the community composition of N fixing microorganisms for the 10 year old
418 Paddy/GCRPS site (Fig. 6). Among the measured abiotic factors, soil E_h was the most
419 important environmental factor influencing the denitrifying and N_2-fixing community
420 composition (P=0.01 and 0.002 for *nosZ* and *nifH* respectively) while soil NH_4^+ content
421 was the only significant factor influencing the *amoA* AOA community composition
422 (P=0.002) as revealed by Monte Carlo permutation test within CCA analysis (data not
423 shown).
424
425

4. Discussion

4.1 Water saving rice production decreases biological nitrogen fixation

The persistent negative effect of water-saving GCRPS on both abundance and expression of the nitrogenase gene *nifH* was one of the most striking findings of this study. Biological N fixation (BNF) as a highly energy demanding process is largely depending on C and light availability as well as on soil anaerobicity (Garten et al 2007, Paul et al 1971). Therefore, lower soil water content and thus better soil aeration in GCRPS (Table 2, Eh values) compared to Paddy may generally impair nitrogenase activity. To meet the high energy demand, BNF depends on photosynthesis of the plants and the provision of ATP. Hence, the significant shading effect induced by the plastic film and likely also the elimination of PAR bands by the film may have reduced the C fixation of the non-symbiontic N fixing microbial community (Belnap, 2003; Millbank, 1978). A decline in BNF at GCRPS fields could result either in less productivity or in a compensatory mining of soil N with adverse effects on associated key soil functions.

Due to the high energy demand, BNF is only advantageous under low N levels, providing the rationale for the inhibitory effects of fertilizer application and high soil mineral N availability on nitrogenase activity (Yoch and Whiting, 1986). In our study, this may explain decreased *nifH* expression and the low abundance in particular in the early stage of rice plant growth following the large N fertilizer input. These results are consistent with a series of previous findings that N fertilizer applications inhibit nitrogenase activity both in pure cultures and environmental samples (Herridge et al., 2008; Ledgard et al., 1996; Rajaramamohan-Rao, 1976).

450 BNF can play a significant role in the N balance of rice systems. Previous studies
451 showed an average input of approx. 30-40 kg N ha^{-1} by BNF when no inorganic N
452 fertilizer was applied (Hayashi et al., 2014; Roger and Ladha, 1992). The application
453 of 100 to 150 kg ha^{-1} fertilizer N reduced BNF to approx. 60% (Rao, 1976) and a
454 complete BNF inhibition was observed when fertilizer N input amounted to 300 (Rao,
455 1976) or 400 kg ha^{-1} (Yoshida et al., 1973). Accordingly, the less pronounced reduction
456 of *nifH* gene expression induced by GCRPS compared to Paddy under fertilizer
457 application (Fig. 3) and the significant interaction between the factors fertilization and
458 cultivation (Table 3b) indicate that under farmers practice of nitrogen fertilizer
459 application, the adverse effect of GCRPS on BNF may be of less importance, with N
460 fertilization then being the dominating regulator of abundance and activity of N fixing
461 microorganisms irrespective of cultivation technique (Keuter et al., 2014; Roger and
462 Ladha, 1992; Tanaka et al., 2006; Vitousek et al., 2002).
463
464 *4.2 Increased N mineralization and nitrification in GCRPS soils*
465 The soil N pool is mainly consisting of organic macromolecules such as chitin and
466 proteins, which require depolymerization and mineralization to form bioavailable
467 mineral N forms such as NH_4^+ (Schimel and Bennett, 2004). Even in fertilized paddy
468 rice fields, more than half up to 2/3 of total N taken up by rice crops can originate from
469 the soil organic N pool (Manguiat et al., 1996; Yeasmin et al., 2012), suggesting that N
470 mineralization is of high importance as a nutrient source for the rice crop. Recently,
471 *chiA* has been chosen as marker gene to study microbial N mineralization (Brankatschk
472 et al., 2013; Gschwendtner et al., 2014; Kielak et al., 2013; Xiao et al., 2005a).
473

474 At the beginning of the growing season when soils were flooded, GCRPS was not found
475 to enhance the *chiA* gene abundance and activity. In contrast, both *chiA* DNA and RNA
476 levels showed a rather small decreasing trend in soils of the GCRPS fields (Fig 3, Fig
477 S2) likely because the chitinase activity was inhibited by anaerobiosis. When the soil
478 moisture content of GCRPS was reduced to ca. 80% WHC, the *chiA* expression was
479 stronger stimulated compared to Paddy soils (Fig 3), likely a result of the facilitation of
480 mineralization due to increased soil Eh. A very similar pattern was observed for *amoA*
481 AOA transcripts (Fig. 3), suggesting both enhanced gross mineralization and
482 nitrification in soils of GCRPS fields with increasing oxygen availability (Table 2, *Eh*
483 values). A tight coupling of mineralization and nitrification can be expected since
484 mineralization provides NH_4^+, the substrate for nitrification (Booth et al., 2005;
485 Davidson et al., 1990). It has been frequently assumed that it is the submergence and
486 anaerobiosis of paddy soil that helps to maintain the relatively high soil organic matter
487 stocks and thus also the soil fertility, e.g., through indigenous N supply to rice
488 (Pampolino *et al.*, 2008). Our study therefore shows that the release of anaerobiosis due
489 to the GCRPS cultivation increases gene expression levels for mineralization.
490 Stimulated mineralization might result in decreases in soil organic matter stocks if
491 mineralization losses are not balanced by additional inputs e.g., due to higher
492 aboveground and belowground residue production and incorporation in GCRPS soils
493 (Liu et al., 2015; Liu et al., 2013; Qu et al., 2012; Tao et al., 2015).
494
495 In this study, AOB expression was only detectable in fertilized soils for the first two
496 sampling dates after N addition (Fig 3), indicating that AOB might be more active for
497 N-rich conditions. Nitrogen fertilizer applied at the begging of the growing season
498 might be rapidly consumed within few weeks in paddy soil (Dobbie et al., 1999; Xing

and Zhu, 1997; Yao et al., 2014). During this period, the activity of AOA was not (3 years GCRPS) or negatively (10 years GCRPS) affected by GCRPS, which might be due to the inhibitory effects induced by the high NH_4^+ contents (Table 2). The AOA community is known to exhibit a relatively higher affinity for ammonia compared to AOB and thus is more adapted to oligotrophic or lower NH_4^+ environments, where they would be more active and responsive to environmental variations (Hansel et al., 2008; Hartmann et al., 2009; Jackson et al., 2009; Schauss et al., 2009; Zhang et al., 2012). Hence, nitrification might have been driven by predominantly AOB under the nitrogen-rich conditions after fertilization (Di et al., 2009; Jia and Conrad, 2009).

4.3 Effects of GCRPS on denitrification

The nitrate produced during nitrification may serve as substrate for denitrification, resulting in the stepwise reduction to gaseous compounds (NO, N_2O, N_2) and consequently in a loss of N from the ecosystem. In the present study, the last three steps of denitrification (the reduction of NO_2^-, NO and N_2O) were investigated by quantifying the transcripts of *nirK*/*nirS*, *qnorB* and *nosZ* genes, respectively. Denitrification is closely linked to labile C and nitrate availability as well as oxygen partial pressure (Barnard et al., 2005; Butterbach-Bahl et al., 2013) and thus can be assumed to be highly sensitive to changes in soil environmental conditions by GCRPS, i.e. increased soil temperature and reduced soil moisture. The decreasing expression of denitrification genes over the growing season both in GCRPS- and Paddy soils (Fig 3, Table 3) might arise from increasing N substrate scarcity (Braker and Conrad, 2011; Butterbach-Bahl and Dannenmann, 2011; Chen et al., 2015; Miller et al., 2008, 2009; Philippot et al., 2007). This decline in N substrate over the growing season likely is mainly caused by plant N uptake.

524

525 The reduction of NO_2^- is catalyzed by two functionally redundant enzymes encoded by
526 the *nirK* and *nirS* genes which do not exist together in one organism (Chen et al., 2010;
527 Coyne et al., 1989). In our study, GCRPS cultivation significantly enhanced *nirS* but
528 not *nirK* expression with the temporal patterns of *nirS* expression resembling those of
529 *cnorB* expression. Furthermore, *nirS* transcript levels but not *nirK* transcript levels were
530 related to N_2O emission (Fig 3 and 5). This might indicate that *nirS*-denitrifiers might
531 be more involved in nitrite reduction compared to *nirK*-denitrifiers in the rice soils
532 under investigation. Neither *cnorB* DNA nor RNA were detected in this study, which
533 is in agreement with an earlier Paddy soil study, in which also *qnorB,* rather than *cnorB*,
534 was detected by sequencing based approaches (Chen *et al.*, 2012). This could on the
535 one hand indicate that *cnorB*-denitrifiers might generally be less involved in
536 denitrification in Paddy soil. On the other hand, another explanation might be that the
537 currently used *cnorB* primers are not covering the species of paddy soils (Braker and
538 Conrad, 2011; Braker and Tiedje, 2003; Chen et al., 2015; Gschwendtner et al., 2014).
539

540 While GCRPS cultivation enhanced the expression of *qnorB* and *nirS* genes compared
541 to Paddy soil (Fig 3), the expression of *nosZ*, catalyzing the last step of denitrification,
542 was overall not significantly affected and rather tended to be reduced during the
543 growing season (Fig 3). This might be related to the higher sensitivity of *nosZ*
544 expression to oxygen availability compared to the earlier steps in the denitrification
545 chain (Zumft, 1997), and the increasing oxygen availability in soil under GCRPS
546 cultivation. Thus, higher ratios of *nirS:nosZ* and *qnorB:nosZ* were found for soils under
547 GCRPS compared to Paddy cultivation (Fig S3). This suggests that increased gross N_2O
548 production in GCRPS soils is not balanced by a similar increase in gross N_2O

549 consumption, overall resulting in increasing net N_2O emissions. Consequently, these
550 gene expression patterns provide a mechanistic rationale for the increased soil N_2O
551 emissions under GCRPS cultivation as observed in this and several other studies
552 (Uchida et al., 2014; Xu et al., 2004; Yao et al., 2014), which was further strengthened
553 by the direct relationships between *nirS* and *qnorB* transcript levels and soil N_2O
554 emissions with their high explanatory power.
555
556 *4.4 Community composition of N_2-fixers, denitrifiers and nitrifiers*
557 With respect to functional redundancy within microbial populations, also community
558 structure was investigated to sharpen our understanding of GCRPS effects on resilience
559 and vulnerability of N cycle processes. Besides fertilization, both 3 years and 10 years
560 of GCRPS changed the composition of the denitrifier and N_2-fixing communities with
561 most pronounced shifts being induced by a decade of GCRPS cultivation. Our CCA
562 analysis showed soil E_h was responsible for compositional shifts in denitrifying and N_2-
563 fixing communities, indicating that soil moisture was a stronger factor than temperature
564 influencing microbial community. Similarly, Liu et al (2012) also found E_h as a
565 dominant variable in controlling/changing denitrifying community composition in two
566 paddy soils. These findings suggest a persistent change of the soil biogeochemical N
567 cycle at GCRPS fields established already after a few years. Furthermore, a return to
568 Paddy cultivation might not result in a quick recovery of soil functions related to the N
569 cycle microbial communities. Compared to the denitrifying and N_2-fixing communities,
570 GCRPS exerted a weaker impact on the nitrifying community composition, indicating
571 that the AOA community possesses a stronger capacity in maintaining the divergence
572 to adapt to changing environmental conditions.

573

574 *4.5 Synthesis: GCRPS effects on soil C and N biogeochemistry and implications for soil*
575 *functions*

576 This study provides for the first time an assessment of GCRPS effects on the key
577 processes of N turnover during the rice growing season. At the intensively investigated
578 site, we found that 3 years of GCRPS cultivation induced increased mineralization,
579 nitrification and denitrification (Fig. 4). Furthermore, the data on gene abundance
580 suggest a GCRPS-induced higher risk of loss of the potent greenhouse gas N_2O due to
581 increased gross N_2O production which is not matched by increased gross N_2O
582 consumption. Direct links between denitrifier abundances and in situ N_2O emissions,
583 which strongly increased at GCRPS fields, strongly underlined this interpretation.
584 Moreover, also biological N fixation significantly decreased over the entire growing
585 season. These alterations of the N cycle seem to be persistent over time as indicated by
586 microbial community changes. Also at the second site with its 10 years of GCRPS
587 cultivation history, similar patterns were observed mainly for the unfertilized treatment.
588 For the unfertilized treatment even a reduction in soil N and C stocks under GCRPS
589 could be observed. Higher N losses through gaseous pathways at the unfertilized, 10
590 years old GCRPS treatment was confirmed by increased $\delta^{15}N$ values. It might be
591 speculated that the site location at the bottom of valley floors cold have promoted high
592 C and N leaching due to groundwater flow, so that the excess dissolved C and N
593 produced by increased mineralization in GCRPS compared to Paddy was easily
594 leached, resulting in higher C and N leaching losses than in Paddy. Consequently,
595 increased N mineralization with associated nutrient mining from soil organic matter
596 might have led to a net loss of SOC and TN. Interestingly this net loss was only

observed for unfertilized treatments, i.e. a practice which was only introduced as a control and is not done by farmers. Under such conditions, the observed increases in yields for GCRPS as compared to Paddy (Qu et al., 2012) were likely due to the promotion of N mineralization of the soil organic matter pool, which finally resulted in reduced C and N stocks. While yield increases under GCRPS has been confirmed by most available studies (Fan et al., 2012; Li et al., 2007; Liu et al., 2015; Liu et al., 2013; Qu et al., 2012; Tao et al., 2015), there are controversial results on GCRPS effects on SOC and TN stocks. A reduction of soil organic matter by 8-25% and total N by 5-22% under GCRPS compared with conventional paddy was reported at five rice field sites in China (Li *et al.*, 2007). In contrast, the regional study of Liu et al. (2015) revealed based on the quantification of SOC and TN stocks down to 1 m depth at 49 pairs of GCRPS/Paddy fields overall significantly increased SOC and TN stocks at GCRPS fields. It is urgently needed to understand which factors determine whether GCRPS results in increased or decreased SOC and TN stocks. Liu et al. (2015) discussed increased above- and belowground carbon inputs due to improved root growth, greater physical protection of soil organic matter against microbial degradation and increased N use efficiency due to a soil coverage-induced minimization of NH_3 loss as potential reasons for increased soil organic matter stocks in GCRPS fields. Vice versa, as GCRPS soils are more aerobic as compared to Paddy soils increased mineralization with associated increased C and N losses along gaseous and hydrological pathways can be expected too, as was confirmed by our study for unfertilized GCRPS treatments. In the rice growing region of the Hubei province, increased plant residue inputs due to increased crop growth appear to generally overcompensate increased C and N losses due stimulated mineralization, so that SOC and TN stocks increased (Liu et al., 2015).

621 Under absence of fertilization however, these advantages of the GCRPS technique may
622 be limited.
623
624 Overall, our study shows in detail at process levels how GCRPS promotes C and N
625 mineralization and nitrification and soil N_2O loss through denitrification while reducing
626 activity of microbes involved in BNF. Still there is the need to better constrain the
627 hydrological, climatic, soil and field management conditions under which gross effects
628 of GCRPS on C and N input, turnover as well as output result in net C and N losses or
629 gain, thus allowing for a more targeted implementation of GCRPS with a better use of
630 the potential environmental and agronomic benefits of this innovative water saving rice
631 cultivation technique.
632
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637

638 # References

639 1. Bannert, A., Kleineidam, K., Wissing, L., Mueller-Niggemann, C., Vogelsang, V., Welzl,
640 G., Cao, Z., Schloter, M., 2011. Changes in Diversity and Functional Gene Abundances of
641 Microbial Communities Involved in Nitrogen Fixation, Nitrification, and Denitrification
642 in a Tidal Wetland versus Paddy Soils Cultivated for Different Time Periods. Applied and
643 Environmental Microbiology 77, 6109-6116.
644 2. Barnard, R., Leadley, P.W., Hungate, B.A., 2005. Global change, nitrification, and
645 denitrification: A review. Global Biogeochemical Cycles 19, GB1007,
646 doi:1010.1029/2004GB002282.
647 3. Belder, P., Bouman, B.A.M., Cabangon, R., Guoan, L., Quilang, E.J.P., Yuanhua, L.,
648 Spiertz, J.H.J., Tuong, T.P., 2004. Effect of water-saving irrigation on rice yield and water
649 use in typical lowland conditions in Asia. Agricultural Water Management 65, 193-210.
650 4. Belnap, J., 2003. Factors Influencing Nitrogen Fixation and Nitrogen Release in Biological
651 Soil Crusts, In: Belnap, J., Lange, O. (Eds.), Biological Soil Crusts: Structure, Function,
652 and Management. Springer Berlin Heidelberg, pp. 241-261.
653 5. Booth, M.S., Stark, J.M., Rastetter, E., 2005. Controls on nitrogen cycling in terrestrial
654 ecosystems: a synthetic analysis of literature data. Ecological Monographs 75, 139-157.
655 6. Bouman, B.A.M., 2007. A conceptual framework for the improvement of crop water
656 productivity at different spatial scales. Agricultural Systems 93, 43-60.
657 7. Bouman, B.A.M., Tuong, T.P., 2001. Field water management to save water and increase
658 its productivity in irrigated lowland rice. Agricultural Water Management 49, 11-30.
659 8. Braker, G., Conrad, R., 2011. Diversity, Structure, and Size of N_2O-Producing Microbial
660 Communities in Soils-What Matters for Their Functioning?, In: Laskin, A.I., Sariaslani,
661 S., Gadd, G.M. (Eds.), Advances in Applied Microbiology, Vol 75, pp. 33-70.
662 9. Braker, G., Fesefeldt, A., Witzel, K.P., 1998. Development of PCR primer systems for
663 amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in
664 environmental samples. Applied and Environmental Microbiology 64, 3769-3775.

10. Braker, G., Tiedje, J.M., 2003. Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. Applied and Environmental Microbiology 69, 3476-3483.
11. Brankatschk, R., Fischer, T., Veste, M., Zeyer, J., 2013. Succession of N cycling processes in biological soil crusts on a Central European inland dune. Fems Microbiology Ecology 83, 149-160.
12. Bremer, C., Braker, G., Matthies, D., Beierkuhnlein, C., Conrad, R., 2009. Plant presence and species combination, but not diversity, influence denitrifier activity and the composition of nirK-type denitrifier communities in grassland soil. FEMS Microbiology Ecology 70, 377-387.
13. Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., Conrad, R., 2007. Impact of plant functional group, plant species, and sampling time on the composition of nirK-Type denitrifier communities in soil. Applied and Environmental Microbiology 73, 6876-6884.
14. Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical transactions of the royal society B 368, http://dx.doi.org/10.1098 /rstb.2013.0122.
15. Butterbach-Bahl, K., Dannenmann, M., 2011. Denitrification and associated soil N_2O emissions due to agricultural activities in a changing climate. Current Opinion in Environmental Sustainability 3, 389-395.
16. Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., Wei, W., 2012. Differentiated Response of Denitrifying Communities to Fertilization Regime in Paddy Soil. Microbial Ecology 63, 446-459.
17. Chen, Z., Luo, X., Hu, R., Wu, M., Wu, J., Wei, W., 2010. Impact of Long-Term Fertilization on the Composition of Denitrifier Communities Based on Nitrite Reductase Analyses in a Paddy Soil. Microbial Ecology 60, 850-861.
18. Chen, Z., Wang, C., Gschwendtner, S., Willibald, G., Unteregelsbacher, S., Lu, H., Kolar, A., Schloter, M., Butterbach-Bahl, K., Dannenmann, M., 2015. Relationships between

denitrification gene expression, dissimilatory nitrate reduction to ammonium and nitrous oxide and dinitrogen production in montane grassland soils. Soil Biology and Biochemistry 87, 67-77.

19. Collins, R.E., Rocap, G., 2007. REPK: an analytical web server to select restriction endonucleases for terminal restriction fragment length polymorphism analysis. Nucleic Acids Research 35, W58-W62.

20. Coyne, M.S., Arunakumari, A., Averill, B.A., Tiedje, J.M., 1989. Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductase in denitrifying bacteria. Applied and Environmental Microbiology 55, 2924-2931.

21. Crouzet, O., Poly, F., Bonnemoy, F., Bru, D., Batisson, I., Bohatier, J., Philippot, L., Mallet, C., 2016. Functional and structural responses of soil N-cycling microbial communities to the herbicide mesotrione: a dose-effect microcosm approach. Environmental Science and Pollution Research 23, 4207-4217.

22. Davidson, E.A., Stark, J.M., Firestone, M.K., 1990. MICROBIAL-PRODUCTION AND CONSUMPTION OF NITRATE IN AN ANNUAL GRASSLAND. Ecology 71, 1968-1975.

23. Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O/'Callaghan, M., Bowatte, S., He, J.Z., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nature Geosci 2, 621-624.

24. Dobbie, K.E., McTaggart, I.P., Smith, K.A., 1999. Nitrous oxide emissions from intensive agricultural systems: Variations between crops and seasons, key driving variables, and mean emission factors. Journal of Geophysical Research: Atmospheres 104, 26891-26899.

25. Fan, M.S., Lu, S.H., Jiang, R.F., Six, J., Zhang, F.S., 2012. Long-term non-flooded mulching cultivation influences rice productivity and soil organic carbon. Soil Use and Management 28, 544-550.

718 26. FAO, 2011. (Food and Agriculture Organization): FAOSTAT online electronic data base,
719 http://faostat.fao.org/.
720 27. Gschwendtner, S., Tejedor, J., Bimueller, C., Dannenmann, M., Knabner, I.K., Schloter,
721 M., 2014. Climate Change Induces Shifts in Abundance and Activity Pattern of Bacteria
722 and Archaea Catalyzing Major Transformation Steps in Nitrogen Turnover in a Soil from
723 a Mid-European Beech Forest. Plos One 9.
724 28. Hansel, C.M., Fendorf, S., Jardine, P.M., Francis, C.A., 2008. Changes in Bacterial and
725 Archaeal Community Structure and Functional Diversity along a Geochemically Variable
726 Soil Profile. Applied and Environmental Microbiology 74, 1620-1633.
727 29. Harris, D., Horwáth, W.R., van Kessel, C., 2001. Acid fumigation of soils to remove
728 carbonates prior to total organic carbon or CARBON-13 isotopic analysis. Soil Science
729 Society of America Journal 65.
730 30. Hartmann, M., Lee, S., Hallam, S.J., Mohn, W.W., 2009. Bacterial, archaeal and eukaryal
731 community structures throughout soil horizons of harvested and naturally disturbed forest
732 stands. Environmental Microbiology 11, 3045-3062.
733 31. Hayashi, K., Tokida, T., Matsushima, M.Y., Ono, K., Nakamura, H., Hasegawa, T., 2014.
734 Free-air CO2 enrichment (FACE) net nitrogen fixation experiment at a paddy soil surface
735 under submerged conditions. Nutrient Cycling in Agroecosystems 98, 57-69.
736 32. Henry, S., Baudoin, E., Lopez-Gutierrez, J.C., Martin-Laurent, F., Brauman, A., Philippot,
737 L., 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time
738 PCR. Journal of Microbiological Methods 59, 327-335.
739 33. Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 2006. Quantitative detection of the
740 *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S
741 rRNA, *narG*, *nirK*, and *nosZ* genes in soils. Applied and Environmental Microbiology 72,
742 5181-5189.
743 34. Herridge, D., Peoples, M., Boddey, R., 2008. Global inputs of biological nitrogen fixation
744 in agricultural systems. Plant and Soil 311, 1-18.

745 35. IRRI, 2011. International Rice Research Institute, Los Banos, Laguna, Philippines.
746 http://irri.org/news/hot-topics/international-land-acquisition-for-rice-production.
747 36. Jackson, C.R., Liew, K.C., Yule, C.M., 2009. Structural and Functional Changes with
748 Depth in Microbial Communities in a Tropical Malaysian Peat Swamp Forest. Microbial
749 Ecology 57, 402-412.
750 37. Jia, Z., Conrad, R., 2009. Bacteria rather than Archaea dominate microbial ammonia
751 oxidation in an agricultural soil. Environmental Microbiology 11, 1658-1671.
752 38. Jin, X., Zuo, Q., Ma, W., Li, S., Shi, J., Tao, Y., Zhang, Y., Liu, Y., Liu, X., Lin, S., Ben-
753 Gal, A., 2016. Water consumption and water-saving characteristics of a ground cover rice
754 production system. Journal of Hydrology 540, 220-231.
755 39. Keuter, A., Veldkamp, E., Corre, M.D., 2014. Asymbiotic biological nitrogen fixation in
756 a temperate grassland as affected by management practices. Soil Biology and Biochemistry
757 70, 38-46.
758 40. Kielak, A.M., Cretoiu, M.S., Semenov, A.V., Sørensen, S.J., van Elsas, J.D., 2013.
759 Bacterial Chitinolytic Communities Respond to Chitin and pH Alteration in Soil. Applied
760 and Environmental Microbiology 79, 263-272.
761 41. Kornaros, M., Zafiri, C., Lyberatos, G., 1996. Kinetics of denitrification by Pseudomonas
762 denitrificans under growth conditions limited by carbon and/or nitrate or nitrite. Water
763 Environment Research 68, 934-945.
764 42. Ledgard, S., Sprosen, M., Steele, K., 1996. Nitrogen fixation by nine white clover cultivars
765 in grazed pasture, as affected by nitrogen fertilization. Plant and Soil 178, 193-203.
766 43. Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I.,
767 Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing
768 prokaryotes in soils. Nature 442, 806-809.
769 44. Li, Y.-S., Wu, L.-H., Zhao, L.-M., Lu, X.-H., Fan, Q.-L., Zhang, F.-S., 2007. Influence of
770 continuous plastic film mulching on yield, water use efficiency and soil properties of rice
771 fields under non-flooding condition. Soil & Tillage Research 93, 370-378.

772 45. Lin, S., Dittert, K., Tao, H.B., Kreye, C., Xu, Y.C., Shen, Q.R., Fan, X.L., Sattelmacher,
773 B., 2002. The ground-cover rice production system (GCRPS): A successful new approach
774 to save water and increase nitrogen fertilizer efficiency? . In: Bouman, B.A.M., Hengsdijk,
775 H., Hardy, B., Bindraban, P.S., Tuong, T.P., Ladha, J.K. (Eds.),
776 46. Water-wise Rice Production: Proceedings of the International Workshop on Water-wise
777 Rice Production, Los Baños, Philippines. 8–11 Apr. IRRI, Los Baños, Philippines, pp.
778 187–196.
779 47. Liu, B., Frostegård, Å., Bakken, L.R., 2014. Impaired reduction of N_2O to N_2 in acid soils
780 is due to a posttranscriptional interference with the expression of *nosZ*. mBio 5.
781 48. Liu, M., Dannenmann, M., Lin, S., Saiz, G., Yan, G., Yao, Z., Pelster, D.E., Tao, H.,
782 Sippel, S., Tao, Y., Zhang, Y., Zheng, X., Zuo, Q., Butterbach-Bahl, K., 2015. Ground
783 cover rice production systems increase soil carbon and nitrogen stocks at regional scale.
784 Biogeosciences 12, 4831-4840.
785 49. Liu, M., Lin, S., Dannenmann, M., Tao, Y., Saiz, G., Zuo, Q., Sippel, S., Wei, J., Cao, J.,
786 Cai, X., Butterbach-Bahl, K., 2013. Do water-saving ground cover rice production systems
787 increase grain yields at regional scales? Field Crops Research 150, 19-28.
788 50. Lueders, T., Manefield, M., Friedrich, M.W., 2004. Enhanced sensitivity of DNA- and
789 rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic
790 centrifugation gradients. Environmental Microbiology 6, 73-78.
791 51. Manguiat, I.J., Watanabe, I., Mascariña, G.B., Tallada, J.G., 1996. Nitrogen mineralization
792 in tropical wetland rice soils. Soil Science and Plant Nutrition 42, 229-238.
793 52. Marstorp, H., Guan, X., Gong, P., 2000. Relationship between dsDNA, chloroform labile
794 C and ergosterol in soils of different organic matter contents and pH. Soil Biology and
795 Biochemistry 32, 879-882.
796 53. Michotey, V., Mejean, V., Bonin, P., 2000. Comparison of methods for quantification of
797 cytochrome cd(1)-denitrifying bacteria in environmental marine samples. Applied and
798 Environmental Microbiology 66, 1564-1571.

54. Millbank, J.W., 1978. The Contribution of Nitrogen Fixing Lichens to the Nitrogen Status of Their Environment. Ecological Bulletins, 260-265.
55. Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. Soil Biology & Biochemistry 40, 2553-2562.
56. Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2009. Influence of liquid manure on soil denitrifier abundance, denitrification, and nitrous oxide emissions. Soil Science Society of America Journal 73, 760-768.
57. Pampolino, M.F., Laureles, E.V., Gines, H.C., Buresh, R.J., 2008. Soil Carbon and Nitrogen Changes in Long-Term Continuous Lowland Rice Cropping. Soil Science Society of America Journal 72, 798-807.
58. Philippot, L., Hallin, S., 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. Current Opinion in Microbiology 8, 234-239.
59. Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural soil, In: Sparks, D.L. (Ed.), Advances in Agronomy, Vol 96, pp. 249-305.
60. Philippot, L., Spor, A., Henault, C., Bru, D., Bizouard, F., Jones, C.M., Sarr, A., Maron, P.-A., 2013. Loss in microbial diversity affects nitrogen cycling in soil. Isme Journal 7, 1609-1619.
61. Qu, H., Tao, H., Tao, Y., Liu, M., Shen, K., Lin, S., 2012. Ground Cover Rice Production System Increases Yield and Nitrogen Recovery Efficiency. Agronomy Journal 104, 1399-1407.
62. Rajaramamohan-Rao, V., 1976. Nitrogen fixation as influenced by moisture content, ammonium sulphate and organic sources in a paddy soil. Soil Biology and Biochemistry 8, 445-448.

824 63. Rao, V.R., 1976. NITROGEN-FIXATION AS INFLUENCED BY MOISTURE-
825 CONTENT, AMMONIUM-SULFATE AND ORGANIC SOURCES IN A PADDY
826 SOIL. Soil Biology & Biochemistry 8, 445-448.
827 64. Ray, D.K., Mueller, N.D., West, P.C., Foley, J.A., 2013. Yield Trends Are Insufficient to
828 Double Global Crop Production by 2050. Plos One 8.
829 65. Roger, P.A., Ladha, J.K., 1992. BIOLOGICAL N2 FIXATION IN WETLAND RICE
830 FIELDS - ESTIMATION AND CONTRIBUTION TO NITROGEN-BALANCE. Plant
831 and Soil 141, 41-55.
832 66. Rosch, C., Mergel, A., Bothe, H., 2002. Biodiversity of denitrifying and dinitrogen-fixing
833 bacteria in an acid forest soil. Applied and Environmental Microbiology 68, 3818-3829.
834 67. Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural
835 gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-
836 oxidizing populations. Applied and Environmental Microbiology 63, 4704-4712.
837 68. Ruser, R., Flessa, H., Russow, R., Schmidt, G., Buegger, F., Munch, J.C., 2006. Emission
838 of N_2O, N_2 and CO_2 from soil fertilized with nitrate: effect of compaction, soil moisture
839 and rewetting. Soil Biology and Biochemistry 38, 263-274.
840 69. Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma,
841 S., Wilke, B.-M., Matthies, M., Smalla, K., Munch, J.C., Amelung, W., Kaupenjohann,
842 M., Schloter, M., Schleper, C., 2009. Dynamics and functional relevance of ammonia-
843 oxidizing archaea in two agricultural soils. Environmental Microbiology 11, 446-456.
844 70. Schimel, J.P., Bennett, J., 2004. NITROGEN MINERALIZATION: CHALLENGES OF
845 A CHANGING PARADIGM. Ecology 85, 591-602.
846 71. Scholefield, D., Hawkins, J.M.B., Jackson, S.M., 1997. Use of a flowing helium
847 atmosphere incubation technique to measure the effects of denitrification controls applied
848 to intact cores of a clay soil. Soil Biology and Biochemistry 29, 1337-1344.
849 72. Schreiner, K., Hagn, A., Kyselkova, M., Moenne-Loccoz, Y., Welzl, G., Munch, J.C.,
850 Schloter, M., 2010. Comparison of Barley Succession and Take-All Disease as

Environmental Factors Shaping the Rhizobacterial Community during Take-All Decline. Applied and Environmental Microbiology 76, 4703-4712.

73. Sessitsch, A., Gyamfi, S., Stralis-Pavese, N., Weilharter, A., Pfeifer, U., 2002. RNA isolation from soil for bacterial community and functional analysis: evaluation of different extraction and soil conservation protocols. Journal of Microbiological Methods 51, 171-179.

74. Tanaka, H., Kyaw, K.M., Toyota, K., Motobayashi, T., 2006. Influence of application of rice straw, farmyard manure, and municipal biowastes on nitrogen fixation, soil microbial biomass N, and mineral N in a model paddy microcosm. Biology and Fertility of Soils 42, 501-505.

75. Tao, Y., Zhang, Y., Jin, X., Saiz, G., Jing, R., Guo, L., Liu, M., Shi, J., Zuo, Q., Tao, H., Butterbach-Bahl, K., Dittert, K., Lin, S., 2015. More rice with less water – evaluation of yield and resource use efficiency in ground cover rice production system with transplanting. European Journal of Agronomy 68, 13-21.

76. Throback, I.N., Enwall, K., Jarvis, A., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology 49, 401-417.

77. Tuong, P., Bouman, B.A.M., Mortimer, M., 2005. More Rice, Less Water—Integrated Approaches for Increasing Water Productivity in Irrigated Rice-Based Systems in Asia. Plant Production Science 8, 231-241.

78. Uchida, Y., Wang, Y., Akiyama, H., Nakajima, Y., Hayatsu, M., 2014. Expression of denitrification genes in response to a waterlogging event in a Fluvisol and its relationship with large nitrous oxide pulses. Fems Microbiology Ecology 88, 407-423.

79. Vitousek, P.M., Cassman, K., Cleveland, C., Crews, T., Field, C.B., Grimm, N.B., Howarth, R.W., Marino, R., Martinelli, L., Rastetter, E.B., Sprent, J.I., 2002. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57, 1-45.

80. Xiao, X., Yin, X., Lin, J., Sun, L., You, Z., Wang, P., Wang, F., 2005a. Chitinase Genes in Lake Sediments of Ardley Island, Antarctica. Applied and Environmental Microbiology 71, 7904-7909.
81. Xiao, X., Yin, X.B., Lin, H., Sun, L.G., You, Z.Y., Wang, P., Wang, F.P., 2005b. Chitinase genes in lake sediments of Ardley Island, Antarctica. Applied and Environmental Microbiology 71, 7904-7909.
82. Xing, G.X., Zhu, Z.L., 1997. Preliminary studies on N2O emission fluxes from upland soils and paddy soils in China. Nutrient Cycling in Agroecosystems 49, 17-22.
83. Xu, Y.C., Shen, Q.R., Li, M.L., Dittert, K., Sattelmacher, B., 2004. Effect of soil water status and mulching on N2O and CH4 emission from lowland rice field in China. Biology and Fertility of Soils 39, 215-217.
84. Yao, Z., Du, Y., Tao, Y., Zheng, X., Liu, C., Lin, S., Butterbach-Bahl, K., 2014. Water-saving ground cover rice production system reduces net greenhouse gas fluxes in an annual rice-based cropping system. Biogeosciences 11, 6221-6236.
85. Yao, Z., Wu, X., Wolf, B., Dannenmann, M., Butterbach-Bahl, K., Brueggemann, N., Chen, W., Zheng, X., 2010. Soil-atmosphere exchange potential of NO and N2O in different land use types of Inner Mongolia as affected by soil temperature, soil moisture, freeze-thaw, and drying-wetting events. Journal of Geophysical Research-Atmospheres 115.
86. Yeasmin, S., Islam, A., Islam, A., 2012. Nitrogen fractionation and its mineralization in paddy soils: a review. Journal of Agricultural Technology 8, 775-793.
87. Yoshida, T., Roncal, R.A., Bautista, E.M., 1973. Atmospheric nitrogen fixation by photosynthetic microorganisms in a submerged Philippine soil. Soil Science and Plant Nutrition 19, 117-123.
88. Yuan, L., Zhang, Z., Cao, X., Zhu, S., Zhang, X., Wu, L., 2014. Responses of rice production, milled rice quality and soil properties to various nitrogen inputs and rice straw

903 incorporation under continuous plastic film mulching cultivation. Field Crops Research
904 155, 164-171.
905 89. Zhang, L.-M., Hu, H.-W., Shen, J.-P., He, J.-Z., 2012. Ammonia-oxidizing archaea have
906 more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly
907 acidic soils. Isme Journal 6, 1032-1045.
908 90. Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology and
909 Molecular Biology Reviews 61, 533-616.
910

Table 1 Site characteristics. Soil data are for 0-20 cm depth.

Field	Location	Mean annual temperature (°C)	Mean annual precipitation (mm)	Elevation (a.s.l.) (m)	Soil texture (sand/silt/clay) (%)	Soil bulk density (g cm^{-3})	Soil pH	Soil total organic carbon (g C kg^{-1})	Soil total nitrogen (g N kg^{-1})
3year GCRPS	N32°07′ E110°43′	14.7	856	440	20/60/20	1.36	6.0	21.3	1.31
10year GCRPS	N32°38′ E110°37′	15.3	834	234	39/59/2	1.32	6.2	10.3	1.20

Table 2 Effects of three years of GCRPS cultivation on soil (a) ammonium (NH_4^+-N), (b) nitrate (NO_3^--N), (c) *Eh* (mV), (d) dissolved organic carbon (DOC), (e) dissolved organic nitrogen (DON), (f) microbial biomass (measured as DNA concentration) for 0-20 cm soil depth and (g) field N_2O flux. Data are shown as means ± standard errors (n = 3). Results from Multivariate analysis of ANOVA testing the effects of cultivation (GCRPS vs Paddy), Fertilization (0 and 150 kg N/ha), date and their interaction are presented. P0, paddy soil without N fertilizer; G0, GCRPS without N fertilizer; P150, paddy soil with fertilization of 150 kg N $ha^{-1}a^{-1}$; G150, GCRPS with fertilization of 150 kg N $ha^{-1}a^{-1}$. Significant differences at $P < 0.05$ are showed in bold. nd: not detectable N concentrations.

	Treatments				Multivariate analysis of ANOVA					
	P0	G0	P150	G150	Culti.	Ferti.	Date	Culti. ×Ferti	Culti. ×Date	Ferti. ×Date
(a) NH_4^+ (mg N kg^{-1})										
May	8.43±1.04	7.15±0.70	11.65±0.54	13.60±3.40	0.686	**0.002**	**<0.001**	0.317	**0.003**	**<0.001**
June	4.49±0.26	2.93±0.56	6.01±0.77	2.8±0.11						
July	3.50±0.22	2.40±0.31	4.06±0.2	2.18±0.16						
Sep.	3.46±0.47	2.78±0.53	3.08±0.42	1.48±0.09						
(b) NO_3^- (mg N kg^{-1})										
May	0.27±0.100	0.20±0.002	0.12±0.02	0.17±0.16	0.719	0.411	**0.014**	0.367	0.846	0.475
June	0.40±0.002	0.32±0.02	0.34±0.06	0.36±0.18						

July	0.12±0.002	0.07±0.02	nd.	nd.						
Sep.	0.10±0.008	nd.	nd.	0.08±0.007						
(c) *Eh* (mV)										
May	-168±31	-32±1	-186±37	-32±6	**<0.001**	**<0.001**	**<0.001**	0.002	**<0.001**	0.136
June	-167±59	392±215	-114±5	833±148						
July	-153±25	392±186	-108±39	853±124						
Sep.	-186±78	74±24	-158±43	580±81						
(d) DOC (mg C kg^{-1})										
May	130.9±3.3	129.7±2.4	116.5±1.0	139.7±7.6	**0.004**	**<0.001**	**<0.001**	0.**02**	**0.004**	**0.014**
June	124.7±3.9	82.2±6.3	116.4±7.7	71.6±11.3						
July	155.7±13.1	124.1±8.0	173.7±5.6	108.1±3.1						
Sep.	130.0±13.0	142.7±18.5	99.8±23.5	75.4±2.8						
(e) DON (mg N kg^{-1})										
May	10.01±0.19	11.58±0.11	8.16±0.19	11.85±0.89	0.089	**0.003**	**<0.001**	0.449	**0.007**	0.091
June	11.89±0.77	8.19±1.10	10.77±1.05	8.08±1.24						
July	15.13±1.46	12.33±1.43	16.44±0.64	11.98±0.11						
Sep.	16.34±1.67	14.88±1.82	12.36±2.48	10.03±0.72						
(f) Microbial biomass (μg DNA g^{-1})										

May	20.5±0.1	17.9±1.2	20.9±0.7	17.5±2.2	0.859	0.268	**<0.001**	0.580	0.083	0.589
June	19.3±1.3	18.3±1.8	19.2±2.5	18.2±1.2						
July	18.8±0.6	20.8±1.8	19.0±2.2	23.9±1.1						
Sep.	13.5±0.8	14.7±1.7	14.8±1.3	15.5±1.5						
(g) N_2O flux (μg N m^{-2} h^{-1})										
May	1.9±0.1	-1.71±2.69	334.7±313.5	1183.1±72.4	**0.008**	**<0.001**	**<0.001**	**0.008**	**0.002**	**<0.001**
June	-1.77±1.64	-0.51±2.80	5.40±7.34	80.09±36.23						
July	2.66±0.88	0.48±1.14	10.24±4.85	-0.41±9.43						
Sep.	-3.15±1.85	-0.58±4.75	-9.41±0.81	1.24±5.21						

Table 3 Results of multivariate ANOVA testing the effects of cultivation (GCRPS vs Paddy), fertilization (0N and 150N), time and their interaction on the microbial parameters quantified in the paired Paddy - 3 years old GCRPS fields. Significant effects are indicated in bold. Vertical arrows show the direction of significant GCRPS effects (increase, decrease).

	Cultivation		Fertilization		Time		Culti. ×Ferti		Culti. ×Time		Ferti. ×Time	
	F value	P	F value	P	F value	P	F value	P	F value	P	F value	P
(a) gene abundance												
nifH	4.34	**0.045 ↓**	4.94	**0.033**	10.72	**<0.0001**	0.47	0.499	1.59	0.211	1.56	0.217
chiA	7.04	0.052	7.55	**0.010**	10.85	**<0.0001**	0.36	0.552	3.57	**0.025**	2.99	**0.045**
AOA	0.01	0.988	0.21	0.648	8.98	**<0.0001**	4.56	**0.040**	0.31	0.818	0.36	0.785
AOB	1.05	0.314	0.05	0.829	10.13	**<0.0001**	9.70	**0.004**	0.08	0.969	0.19	0.901
nirK	1.17	0.288	0.27	0.607	16.76	**<0.0001**	0.01	0.927	0.48	0.697	0.99	0.410
nirS	10.50	**0.003 ↑**	0.03	0.860	10.32	**<0.0001**	0.13	0.724	0.58	0.633	2.06	0.125
qnorB	0.01	0.985	0.48	0.494	11.19	**<0.0001**	0.09	0.767	4.48	**0.010**	1.07	0.377
nosZ	5.49	**0.026 ↓**	5.79	**0.022**	6.88	**0.001**	5.57	**0.025**	1.07	0.378	0.65	0.589
(b) gene transcripts												
nifH	19.14	**<0.0001 ↓**	25.21	**<0.0001**	1.88	0.153	8.65	**0.006**	1.22	0.318	1.85	0.158

chiA	14.50	**0.001** ↑	12.57	**0.001**	4.78	**0.007**	2.78	0.105	8.91	**<0.0001**	4.28	0.105
AOA	36.77	**<0.0001** ↑	8.33	**0.008**	4.73	**0.009**	7.70	**0.010**	10.64	**<0.0001**	2.97	**0.050**
nirK	1.25	0.273	1.57	0.219	39.857	**<0.0001**	3.30	0.079	1.02	0.397	1.14	0.346
nirS	10.32	**0.003** ↑	0.059	0.810	27.93	**<0.0001**	0.44	0.510	0.52	0.673	0.83	0.489
qnorB	22.19	**<0.0001** ↑	12.93	**0.001**	40.69	**<0.0001**	0.457	0.504	4.47	**0.010**	7.15	**0.001**
nosZ	0.20	0.656	5.72	**0.023**	11.21	**<0.0001**	0.80	0.378	0.07	0.978	0.96	0.426

Figure captions

Fig. 1. The dynamics of (a) daily precipitation and mean air temperature, (b) daily mean soil temperature and the temperature difference for the conventional paddy and ground cover rice production system (GCRPS) during the rice growth season of 2014. The arrows in (b) indicate the fertilization immediately followed by transplanting at April 29 (F&T), soil sampling times on May 25 (middle tillering, MiT), June 22 (maximum tillering, MaT), July 25 (panicle initiation, PI), and September 5 (maturity, MA).

Fig. 2. GCRPS-induced changes in soil organic C and total N concentrations and soil $\delta^{15}N$ isotopic signature at the sites with 10 years (left column) and 3 years (right column) old GCRPS fields. Error bars represent standard errors of the mean. Indices indicate statistical significant differences ($P<0.05$).

Fig. 3. Gene transcripts of (a) *nifH*, (b) *chiA*, (c) AOA, (d) AOB, (e) *nirK*, (f) *nirS*, (g) *qnorB*, (h) *nosZ* (mRNA level, n = 3) are shown for 3 years old GCRPS and Paddy control treatments during the growing season. P0, paddy soil without N fertilizer; G0, GCRPS without N fertilizer; P150, paddy soil with 150 kg N $ha^{-1}a^{-1}$; G150, GCRPS with 150 kg N $ha^{-1}a^{-1}$. Different letters above bars indicate signiciant differences between the GCRPS and Paddy treatments at a given sampling time ($P < 0.05$, LSD test).

Fig. 4. Scheme of GCRPS effects on the soil microbial N cycle as derived from the percentage of change of gene transcript data at the 3 years old GCRPS site. Decreased N turnover processes under GCRPS compared to Paddy are indicated in grey, while increased N turnover processes are shown in black bold letters and arrows.

Fig. 5. Relationships between (a) *nirS* mRNA transcript abundance and N_2O flux and (b) between *qnorB* mRNA transcript abundance and N_2O flux. Regression analyses were conducted separately for Paddy and GCRPS treatments. P0, paddy soil without N fertilizer; G0, GCRPS without N fertilizer; P150, paddy soil with fertilizer application of 150 kg N $ha^{-1}a^{-1}$; G150, GCRPS with fertilizer application of 150 kg N $ha^{-1}a^{-1}$.

Fig. 6. Between-group analysis on Correspondence Analysis (CA) of the T-RFLP data set for nitrogen fixation (*nifH*), denitrification (*nosZ*) and nitrification (*amoA* AOA) gene fragments from 3year-GCRPS (left column) and 10year-GCRPS (right column). Symbols illustrate the three field replicates for each treatment. P0, paddy soil without N fertilizer; G0, GCRPS without N fertilizer; P150, paddy soil with 150 kg N $ha^{-1}a^{-1}$; G150, GCRPS with 150 kg N ha^{-1} a^{-1}.

Figure 1

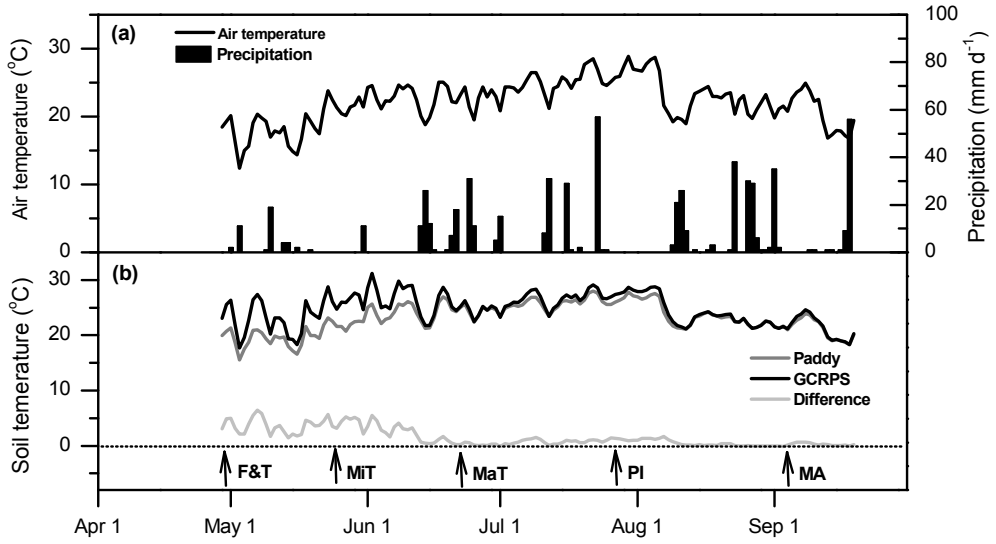

(a)
Air temperature
Precipitation
Air temperature (°C)
Precipitation (mm d-1)
(b)
Soil temerature (°C)
Paddy
GCRPS
Difference
F&T
MiT
MaT
PI
MA
Apr 1
May 1
Jun 1
Jul 1
Aug 1
Sep 1

Figure 2

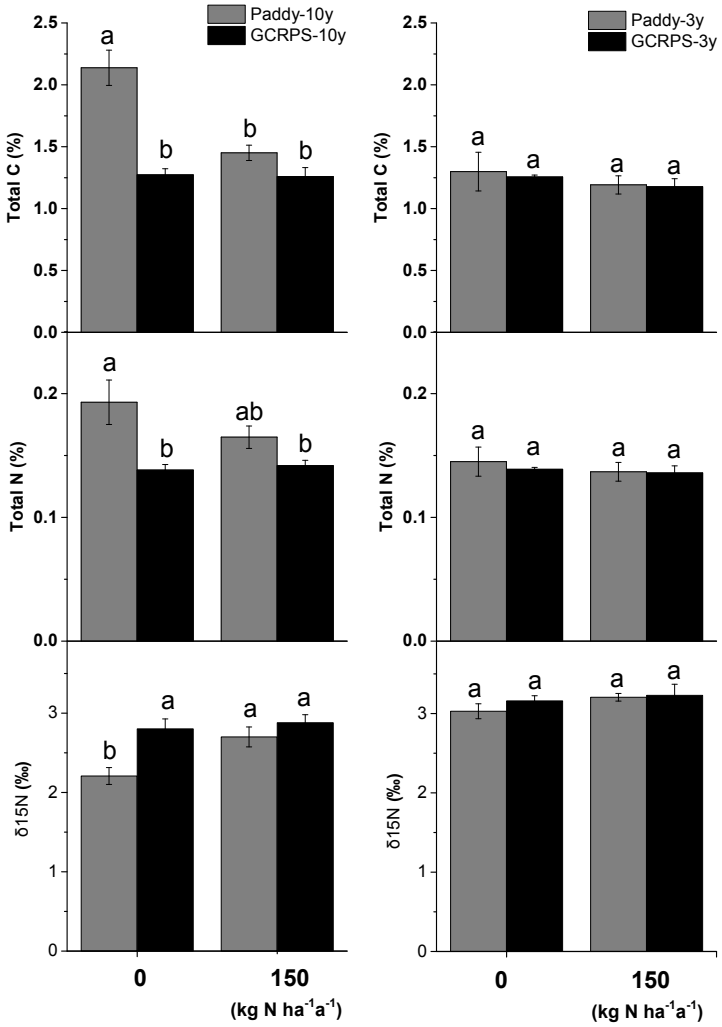

Paddy-10y
GCRPS-10y
Paddy-3y
GCRPS-3y
Total C (%)
Total N (%)
δ15N (‰)
0
150
(kg N ha-1a-1)

Figure 3

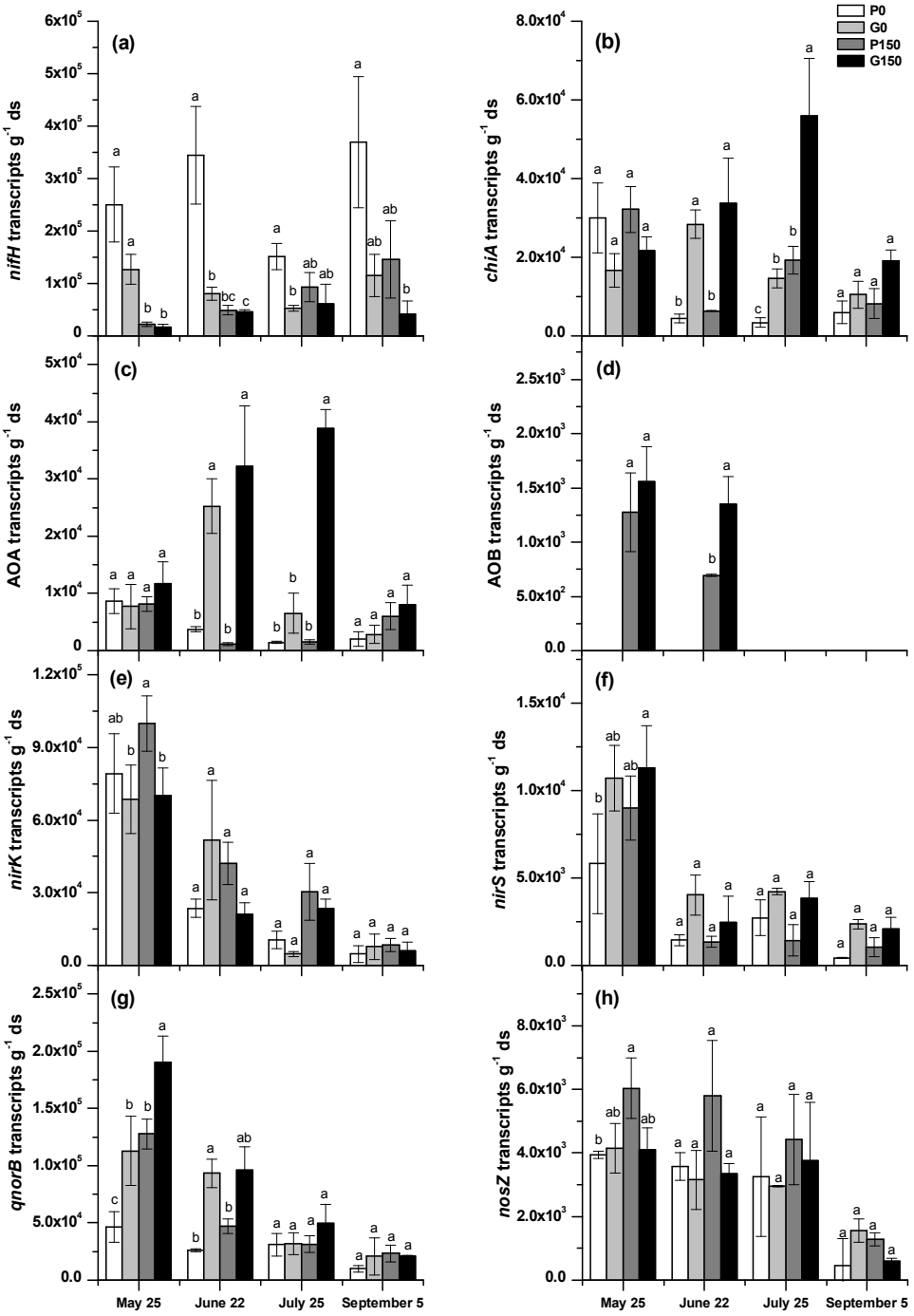

P0
G0
P150
G150
(a)
nifH transcripts g-1 ds
(b)
chiA transcripts g-1 ds
(c)
AOA transcripts g-1 ds
(d)
AOB transcripts g-1 ds
(e)
nirK transcripts g-1 ds
(f)
nirS transcripts g-1 ds
(g)
qnorB transcripts g-1 ds
(h)
nosZ transcripts g-1 ds
May 25
June 22
July 25
September 5

Figure 4

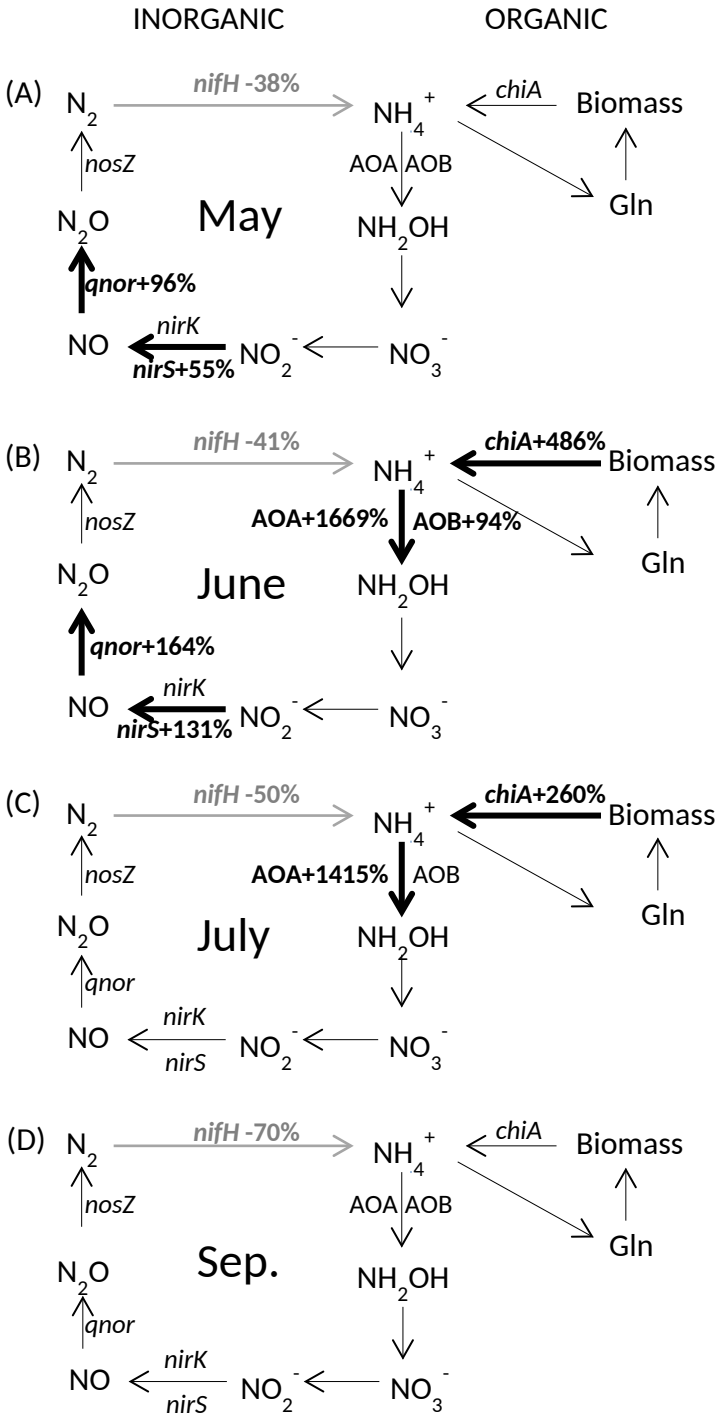

INORGANIC
ORGANIC
(A)
nifH -38%
chiA
Biomass
N2
NH4+
nosZ
AOA AOB
Gln
May
N2O
NH2OH
qnor+96%
nirK
NO
nirS+55%
NO2-
NO3-
(B)
nifH -41%
chiA+486%
Biomass
N2
NH4+
nosZ
AOA+1669%
AOB+94%
Gln
June
N2O
NH2OH
qnor+164%
nirK
NO
nirS+131%
NO2-
NO3-
(C)
nifH -50%
chiA+260%
Biomass
N2
NH4+
nosZ
AOA+1415%
AOB
Gln
July
N2O
NH2OH
qnor
nirK
NO
nirS
NO2-
NO3-
(D)
nifH -70%
chiA
Biomass
N2
NH4+
nosZ
AOA AOB
Gln
Sep.
N2O
NH2OH
qnor
nirK
NO
nirS
NO2-
NO3-

Figure 5

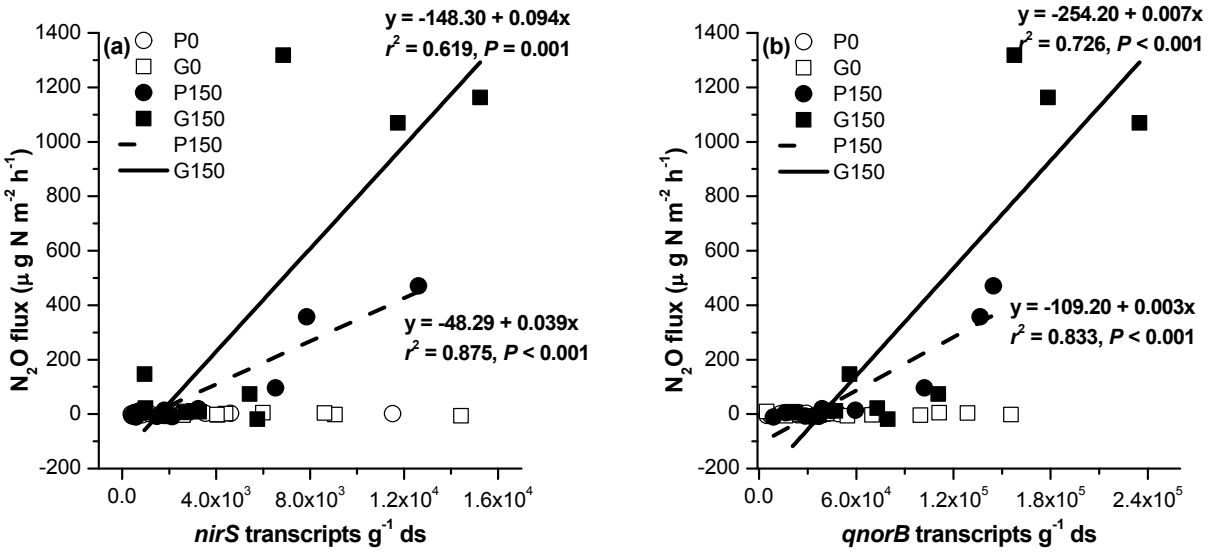
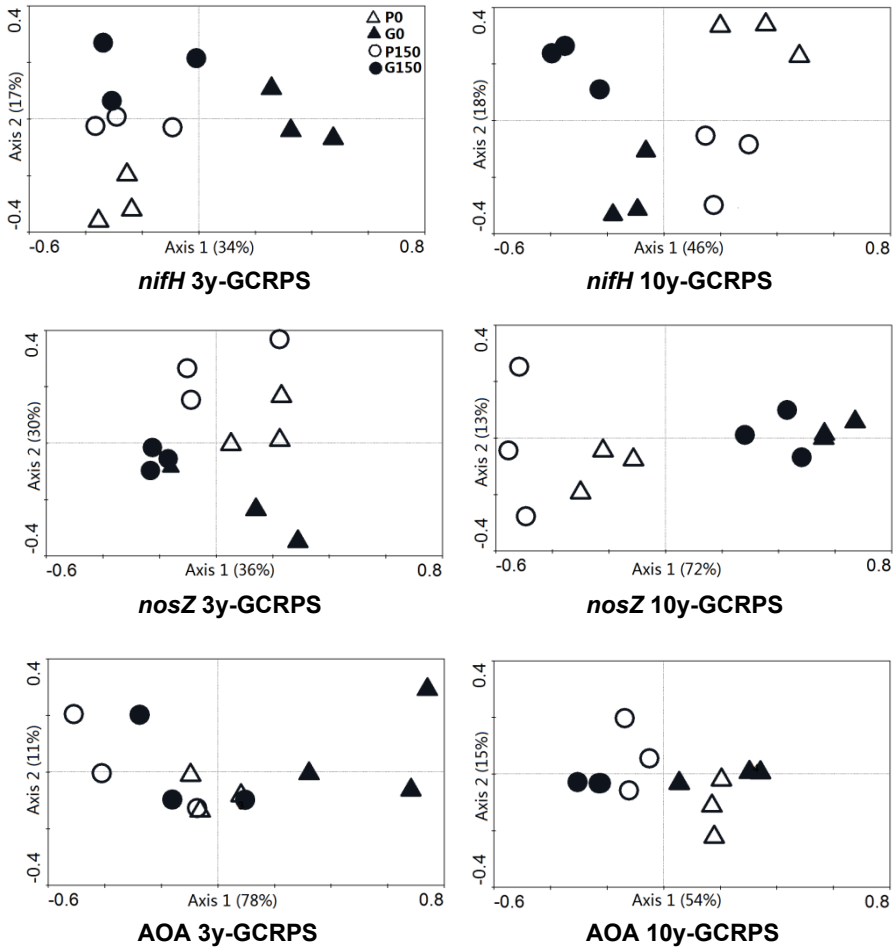

(a)
P0
G0
P150
G150
P150
G150
y = -148.30 + 0.094x
r² = 0.619, P = 0.001
y = -48.29 + 0.039x
r² = 0.875, P < 0.001
N2O flux (μ g N m-2 h-1)
nirS transcripts g-1 ds
(b)
y = -254.20 + 0.007x
r² = 0.726, P < 0.001
y = -109.20 + 0.003x
r² = 0.833, P < 0.001
qnorB transcripts g-1 ds

Figure 6


P0
G0
P150
G150
Axis 2 (17%)
Axis 1 (34%)
nifH 3y-GCRPS
Axis 2 (18%)
Axis 1 (46%)
nifH 10y-GCRPS
Axis 2 (30%)
Axis 1 (36%)
nosZ 3y-GCRPS
Axis 2 (13%)
Axis 1 (72%)
nosZ 10y-GCRPS
Axis 2 (11%)
Axis 1 (78%)
AOA 3y-GCRPS
Axis 2 (15%)
Axis 1 (54%)
AOA 10y-GCRPS
0.4
-0.4
-0.6
0.8

Highlights of the article "Enhanced nitrogen cycling and N_2O loss in water-saving ground cover rice production systems (GCRPS)"

(1) GCRPS cultivation changed the community composition of N fixers and denitrifiers.

(2) GCRPS decreased *nifH* and increased *qnorB* and archaeal *amoA* transcripts levels.

(3) Increased *qnorB* expression was closely correlated with increased soil N_2O emissions.

(4) Despite enhanced N turnover, no net N loss occurred in fertilized GCRPS.

Supporting information

Table S1. Thermal profiles and primers used for real-time PCR quantification of functional genes[a]

Target gene	Source of standard	Termal profile	No. of cycles	Primers (reference)	DMSO[c]
chiA	*Streptomyces griseus*	94°C, 30 s; 60°C, 30 s; 72°C, 30 s	40	chiF2, chiR (Xiao *et al.*, 2005b)	
nifH	*Azospirillum irakense*	95°C, 30 s; 55°C, 30 s; 72°C, 30 s	40	nifHF (Rosch *et al.*, 2002), nifHR (Rosch *et al.*, 2002)	
AOA	*Fosmid clone* 54d9	94°C, 30 s; 55°C, 30 s; 72°C, 30 s	40	amo19F (Leininger *et al.*, 2006), CrenamoA616r48x (Schauss *et al.*, 2009)	
AOB	*Nitrosomonas europaea*	94°C, 30 s; 58°C, 30 s; 72°C, 30 s	40	amoA1F, amoA2R (Rotthauwe *et al.*, 1997)	
nirK	*Azospirillum irakense*	95°C, 15 s; 63-58°C, 30 s; 72°C, 30 s	5	nirK876 (Henry *et al.*, 2004), nirK5R (Braker *et al.*, 1998)	0.5
		95°C, 15 s; 58°C, 30 s; 72°C, 30 s	40		
nirS	*Pseudomonas stutzeri*	95°C, 30 s; 57°C, 30 s; 72°C, 30 s	40	cd3aF (Michotey *et al.*, 2000), R3cd (Throback *et al.*, 2004)	0.5
qnorB	*Ralstonia eutropha*	95°C, 15 s; 60-55°C, 30 s; 72°C, 30 s	5	qnorB2f (Braker and Tiedje, 2003)	
		95°C, 15 s; 55°C, 30 s; 72°C, 30 s	40	qnorB5r (Braker and Tiedje, 2003)	
nosZ	*Pseudomonas stutzeri*	95°C, 15 s; 65-60°C, 15 s; 72°C, 30 s	5	nosZ2F (Henry *et al.*, 2006), nosZ2R (Henry *et al.*, 2006)	
		95°C, 15 s; 60°C, 15 s; 72°C, 30 s	40		

[a] PCR mixtures consisted of Power SYBR green master mix (12.5μ L), BSA (3%, 0.5 μL), and template (2 μL, 2 to 5 ng μL^{-1}), as well as primer (10 μM, 0.5μL for each Forward and Reverse) and DMSO, as referenced in the table

[b] Touchdown: -1°C $cycle^{-1}$

[c] DMSO, dimethyl sulfoxide

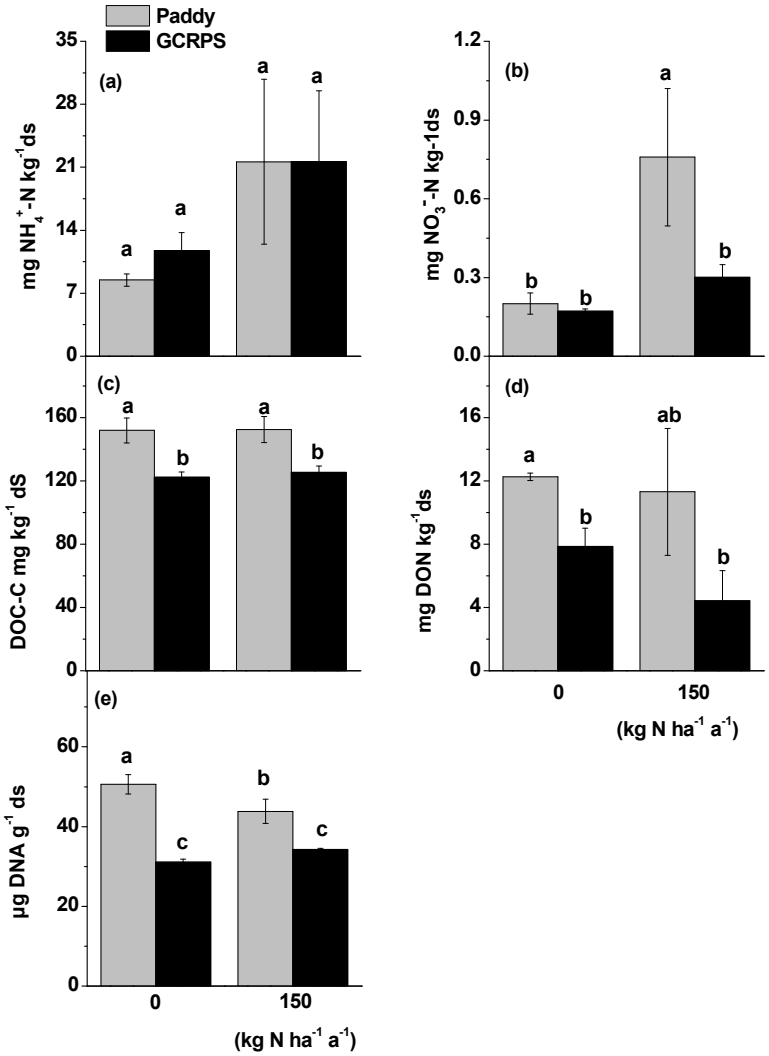


Fig. S1. Changes in soil ammonium (a), nitrate (b), DOC (c), DON (d) and microbial biomass (e, measured as microbial DNA concentration) for paired Paddy and 10-year old GCRPS fields. Sampling was conducted in May when soil was flooded. Error bars represent standard errors of triplicate samples. Different letters above the bars indicate a significant difference ($P<0.05$).

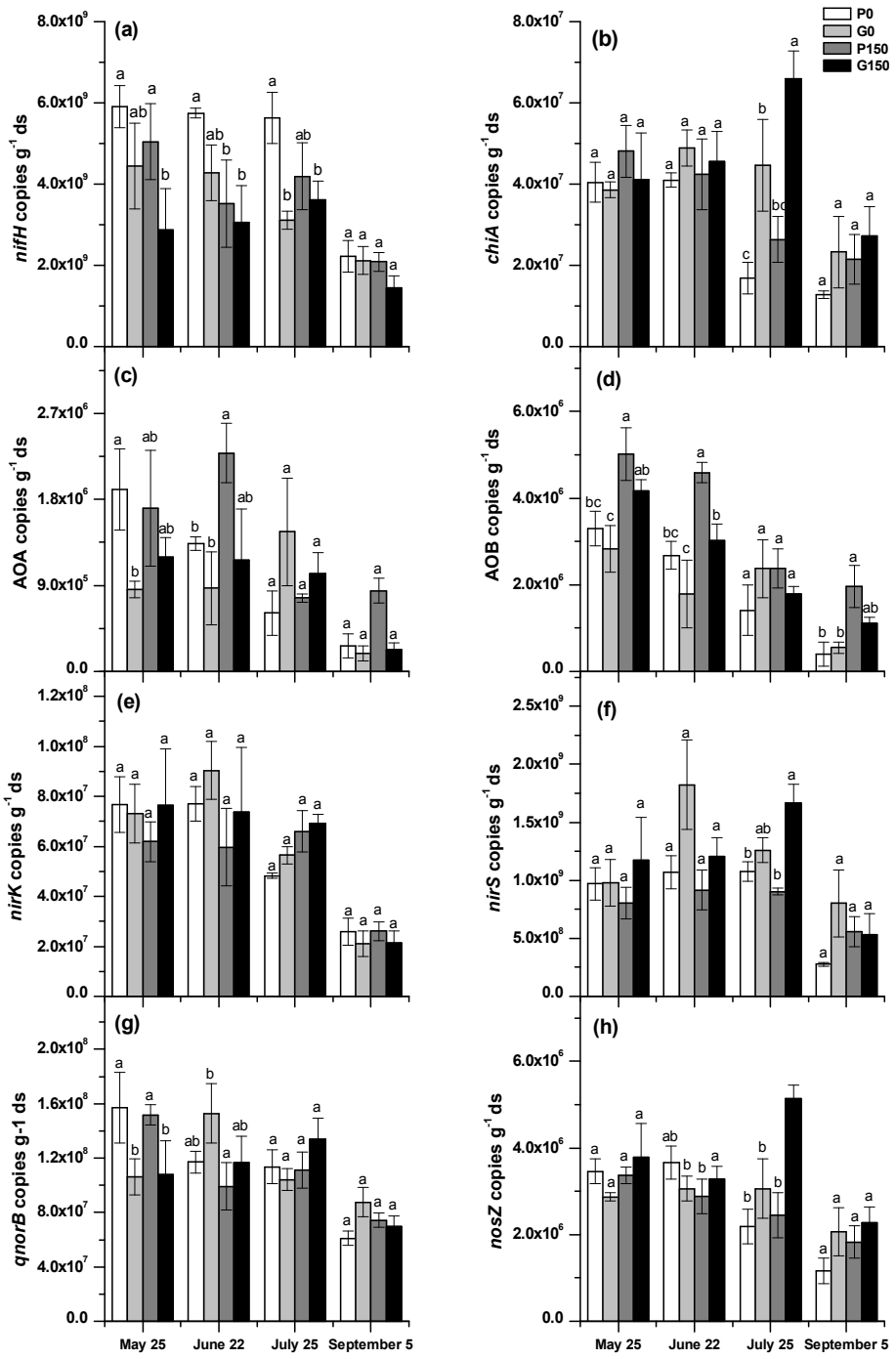


Fig. S2. Gene abundance of (a) *nifH*, (b) *chiA*, (c) AOA, (d) AOB, (e) *nirK*, (f) *nirS*, (g) *qnorB*, (h) *nosZ* (DNA level, n = 3) are shown for 3 year old GCRPS fields and adjacent Paddy controls during the growing seasons. P0, paddy soil without N fertilizer; G0, GCRPS without N fertilizer; P150, paddy soil with 150 kg N $ha^{-1}a^{-1}$; G150, GCRPS with 150 kg N $ha^{-1}a^{-1}$. Different letters above bars indicate signiciant differences among the GCRPS and Paddy treatments for each sampling time separately ($P < 0.05$, LSD test).

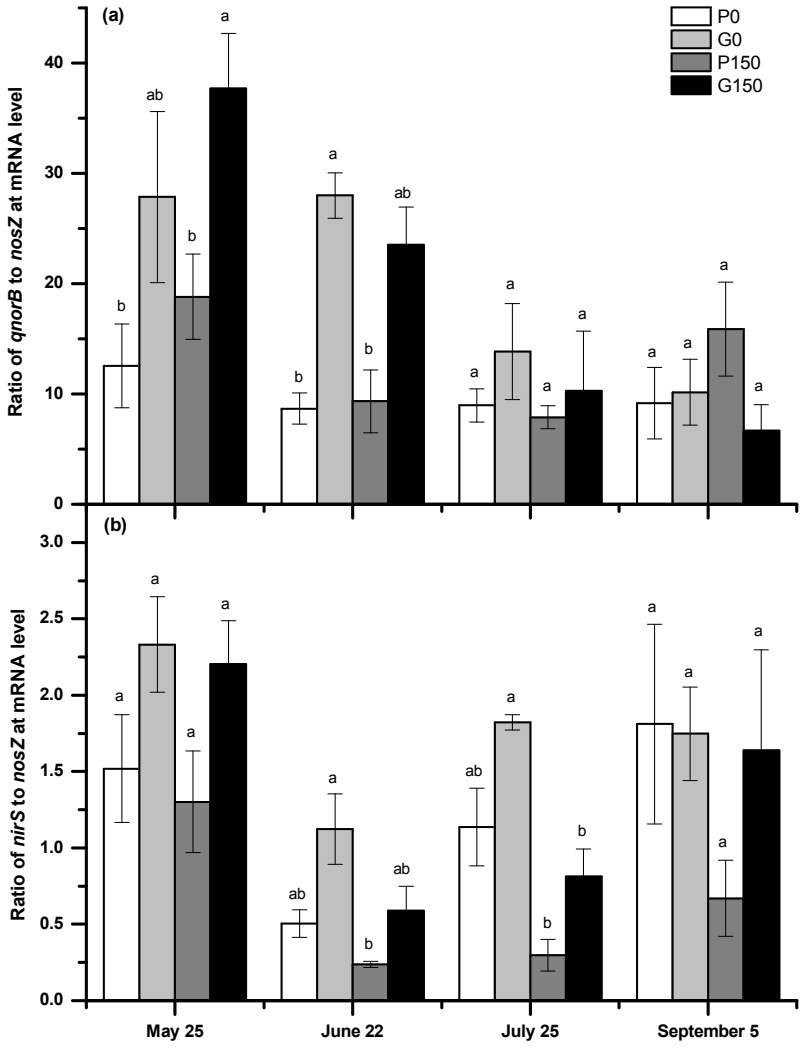


Fig S3. Effects of GCRPS on the (a) ratio of *qnorB* to *nosZ* transcripts at mRNA level, (b) ratio of *nirS* to *nosZ* transcripts at mRNA level over the rice growing season for Paddy and 3-years old GCRPS treatments. Error bars represent standard errors of triplicate samples. Different letters above the bars indicate a significant difference ($P<0.05$).

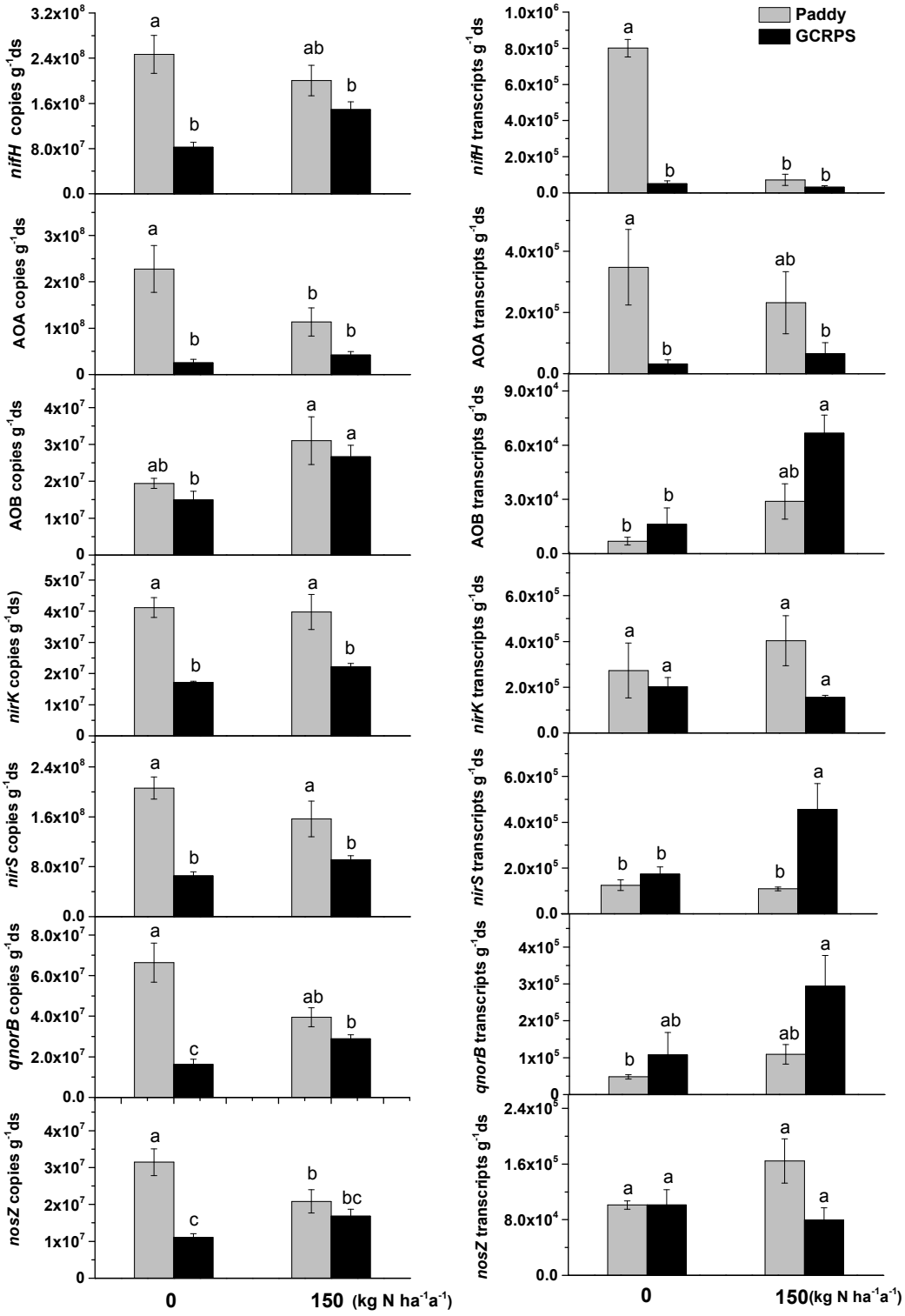


Fig. S4. Changes in abundance (left column) and transcripts (right column) of functional genes involved in the nitrogen fixation (*nifH*), denitrification (*nirK* and *nosZ*) and nitrification (AOA and AOB) for Paddy and 10-year GCRPS (sampling at May and soil was flooded). Error bars represent standard errors of triplicate samples. Different letters above the bars indicate a significant difference ($P<0.05$). ds: dry soil.