Differential Responses of Nitrate Reducer Community Size, Structure, and Activity to Tillage Systems††

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The main objective of this study was to determine how the size, structure, and activity of the nitrate reducing community were affected by adoption of a conservation tillage system as an alternative to conventional tillage. The experimental field, established in Madagascar in 1991, consists of plots subjected to conventional tillage or direct-seeding mulch-based cropping systems (DM), both amended with three different fertilization regimes. Comparisons of size, structure, and activity of the nitrate reducer community in samples collected from the top layer in 2005 and 2006 revealed that all characteristics of this functional community were affected by the tillage system, with increased nitrate reduction activity and numbers of nitrate reducers under DM. Nitrate reduction activity was also stimulated by combined organic and mineral fertilization but not by organic fertilization alone. In contrast, both negative and positive effects of combined organic and mineral fertilization on the size of the nitrate reducer community were observed. The size of the nitrate reducer community was a significant predictor of the nitrate reduction rates except in one treatment, which highlighted the inherent complexities in understanding the relationships between size, diversity, and structure of functional microbial communities along environmental gradients.

The transition from intensive tillage to various forms of conservation tillage began more than 50 years ago with the development of herbicides which have replaced mechanical cultivation. Since then, the principles of no-till cropping have been extensively adopted by farmers worldwide. This cropping system, also known as direct seeding, mimics natural systems by leaving the soil mostly undisturbed and permanently covered with crop residues or living plants. The benefits of reducing tillage in sustainable agriculture are now well recognized for various environmental and economic reasons (14). Leaving all residues of the previous crop on the soil surface protects against evaporative water loss, wind erosion, and surface water runoff. Concomitant with reduced erosion, no-till cropping can also result in enhanced soil carbon storage in the topsoil layer, with estimated carbon sequestration rates of 30 to 60 g C m⁻² year⁻¹ (27, 50). In turn, these changes in soil organic matter and soil structure under a no-till cropping system can affect microbial communities (20). Thus, the microbial biomass is most often higher in no-till systems than in conventional tillage systems (11, 26). Analysis of the structure or activity of soil microbial communities has also revealed significant differences between conventional tillage and minimal tillage or no-tillage systems (25, 29). However, although the effect of tillage practices on the total soil microbial community in relation to soil organic matter management has frequently been investigated, knowledge of the changes in N-cycling microbial communities induced by no-till management is limited and is mainly focused on N process rates (3, 11, 32).

The aim of this work was to determine how conversion from conventional tillage to no-till affects microorganisms involved in the N cycle. For this purpose, we used the nitrate reducing community as a model functional guild (40). Prokaryote nitrate reducers constitute a wide taxonomic group with a shared ability to produce energy from the dissimilatory reduction of nitrate to nitrite, the first step of denitrification and of the dissipatory processes of reduction of nitrate to ammonium (39). Nitrate reduction by denitrification is of great importance, since the resulting nitrite is then reduced to N₂O or N₂ gases, which can lead to considerable nitrogen losses in agriculture and emissions of the N₂O greenhouse gas (4, 13). We hypothesized that higher C and N contents in the no-till system will result in increased nitrate reduction rates and nitrate reducer abundance combined with shifts in the community composition. Relationships between the size, activity, and structure of the nitrate reducing community in the studied cropping systems were also investigated. The structure and size of the nitrate reducing community were assessed by fingerprinting and real-time PCR using the napA and narG genes, encoding the membrane-bound and periplasmic nitrate reductases, respectively, as molecular markers (40, 41). The potential activity of the nitrate reducing community was determined by colorimetric measurement of the nitrite produced during nitrate reduction.

MATERIALS AND METHODS
Experimental field and sampling. This study was conducted in the experimental station of Tany sy Fampandrosoana, district of Bemasoandro (19°46′45″ south, 47°06′25″ east), Antsirabe region, in the highlands of Madagascar. This area has a cold tropical high-altitude climate with 10 to 20 days of frost annually and a mean temperature of 16.9°C. The site is at an altitude of 1,600 m above sea level.
with an annual average rainfall of 1,450 mm. The soil is described as an andic
dystrustep, and the physical properties were as follows: clay, 61.90%; bulk
density, 0.76 g cm\(^{-3}\); pH (H\(_2\)O), 5.72; CEC; 17.32 cmol kg\(^{-1}\) soil). The experi-
ment was set up in 1991, with annual soybean (Glycine max L.)-rice (Oriza sativa)
rotations and conventional tillage or direct-seeding mulch-based cropping (DM)
dystems. DM is based on the absence of soil tillage, maintenance of a mulch of
crop residues at all times, direct seeding into crop residues, and use of suitable
crop successes. Both DM and conventional tillage systems were either not
fertilized (F0), fertilized with farmyard manure at 5 tons ha\(^{-1}\) year\(^{-1}\) (F1), or
fertilized with farmyard manure at 5 tons ha\(^{-1}\) year\(^{-1}\) combined with mineral
fertilizer (F2), which resulted in six management regimes. Mineral fertilizers
were used at the recommended rates of 30 N, 30 P, and 40 K kg ha\(^{-1}\) year\(^{-1}\)
for soybeans and 70 N, 30 P, and 40 K kg ha\(^{-1}\) year\(^{-1}\) for rice. The direct-seeding
mulch-based cropping system consisted of sowing in a mulch of crop residue with
no tillage. The experimental site consists of three randomized blocks, each block
containing six plots (3 by 5 m), corresponding to the six management regimes.
Soil was collected from the 0- to 5-cm horizon on 28 January 2005 and on 12
February 2006, air-dried, and sieved to <2 mm. In each of the 18 plots, we
collected five soil cores at three different locations, which were pooled to obtain
three composite bulk soil samples per plot and nine samples per treatment. In
total, we therefore collected 270 soil cores in the experimental field on each
sampling date, and 54 composite soil samples were used for the subsequent
analysis.

Soil organic carbon, total nitrogen, nitrate, and ammonium. Total organic
carbon (C) and total nitrogen (N) were determined by dry combustion in a CHN
autoanalyzer (EA1112 Thermofinnigan Series) using dried (105°C, 48 h) and ground
soil samples (\(<200 \mu m\)). Results were expressed in mg g\(^{-1}\) soil. Nitrate and
ammonium concentrations were determined using a colorimetric analysis using an
automated analyser (Technicon II; Bran-Luebbe, Plaisir, France) after extraction from
10 g of soil with 100 ml of 1 M KCl according to the method of Bremner (5).

Measurement of potential nitrate reductase activity. Potential nitrate reducta-
tase activity was determined by soil anaerobic incubation, following the slightly
modified protocol of Kandeler (31). The method involved determination of the
NO\(_3\)\(^-\)N production after addition of nitrate as a substrate and 2,4-dinitrophenol
(DNP) as a nitrite inhibitor, with the nitrate reductase activity measured as ni-
tron transfer but allowed nitrate reduction to continue. Substrate as well as DNP
inhibitor concentrations were optimized in preexperiments. In detail, for each of
the three replicates from each plot, soil (0.2 g) was weighed and divided into four
replicates and then was incubated in a total volume of 1 ml containing 1 m M
potassium nitrate. The optimum inhibitory concentration of DNP (0.9 mM) was
then added to inhibit nitrite reduction. After 24 h of incubation at 20°C, the soil
mixture was extracted with 4 M KCl and centrifuged for 1 min at 13,000 x g. The
accumulated nitrite in the supernatant was determined by colorimetric reaction
at 520 nm using a reagent composed of phosphoric acid (1% [vol/vol]), N\(_2\)-
naphthil ethylenediamine dichloride (2 g liter\(^{-1}\)), and sulfanilamide (40 g
liter\(^{-1}\)).

Soil DNA extraction. DNA was extracted from the three composite soil
samples from each plot according to the Ultra Clean Soil DNA kit protocol (Protocol
12800-100; Oxyme, Mo Bio, France). The 54 DNA extracts for each sampling
date were then quantified by spectrophotometry at 260 nm using a biophotometer
(Eppendorf, Hamburg, Germany).

Genetic structure analysis of the nitrate reducer community. The structure of
the nitrate reducer community was assessed using the narG and napA genes,
encoding the membrane-bound and periplasmic nitrate reductase, respectively,
as molecular markers. The three DNA extracts from triplicate plots were pooled,
resulting for each sampling date in a total of 18 DNA samples, which were used
as a PCR template. The narG and napA genes were amplified using the
narG1960F (5'-TTTCTC-3') and napA1960R (5'-TTYTCR-3') primers (45) and the
narG675 (5'-AAYATGGCVGAR-3') and napA675 (5'-GRTRAAARCCCATSGTCCA-
3') primers using previously described PCR conditions (28, 45). PCR products
were carried out in a 50-μl mixture containing 1.5 mM Mg\(_2\)\(^2\)O, 200 mM of each
deoxyribonucleoside triphosphate, 5 mM of each primer, and 1.25 U of Taq
polymerase (Qbiogene, France). At least three independent PCRs were
performed, and the PCR products were then pooled for each replicate to minimize
the effect of PCR bias. PCR products were purified using the MinElute gel
extraction kit (Qiagen, France). Purified narG and napA PCR products were
digested with the AluI restriction enzyme at 37°C for 4 h as described previously
(28, 45). For the narG and napA restriction fragment length polymorphism (RFLP)
fingerprints were obtained after separation by electrophoresis on a native 6%
carcamide-bisacrylamide (29:1) gel for 11 h at 5 mA. DNA staining was done
using SYBR green, and the resulting fluorescence was scanned with a PhosphoImager
(Storm 860; Molecular Dynamics, Sunnyvale, CA).

Quantitative PCR assays for narG and napA gene copy enumeration. The
real-time PCR assay was carried out in a 20 μl reaction volume containing SYBR
green PCR master mix (Absolute QPCR SYBR green ROX; ABgene, France),
1 μM of each primer, 100 ng of T4 gene 32 (OBIgene, France), and 12.5 ng of
DNA. Fragments of the narG and napA genes were amplified using the previ-
ously described primers and thermal cycling conditions (8). Thermal cycling,
fluorescent data collection, and data analysis were carried out with the ABI
Prism 7900HT sequence detection system according to the manufacturer's in-
structions. Two independent quantitative PCR assays were performed for each
gene and for the three DNA extracts from each plot. Two to three no-template
controls were run for each quantitative PCR assay. Serial dilution of linearized
plasmids containing the narG and napA genes from Pseudomonas aeruginosa
PA01 were used to generate standard curves (8). The copy number of standard
DNA was calculated using the following formula: X = g plasmid DNA(plasmid
length (bp)) × 660 × 10\(^{-3}\) = X molecule/μl, where X is the concen-
tration of linearized plasmid.

All narG and napA assays were run with DNA from P. aeruginosa PA01
containing one copy of each of these two genes as an external standard. The
specificity of the assay was verified by melting-curve analysis and gel elec-
rophoresis. Tests for the potential presence of PCR inhibitors in DNA extracted
from soil were performed by diluting soil DNA extracts and spiking soil DNA
extracts with a known amount of standard DNA prior to quantitative PCR. In all
cases, no inhibition was detected.

Statistical analysis. When required, data were normalized by log or Box-Cox
transformation. A Student t test was used to analyze the effects of the tillage
system and fertilization regime on the size and activity of the nitrate reducer
community. The narG and napA fingerprint gels were analyzed using the Total-
 fingerprints software (Nonlinear Dynamics Ltd.). This software converted the
fluorescence of DNA fragments of different sizes into electropherograms. Data
obtained from the software were converted into a table summarizing the band
presence and intensity. Hierarchical cluster analysis was based on Bray-Curtis
dissimilarity matrices of the fingerprint data using the XLSTAT software pro-
gram (Addinsoft SARL, France). Pearson correlation analyses were performed
to test the relationships between gene copy numbers and nitrate reduction rates,
on each of the 18 DNA samples in the experimental field on each sampling date.

RESULTS

Effect of tillage system and fertilization regime on soil carbon and
nitrogen contents. The tillage system significantly
influenced soil C and N, since contents were higher under DM
than under conventional tillage regardless of the fertilization
regime or sampling year (Table 1). Similarly, higher nitrate and
ammonium concentrations were observed for DM than for
conventional tillage in the F0 and F1 plots (Table 1). The
fertilization regime also had a significant impact on soil C
and N contents, which were modulated by both the sampling
year and the tillage system (P < 0.03). In general, the C and N
content followed the gradient of fertilization, with higher
contents in plots amended with farmyard manure combined with
mineral fertilizers (F2) than in plots amended with farmyard
manure alone (F1) or without fertilizers (F0). However, in
2005, the N content was not affected by the fertilization regime
in the DM system.

Effect of tillage system and fertilization regime on nitrate reduction
activity. The potential nitrate reduction activity,
was measured in each of the 18 plots using a biophotometer
(Eppendorf, Hamburg, Germany).

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was between 2.5 and 4.4 times higher in DM than in the conventional tillage system for both years and for all fertilization regimes, except the no-fertilization treatment in 2006 (Fig. 1). A significant effect of the fertilization regime on potential nitrate reduction activity was also observed, with higher rates in the farmyard manure combined with mineral fertilizer treatment (F2) than with the other fertilization regimes (Fig. 1). This stimulating effect of combined organic and mineral fertilization was stronger in 2006 than in 2005, the rates being 2.5 times and 3.4 times higher in 2006 under the DM and conventional tillage systems, respectively (Fig. 1).

Structure of nitrate reducer community in relation to the agricultural management regime. In order to reduce the impact of gel-to-gel variations on gel analysis, all samples from the same sampling date were run on the same gel. Twelve to 27 bands were used for analysis of the `narG`or `napA` RFLP gels (data not shown). Hierarchical cluster analysis of both `narG` and `napA` RFLP fingerprints showed that most samples from DM separated from those from the conventional tillage system (Fig. 2) but with weak differences between the two types of agricultural practices (with total variance explained by principal component analysis ranging from 20.5 to 47.6%; data not shown). The fertilization regime had the strongest effect on the `napA` community structure, as shown by the branching of the F2 samples from DM (Fig. 2C and D). However, in all the other cases, the effect of the fertilization was very weak or not significant.

Size of nitrate reducer community in relation to the agricultural management regime. The size of the nitrate reducer community, estimated by real-time PCR quantification of the `narG` and `napA` gene copies, is presented in Fig. 3. The average gene copy number for `narG` was slightly higher than that for `napA`, with densities between $0.76 \times 10^5$ and $11.7 \times 10^5$ copies per ng of extracted DNA, which corresponds to $0.7 \times 10^8$ and $9.0 \times 10^8$ copies per gram of dry soil. The copy numbers of `narG` and `napA` per gram of soil were significantly higher in DM than in the conventional tillage system for all fertilization regimes and both years except for F2 in 2005, where no significant differences were observed. The impact of the fertilization regime on gene copy number was dependent on the tillage system and sampling year and also on the gene targeted (Fig. 3). Thus, the fertilization regime had no effect on `narG` density except in 2005 under DM, when the `narG` gene copy numbers per gram of soil were significantly lower with F2 than with F0 or F1. The same decrease of `napA` density was observed with

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**TABLE 1.** Carbon and nitrogen contents in conventional-tillage and DM systems subjected to three different fertilization regimes

| Tillage system                  | Fertilization regime | Carbon (%) | Nitrogen (%) | Nitrate (mg N g⁻¹ soil) | Ammonium (mg N g⁻¹ soil) |
|---------------------------------|---------------------|------------|--------------|-------------------------|--------------------------|
|                                 |                     | 2005       | 2006         | 2005                    | 2006                     |
| Conventional                    | F0                  | 2.3 (0.3) a| 2.5 (0.3) a | 0.16 (0.02) a           | 0.18 (0.03) a            | 6.8 (1.0) a ND           | 6.2 (2.0) a ND           |
|                                 | F1                  | 2.4 (0.2) ab| 2.7 (0.2) a | 0.17 (0.02) ab          | 0.20 (0.02) a            | 4.2 (1.0) b ND           | 8.5 (2.4) a ND           |
|                                 | F2                  | 2.6 (0.3) b| 2.9 (0.2) a | 0.19 (0.03) b           | 0.23 (0.02) b            | 4.0 (1.0) b ND           | 15.2 (2.7) b ND          |
| Direct-seeding mulch based      | F0                  | 3.5 (0.3) c| 3.5 (0.3) c | 0.25 (0.03) c           | 0.28 (0.03) c            | 9.9 (1.9) c ND           | 14.2 (2.5) b ND          |
|                                 | F1                  | 3.7 (0.4) cd| 3.9 (0.3) d | 0.25 (0.03) c           | 0.31 (0.03) c            | 6.3 (2.5) a ND           | 13.1 (2.6) b ND          |
|                                 | F2                  | 3.9 (0.5) d| 4.5 (0.5) c | 0.25 (0.05) c           | 0.37 (0.05) d            | 5.2 (3.0) ab ND          | 15.3 (3.4) b ND          |

*Within each year, means followed by the same letter are not significantly different at P values of <0.05. ND, not determined.*
F2 under DM in 2005. On the contrary, a significant stimulatory effect of the F2 fertilization regime on napA density was observed for both years in the conventional tillage system and in the DM in 2006 (Fig. 3).

Relationships between activity, size, and composition of nitrate reducer community in different cropping systems. Weak or no correlation was observed between narG (r² = 0.046), napA (r² = 0.148; P < 0.01), or narG plus napA (r² = 0.097; P < 0.05) gene copy numbers and potential nitrate reduction activity (Fig. 4). However, when the data were analyzed without the F2 plots, significant stronger correlations between activity and the size of the nitrate reducer community were found for narG (r² = 0.539; P < 0.001), napA (r² = 0.556; P < 0.001), and narG plus napA (r² = 0.555; P < 0.001) gene copy numbers (Fig. 4). Mantel tests revealed that activity of the nitrate reducer community was also significantly related to the structure of this community, which was due mainly to the larger differences observed in the F2 treatment (Fig. 5). Thus, differences between the napA and narG community composition were correlated with differences in potential nitrate reduction activity, with the highest correlations of R values of 0.729 and 0.794 between napA community composition and potential nitrate reduction activity being observed in 2005 and 2006, respectively (Fig. 5). Differences in nitrate reducer community composition were also related to differences in nitrate reducer community size (P < 0.05), except for narG in 2005 (R = 0.063). Thus, correlations of R values of 0.212 and 0.34 were observed for differences between napA community composition and napA gene copy numbers in 2005 and 2006, respectively, and an R value of 0.412 for differences between narG community composition and narG gene copy numbers in 2006 (data not shown).

DISCUSSION

Leaving soils mostly undisturbed and covered with crop residues is known to improve the soil nutrient status in the top layer (14). Accordingly, a significant increase in C and N contents was observed under DM compared to results with the conventional cropping system in this study (Table 1). This increase of C and N under DM was concomitant with a significant stimulation (P < 0.001) of the potential nitrate reduction activity in DM both in 2005 and 2006, with rates in the same range as those previously reported for a grassland soil (18). A higher potential denitrification activity under DM than under the conventional cropping system had also been observed in the same experimental field (E. Baudoin, L. Philippot, D. Cheiney, L. Chapuis-Lardy, N. Fromin, D. Bru, B. Rabary, and A. Brauman, submitted for publication). That nitrate reduction and denitrification are correlated with C content in soil is well known and has been demonstrated in several studies (6). However, no correlation was observed between nitrate concentrations and potential nitrate reduction rates. In our field ex-
experiment, it cannot be excluded that factors other than the higher C and N contents also contributed to the promotion of nitrate reduction activity in DM compared to results in the conventional tillage system. Indeed, a previous study reported a higher soil water content in the DM than in the conventional tillage system at the same experimental site (45a). Investigations of the effect of tillage on N-cycle microbial processes have mainly focused on denitrification or nitrification. Although it was shown that a tillage event could temporally increase denitrification rates for a few days (10, 30), it is apparent from most values cited in the literature that no-till soils, as compared to conventionally tilled soils, stimulate denitrification rates in the long term (1, 24, 37, 49), which is in agreement with our results. We found that the fertilization regime also had an effect on potential nitrate reduction, with the rates being significantly increased in plots fertilized with farmyard manure combined with mineral fertilizer. This increase was stronger in DM than in the conventional tillage system (Fig. 1), which suggests that nitrate reduction was more stimulated by the addition of combined fertilizer when soil aggregation and the nitrate reducer community were not affected by tillage. In contrast, addition of farmyard manure alone had no effect on potential nitrate reduction activity, whereas manure has been shown to promote denitrification activity (17, 23, 36, 38). Altogether, our results on nitrate reduction activity are in agreement with reports of previous studies with increases of denitrification activity in reduced tillage systems or in response to higher fertilization levels (reviewed in reference 44).

Analysis of the nitrate reducer community structure using the narG and napA genes encoding the catalytic subunits of the two types of respiratory nitrate reductases as molecular markers revealed small differences between the DM and conventional cropping systems (Fig. 3). Agricultural practices, such as tillage or fertilization, have already been reported to be important factors driving the structure of soil microbial communities (10, 15, 20, 21). Salles et al. (48) even found that fertilization and tillage were more effective than the agricultural management regime in changing the Burkholderia community structure. Studies of the effect of tillage practices on the structure of microbial communities involved in N cycling are rare (9). However, more information is available about how the structure of these communities can be affected by the fertilization regime. Thus, slight to important modifications in the structure of the denitrifier community in response to fertilizer addition have been reported, depending on the type of fertilizer and also on the time scale of the field experiment (2, 17, 23, 51; reviewed in reference 44). We found that fertilization with farmyard manure combined with mineral fertilizer had the strongest effect on the napA community structure (Fig. 2C and D), while tillage practice was the primary driver of the narG community structure (Fig. 2A and B). This suggests that

![Graphs showing correlation between nitrate reducer community structure and activity dissimilarity matrices.](http://aem.asm.org/)
different microbial populations are carrying the \( \text{narG} \) and \( \text{napA} \) genes and that these populations are differentially affected by the studied agricultural practices. However, the differences observed in this study were minor, and after 15 years, neither tillage practices nor fertilization regimes caused important shifts in the composition of the nitrate reducer community.

The size of the nitrate reducer community in the different cropping systems was estimated by quantifying the \( \text{narG} \) and \( \text{napA} \) genes by real-time PCR. Because it is likely that all DNA was not successfully extracted from the soil samples, the gene copy numbers were calculated both as nanograms of extracted DNA (see Fig S1 in the supplemental material) and per gram of soil (Fig. 3). However, since potential nitrate reductase activities are expressed per gram of soil, only the gene copy numbers per gram of soil were used for further analyses of the relationships between the size, structure, and activity of the nitrate reducer community. Besides, while most nitrate reducing bacteria possess either \( \text{narG} \) or \( \text{napA} \), a significant proportion of nitrate reducing bacteria also possess both \( \text{narG} \) and \( \text{napA} \) (39, 47), which makes it difficult to convert the \( \text{narG} \) and \( \text{napA} \) gene copy numbers into nitrate reducer cell numbers. Therefore, we used the \( \text{narG} \) and \( \text{napA} \) real-time PCR data separately but also the sum of the \( \text{narG} \) and \( \text{napA} \) gene copy numbers to investigate relationships between the size, structure, and activity of the nitrate reducer community. The \( \text{narG} \) and \( \text{napA} \) gene copy numbers in the upland soil of Madagascar were similar to densities previously found in agricultural soils (8, 42). Studies investigating the effect of tillage on the sizes of microbial communities were based mostly on microbial biomass measurements and reported increases in microbial biomass after the change from conventional to reduced or minimum tillage (12, 16, 19, 20). However, later studies demonstrated that this stimulating effect of reduced tillage was limited to the topsoil, with no consistent effect in the 2.5- to 20-cm (52) or 20- to 30-cm layer (33). Reduced tillage on its own was not responsible for enhanced microbial biomass, but rather the combination of reduced tillage and residue amendment was responsible. Stimulatory effects were attributed mainly to increased soil moisture and aeration, cooler temperature, and a higher carbon content in surface soil (20). A few studies also reported that cultivable soil denitrifiers populations tended to increase with less tillage (7, 22). Our study showing significantly higher \( \text{narG} \) and \( \text{napA} \) gene copy numbers in the 0- to 5-cm layer under DM than under conventional tillage (\( P < 0.001 \)) confirmed these findings. Addition of farmyard manure alone (F1) had no impact on either the \( \text{narG} \) or \( \text{napA} \) gene copy numbers, whatever the sampling year, whereas a combined farmyard manure and mineral fertilizer amendment (F2) significantly affected the gene copy numbers either positively or negatively (Fig. 4). Further investigations are required to understand how combined organic and mineral fertilization can sometimes decrease the numbers of nitrate reducers.

Functional communities involved in N cycling provide good models in microbial ecology for studying the role of the size and structure of microbial communities in corresponding process rates and ecosystem functioning (34, 43). To analyze the relationships between the size and structure of the nitrate reducer community and nitrate reduction activity, we performed Mantel tests of correlation between dissimilarity matrices and calculated Pearson correlations. Since nitrate reduction is a facultative respiratory process, the presence of nitrate and carbon and the absence of oxygen are the factors primarily regulating the nitrate reduction activity. Nevertheless, when conditions are favorable to nitrate reduction, we can hypothesize that the nitrate reduction activity is related to the size of the nitrate reducer community. In this study, a significant correlation was observed between the size and the activity of the nitrate reducer community but only when values from the F2 treatment were excluded from the analysis (Fig. 4). The slope of the regression lines indicates that a 10-fold increase in the number of nitrate reduction genes corresponded to a threefold increase in nitrate reduction rates. This suggests that only a fraction of the nitrate reducers were present in niches where nitrate reduction could occur and/or that several copies of the targeted genes were present in a single cell. Indeed, up to three copies of the \( \text{narG} \) gene can be present in the same bacterium (39). The loss of correlation between size and activity of the nitrate reducer community in plots amended with farmyard manure combined with mineral fertilizers (F2) could be attributed to the higher substrate concentration leading to changes in cellular regulation, as suggested by Röling (46). Other explanations might be that nitrate reducer populations, which are not targeted with our primers or with different specific activities, are selected in the F2 plots. This last hypothesis is supported by analysis of nitrate reducer community structure, which shows the separate clustering of samples from DM fertilized with F2. Thus, the significant correlations observed between differences in structure and activity of the nitrate reducer community were mainly due to the fact that samples from the F2-amended DM system differed the most in both cases (Fig. 5).

This was also true for the correlations between differences in the structure and size of the nitrate reducer community (data not shown), which prevents us from drawing robust conclusions about the putative relationships between structure and activity or size of the nitrate reducer community. These results illustrate the importance of using a large gradient of environmental conditions to analyze the relationships between the size, structure, and activity of functional microbial communities.

In conclusion, we found that all of the characteristics of the nitrate reducer community (size, structure, and activity) were affected by the tillage system. While the use of direct seeding is more sustainable because it improves the soil nutrient status and allows farmers to cut costs and save time and fuel, we showed, along with previous studies, that it also can favor N losses. In the highlands of Madagascar, nitrate reduction activity was stimulated by combined organic and mineral fertilization but not by organic fertilization alone. However, both negative and positive effects of combined organic and mineral fertilization were observed on the size of the nitrate reducer community.

The size of the nitrate reducer community was a significant predictor of the nitrate reduction rates but not in all treatments, which highlights the inherent complexities in understanding the relationships between the size, diversity, and activity of functional microbial communities along environmental gradients.

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