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Shifts of nitrogen transforming microbial communities during paddy soil evolution

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„Wir müssen unbedingt Raum für Zweifel lassen, sonst gibt es keinen Fortschritt, kein Dazulernen. Man kann nichts Neues herausfinden, wenn man nicht vorher eine Frage stellt. Und um zu fragen, bedarf es des Zweifelns.“

(Richard P. Feynman
– Physiker und Nobelpreisträger des Jahres 1965)

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List of publications and contributions

Publications

Research article:

I.   **Bannert, A.**, Kleineidam, K., Wissing, L., Müller-Niggemann, C., Vogelsang, V., Welzl, G., Cao, Z.-H., Schloter, M., 2011. Changes in diversity and functional gene abundances of microbial communities involved in nitrogen fixation, nitrification and denitrification comparing a tidal wetland to paddy soils cultivated for different time periods. *Applied Environmental Microbiology* 77, 6109-6116.

II.   **Bannert, A.**, Müller-Niggemann, C., Kleineidam, K., Wissing, L., Cao, Z.-H., Schwark, L., Schloter, M., 2011. Comparison of lipid biomarker and gene abundance characterizing the archaeal ammonia-oxidizing community in flooded soils. *Biology and Fertility of Soils* 47, 839-843.

III.   Roth, P., Lehndorff, E., Cao, Z.-H., Zhuang, S., **Bannert, A.**, Wissing, L., Schloter, M., Kögel-Knabner, I., Amelung, W., 2011. Accumulation of nitrogen and microbial residues during 2000 years of rice paddy and non-paddy soil development in the Yangtze River Delta, China. *Global Change Biology* 17, 3405-3417.

IV.   Müller-Niggemann, C., **Bannert, A.**, Schloter, M. Lehndorff, E. Schwark, L., 2012. Intra-versus inter-site macroscale variation in biogeochemical properties along a paddy soil chronosequence. *Biogeosciences*, doi:10.5194/bgd-8-10119-2011.

V.   Töwe, S., Wallisch, S., **Bannert, A.**, Fischer, D., Hai, B., Häsler, F., Kleineidam, K., Schloter, M., 2011. Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. *Journal of Microbiological Methods,* 84, 406-412.

Review:

VI.   Ollivier J., Töwe, S., **Bannert, A.**, Hai, B., Kastl, E.M., Meyer, A., Su, M., Kleineidam, K., Schloter, M., 2011. Nitrogen turnover in soil and global change. *FEMS Microbiology Ecology* 78, 3-16.

My contribution to the publications

I.+II.   I was involved in planning and conducting the experiment and the sampling. Only the GDGT analysis in Publication II was performed by Cornelia Müller-Niggemann, University Kiel. The published data were ascertained by me. For the analysis of T-RFLP data in Publication I the help of a statistician was consulted. The manuscripts are mainly based on my input.

III.+IV   I was involved in the sampling and I performed the determination of N_{mic}, C_{mic}, NO_3^-, NH_4^+, DON and DOC contents.

V.   I was mainly involved in planning and conducting the T-RFLP analysis and performing the connected statistics. Regarding the manuscript, I was responsible for the material and methods part for the T-RFLP and I was involved in the discussion of this experiment.

VI.   I was responsible for the chapter "Changing land-use pattern".

Summary

Microbes are main players in soil nutrient turnover and therefore important for soil health. Thus, effects of land-use changes on soil microbial communities have been studied with great interest. However, most of these studies describing changes in the soil microbial community structure and function are focusing on short-term effects. Hence, it is not clear if the observed changes are an adaptation to the new conditions or just reflect a response to the disturbance of an equilibrated ecosystem. To answer this question, we studied land-use changes from natural tidal wetlands to cultivated paddy soils and long-term paddy soil management. The common practice of land reclamation through sea dyke building in the Yangtze River Delta in China, provided the unique opportunity to study a paddy soil chronosequence ranging from 0 (tidal wetland) to 2000 years of continuous management. Paddy soils are an important agricultural system, as rice is the main source of food for more than half of the world's population. Flooded rice fields are known to be strongly nitrogen deficient. In terms of feeding the world, the inorganic nitrogen cycle is therefore of special scientific interest.

The first and main part of this Ph.D. thesis was the molecular analysis of nitrogen-transforming microbial communities in the chronosequence soils. We measured functional gene abundances and diversity pattern in soil samples from sites being under rice cultivation for 0 (tidal wetland), 50, 100, 300 and 2000 years. The processes and respective functional genes being investigated were nitrogen fixation (*nifH*), nitrification (*amoA* AOA, *amoA* AOB) and denitrification (*nirK*, *nirS*, *nosZ*). Overall, changes in abundance and diversity of the functional groups could be observed, reflecting the different chemical and physical soil properties, which changed in terms of soil development. However, pronounced were changes between the tidal wetland and the paddy soils. This might be explained by alteration

from salt- to freshwater conditions and the beginning effects of the rice plants. The latter might be driver for the significant increase of *nifH* gene copy numbers in the 50 years cultivated paddy soil compared to the tidal wetland and the other paddy soils. They might also influence gene abundances of bacterial ammonia oxidizers, which significantly declined in the paddy soils, while their archaeal counterparts remained at a constant level throughout the chronosequence. This might indicate that ammonia-oxidizing archaea are able to adapt very fast to the changing environmental conditions. Abundant genes of denitrification (*nirK*, *nosZ*) showed only changes after 300 years of paddy soil development, suggesting that accumulation effects might have strong influences on these genes.

In general, changes in diversity pattern were more pronounced than in abundance pattern. T-RFLP results showed significant differences between the tidal wetland and the paddy soils for all genes under investigation (*nifH*, *amoA* AOA, *nosZ*), indicating that changes in community structure occurred rapidly over a time period of less than 50 years. No significant differences were found for *amoA* AOA genes between the 100 and the 300 years cultivated paddy soils, while differences between the 2000 years cultivated and the younger paddy soils were again significant for all genes analyzed. This shows that accumulation effects of agricultural management practices over such a long time period thoroughly impact the soil microbial community structure.

The second part of this thesis was a comparison of phenotypic and genotypic markers for the dominant nitrifying organisms in the chronosequence: ammonia-oxidizing archaea. The glycerol dialcyl glycerol tetraether lipid crenarchaeol was suggested to be specific for the recently identified archaeal phylum Thaumarchaeota to which known archaeal ammonia oxidizers belong. Surprisingly, the ratios of *amoA* AOA gene abundances to crenarchaeol were not constant, as shown for different aerated agricultural soils, but were significantly higher in the paddy soils compared to the tidal wetland and upland soils. This leads to the hypothesis, that ammonia-oxidizing archaea different from crenarchaeol containing Thaumarchaeota play an important role in paddy soils.

One of the major problems of the chronosequence study is that there might be factors influencing soil microbial communities, which are not related to soil age. But statistical evaluation revealed that the intrinsic heterogeneity of paddy soil organic and minerogenic components per field was smaller than between study sites. Furthermore, a greenhouse experiment under defined conditions was conducted as third part of the thesis, comparing the 50 and the 2000 years cultivated paddy soils. In this context an improved protocol for simultaneous extraction of DNA and RNA from soil was developed.

Overall, we could show that during ongoing paddy cultivation microbial communities adapted to the changes in soil structure and organic matter quality and consequently, selected functional groups became dominant. Thus, these changes are due to the type of management and not a response towards the disturbance of an equilibrated system. Furthermore, changes in microbial community structure and function were observed at very different points in time along the chronosequence. Therefore, we hypothesized paddy soil development occurring in three different phases: (I) the change from tidal wetlands to initial paddy soils, (II) ongoing paddy soil cultivation, and (III) long-term cultivation for hundreds and thousands of years, including accumulation effects.

Zusammenfassung

Mikroorganismen spielen eine wichtige Rolle für die Stoffkreisläufe im Boden. Deshalb ist die Untersuchung der Auswirkungen von Landnutzungsänderungen auf die mikrobielle Gemeinschaft von Interesse. Die meisten Studien, die sich in diesem Zusammenhang mit Veränderungen von mikrobieller Funktion und Diversität im Boden beschäftigen, beschreiben allerdings nur Kurzzeiteffekte. Deshalb ist unklar, ob die beobachteten Veränderungen der mikrobiellen Gemeinschaft wirkliche Anpassungen an die neuen Bedingungen sind, oder ob sie lediglich eine kurzfristige Reaktion auf die Störung des Ökosystemgleichgewichts darstellen. Um diese Frage zu klären, wurden Landnutzungsänderungen von der Salzmarsch zum Nassreisboden und die Langzeitkultivierung dieser Reisböden untersucht. Im Yangtze Flussdelta in China wird neues Ackerland sukzessive durch Deichbau gewonnen. Dies ermöglichte die Untersuchung einer Chronosequenz von Nassreisböden, die bis zu 2000 Jahre kontinuierlich bewirtschaftet wurden. Reisböden sind wichtige landwirtschaftliche Nutzflächen, weil für mehr als die Hälfte der Weltbevölkerung Reis das Hauptnahrungsmittel ist. In Nassreisböden ist Stickstoff limitierender Faktor für das Pflanzenwachstum. In Bezug auf die Welternährung ist das Verständnis des anorganischen Stickstoffkreislaufs in Nassreisböden deshalb von besonderem wissenschaftlichem Interesse.

Hauptteil der Doktorarbeit war die molekulare Analyse der am Stickstoffkreislauf beteiligten Mikroorganismen in den Böden der Chronosequenz. Gemessen wurden Abundanzen und Diversitätsmuster funktioneller Gene in 0 (Salzmarsch), 50, 100, 300 und 2000 Jahre kultivierten Nassreisböden. Die folgenden Prozesse und die entsprechenden funktionellen Gene wurden untersucht: Stickstofffixierung (*nifH*), Nitrifikation (*amoA* AOA, *amoA* AOB) und Denitrifikation (*nirK*, *nirS*, *nosZ*). Sowohl Abundanz als auch Diversität der entsprechenden funktionellen Gruppen änderten sich über die Chronosequenz und spiegelten die Entwicklung der chemischen und physikalischen Eigenschaften der Böden wieder. Am stärksten waren die Unterschiede zwischen der Salzmarsch und den Nassreisböden. Eine

mögliche Erklärung ist der Wechsel von Salz- zu Süßwasserbedingungen und der beginnende Einfluss der Pflanzen in den Reisböden. Die Reispflanzen bedingen möglicherweise den signifikanten Anstieg in den *nifH* Genkopienzahlen im 50 Jahre kultivierten Boden im Vergleich zur Salzmarsch und den anderen Reisböden. Sie könnten auch Einfluss auf die Genkopienzahlen der bakteriellen Ammoniumoxidierer haben, die in den Reisböden signifikant abnehmen, während die Genabundanzen Ammonium oxidierender Archaeen über die gesamte Chronosequenz auf einem gleichen Niveau bleiben. Die Ammonium oxidierenden Archaeen scheinen also die Möglichkeit zu haben, sich sehr schnell an die veränderten Umweltbedingungen anzupassen. Veränderungen in den Genabundanzen dominanter Gene der Denitrifikation (*nirK*, *nosZ*), konnten erst nach 300 Jahren gemessen werden. Das zeigt, dass Akkumulationsprozesse möglicherweise einen großen Einfluss auf diese Gene haben. Generell wurden mehr signifikante Veränderungen in den Diversitätsmustern gemessen als in den Genabundanzen. Die Ergebnisse der T-RFLP zeigten signifikante Unterschiede zwischen der Salzmarsch und den Nassreisböden für alle untersuchten Gene (*nifH*, *amoA* AOA, *nosZ*). Die Diversität veränderte sich also in einem Zeitraum von weniger als 50 Jahren. Für *amoA* AOA wurden keine signifikanten Unterschiede in den Diversitätsmustern für Proben des 100 und 300 Jahre kultivierten Reisbodens gefunden. Unterschiede zwischen dem 2000 Jahre alten Boden und den anderen Reisböden waren hingegen für alle untersuchten Gene wieder signifikant. Dies zeigt, dass Akkumulationseffekte durch landwirtschaftliche Bewirtschaftungsmethoden über einen entsprechend langen Zeitraum einen großen Einfluss auf die Struktur der mikrobiellen Gemeinschaft haben.

Der zweite Teil der Doktorarbeit bestand in einem Vergleich zwischen genotypischen und phenotypischen Markern für die dominierenden Nitrifizierer in der Chronosequenz: Ammonium oxidierende Archaeen. Bekannte Ammonium oxidierende Archaeen gehören zu dem kürzlich postulierten Phylum Thaumarchaeota. Das Gylcerol Dialkyl Glycerol Tetraether Lipid Crenarchaeol ist nach bisherigen Erkenntnissen für diese Gruppe spezifisch.

Überraschenderweise war das Verhältnis der *amoA* AOA Genabundanzen zu Crenarchaeol für die Chronosequenz nicht konstant, wie es in anderen Arbeiten mit landwirtschaftlichen Böden gemessen wurde, sondern in den Reisböden signifikant höher als in der Salzmarsch und generell höher als in durchlüfteten landwirtschaftlichen Böden. Dies führt zu der Hypothese, dass in Reisböden andere Ammonium oxidierende Archaeen als Crenarchaeol enthaltende Thaumarchaeota eine wichtige Rolle spielen.

Eines der Hauptprobleme der Chronosequenzstudie ist der mögliche Einfluss von Faktoren, die nicht mit dem Alter der Böden korreliert sind. Statistische Analysen konnten jedoch zeigen, dass die Heterogenität organischer und minerogener Komponenten innerhalb eines Reisfeldes geringer war, als zwischen den unterschiedlich lange kultivierten Böden. Der dritte Teil der Doktorarbeit bestand in einem Gewächshausexperiment unter kontrollierten Bedingungen. Hier wurden der 50 und der 2000 Jahre kultivierte Boden verglichen. In diesem Zusammenhang wurde ein verbessertes Protokoll für die Coextraktion von DNA und RNA aus Boden entwickelt.

Zusammenfassend konnten wir zeigen, dass sich die Mikroorganismen im Laufe der Bodenentwicklung an die veränderten Bedingungen in Bodenstruktur und Qualität der organischen Substanz anpassen und ausgewählte Gruppen im Zuge der Bodengenese dominant werden. Dies zeigt, dass die Veränderungen in Funktion und Struktur der mikrobiellen Gemeinschaft durch die unterschiedliche Landnutzung und kontinuierliche Bewirtschaftung entstehen und nicht aufgrund der Störung eines sich im Gleichgewicht befindenden Ökosystems. Außerdem veränderten sich die verschiedenen funktionellen Gruppen zu sehr unterschiedlichen Zeitpunkten der Bodenentwicklung. Dies führt zu der Hypothese, dass diese in drei Phasen abläuft: (I) die Landnutzungsänderung von der Salzmarsch zum Nassreisboden, (II) die weitere Bewirtschaftung und schließlich (III) die Langzeitkultivierung mit entsprechenden Akkumulationseffekten.

Introduction

1. The paddy soil chronosequence

1.1. Global relevance of wetlands

The global wetland area is estimated to range around 700 Mha (Mitsch et al. 2007). Almost half of it is situated in the tropics. Wetlands are in general terms lands where water saturation is the dominant factor determining the nature of soil development. They are seasonally or permanently water-saturated and occur therefore in areas where soils are naturally or artificially flooded due to high groundwater or surface water (Neue et al. 1997). Wetland ecosystems may be differentiated on the basis of hydrology, soil type and vegetation (Cowardin et al. 1979). Both, natural and agricultural wetlands are important sources for atmospheric methane (CH_4) (Neue et al. 1997). Estimates of the total annual emission of CH_4 amount to 100 to 200 Tg a^{-1} for natural wetlands and to 20 to 100 Tg a^{-1} for paddy soils (Houghton et al. 1992). The latter accounts for approximately 15% of the global methane emission and may therefore be one of the major biogenic sources of this atmospheric greenhouse gas (Neue et al. 1997, Liesack et al. 2000, Denier van der Gon et al. 2002). Additionally, paddy fields are also a relevant source for the greenhouse gas nitrous oxide (N_2O). Around 1% of the nitrogen applied is lost through N_2O emission (Cai et al. 1997, Denier van der Gon et al. 2002; summarized in reviews: Frenzel 2000, Majumdar 2005).

The importance of wetlands to global biogeochemistry, water balance, wildlife, and human food production is much greater than their proportional surface area on earth (Neue et al. 1997). Tropical wetlands, e.g., contain the most productive agricultural and natural ecosystems on earth (Downing et al. 1993). Most important agricultural wetlands are paddy soils, as rice is the main source of food for more than half of the world's population (Fukai et al. 1985).

1.2 The wetland rice ecosystem

Worldwide around 158 million hectares of land are under rice cultivation (FAOSTAT 2009) and about 75% of this rice grows under flooded conditions (Roger et al. 1993). For comparison, the worldwide amount of land used for wheat cultivation is around 226 million hectares (FAOSTAT 2009).

Paddy soils are man-made wetlands and belong to the Hydragric Anthrosols (Zhang et al. 2003, IUSS Working Group WRB 2006).

The biogeochemistry of paddy soils differs from aerated agricultural soils as it is controlled by flooding and the resulting pattern of oxidation and reduction (Neue et al. 1997). The diffusion of atmospheric oxygen decreases by a factor of 10^5 due to submergence. Oxygen only penetrates the first millimetres of the soil where it is rapidly consumed and is therefore a limiting factor in flooded paddy soils (Frenzel et al. 1992, Revsbech et al. 1999). This leads to a coexistence of oxidized and reduced zones forming a compartmentalized system with three major parts: (I) the anoxic bulk soil, (II) the oxic surface soil and (III) the partially oxic rhizosphere with increased substrate concentrations. Diffusive transport of oxygen through the aerenchyma of rice roots leads to the availability of oxygen in the rhizosphere (Armstrong 1971, Gilbert et al. 1995, Revsbech et al. 1999). Between the compartments, well-defined chemical gradients can be measured and form different habitats for microorganisms (Revsbech et al. 1999, Liesack et al. 2000). Anaerobic microorganisms, such as fermentative bacteria and methanogenic archaea, predominate within the microbial community. Consequently, methane is the final product of anaerobic degradation of organic matter in paddy soils (Liesack et al. 2000). Oxygen at the soil surface and along the roots is a key factor controlling the gradients of other electron acceptors such as nitrate, ferric oxide and sulphate. In the anoxic zone alternative electron acceptors are used according to their redox potential. Nitrate is the first electron acceptor reduced after oxygen depletion (E_0'=750 mV) followed by Mn(IV), Fe(III), SO_4^{2-}, and CO_2 (Ponnamperuma 1972, Zehnder et al. 1988, Vepraskas et al. 2001; Figure 1).

E$_0$´ [mV]

- 810 — O$_2$/H$_2$O
- 750 — NO$_3^-$/N$_2$
- 510 — MnO$_2$+HCO$_3^-$/MnCO$_3$
- 200 — Fe(OH)$_3$+HCO$_3^-$ /FeCO$_3$
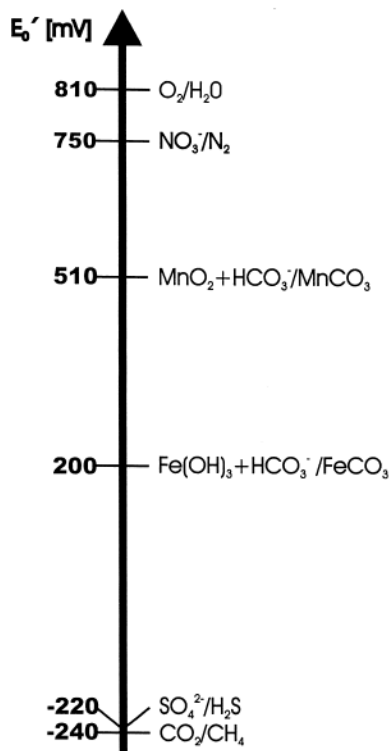- -220 — SO$_4^{2-}$/H$_2$S
- -240 — CO$_2$/CH$_4$

Figure 1: Oxidation-reduction couples of various electron acceptors occurring in paddy soils arranged from the strongest oxidants at the top to the strongest reductants at the bottom (Liesack et al. 2000).

In the presence of oxygen these electron acceptors are regenerated by an oxygen-dependent oxidation of the reduced products (ammonium, iron (II) or sulfide). Consequently, redox cycling of nitrogen (N), iron (Fe) and sulphide (S) takes place (Liesack et al. 2000, Conrad et al. 2002).

The microbial food web is driven by an input of organic matter into the rice soil. Besides soil organic matter, the organic carbon originates from decay of plant material (addition of rice straw represents a common fertilization method) or is released from the plant through root exudation (Hartmann et al. 2009).

1.3. Characteristics of the paddy soil chronosequence in Cixi, Zhejiang Province, China

A chronosequence is defined as a sequence of related soils that differ in their degree of profile development because of differences in their age (Allaby 2004). There are many reports about different, investigated chronosequences, such as glacier forelands (Haugland 2004, Brankatschk et al. 2010), marine terraces (Scarciglia et al. 2006), mining area (Frouz et al. 2001) or pasture (Numata et al. 2003). However, few reports are related to paddy soils. There are 112 rice producing countries. But according to the Food and Agriculture Organization (FAO) of the U.N., 80% of the world rice production comes from only 7 countries. Most of the paddy soils are located in China, which contributes 32.7% to the worldwide rice production (2009-2010). They total more than 30 million hectares and occur mainly in the area south of the Yangtze River (Zhang and Gong 2003). The Yangtze River Delta is one of the earliest and major rice production areas, where about 70% of the ancient rice relict sites in China have been found (Cao et al. 2006). In this area, it is a common practice to create new farmland through consecutive land reclamation by protective dykes. Once a dyke is built, the land inside the dyke gradually becomes arable as the salts are leached off by rain. Rice cultivation begins when the salt concentration decreases to a certain level. A historically well-dated construction of the dykes can provide a unique chronosequence setting of soil formation under agricultural use. This is the case in the Chinese Zhejiang Province, near Cixi, a subtropical monsoon area, with a mean annual temperature of 16.3°C and precipitation of 1325 mm (Zhang et al. 2004). The chronosequence there ranges between day zero of soil development, represented by a tidal wetland being the soil parent material, and 2000 years of continuous paddy soil cultivation. For this thesis a tidal wetland (P0) and paddy soils being under rive cultivation for 50 (P50), 100 (P100), 300 (P300) and 2000 (P2000) years, respectively, were investigated. All sites are located within 40 km. Coordinates of sampled sites were: P0: 30° 19' N, 121° 09' E; P50: 30° 11' N, 121° 22' E; P100: 30° 09' N, 121° 21' E; P300: 30° 06' N, 121° 31' E and P2000: 30° 05' N, 121° 27' E (Figure 2). Points in time of sea dyke construction are well documented

in Cixi County Annals. Abstracted information is available in Chinese at www.cixi.gov.cn and was summarized by Cheng et al. (2009).

Figure 2: Geographic location of the sampling site showing chronosequence recognition. Dark lines in the close-up map show the dike positions and the year of construction. Modified from Cheng et al., (2009).

Due to the nature of the marine sediments, the texture is similar between all age groups and strongly dominated by silt-sized particles. A continuously increasing accumulation of total organic carbon in the topsoils with cultivation time from 1.7% in P50 to 3.1% in P2000 could be shown. Similarly, total nitrogen content of the soils increased with cultivation time. The pH$_{(KCl)}$ value of soil decreased from neutral to slightly acidic with increasing duration of rice cultivation due to continuous decalcification. This process is very fast and firstly appears in the topsoils of the paddy chronosequence because of abundant irrigation water input in the paddy fields (Zou et al. 2011). Rice cultivation in summer and cultivation of wheat or vegetables in winter is the major crop rotation system in this area and was also practiced on the investigated fields. Types, rates and methods of application of fertilizers and pesticides were similar between all sites. Irrigation is needed to maintain standing water in certain periods of rice growing. Water regimes were also comparable between all sites of the investigated chronosequence. In fact, the study of Cheng et al. (2009) comparing the paddy

soil chronosequence with a corresponding non-paddy chronosequence revealed that paddy management had a profound impact on soil formation.

2. The microbial nitrogen cycle

2.1. The importance of nitrogen for rice growth

Nitrogen (N) is the most important determinant for plant growth and crop yield (Hooper et al. 1999), as it is an essential component of proteins and nucleotides. It is also part of co-enzymes, photosynthetic pigments, secondary metabolites or other molecules. However, the most abundant form N_2 (3.9×10^9 million tonnes on earth) accounting for 78% of the atmosphere (Jenkinson 2001) is an inert gas and thus not directly available for plants. Nitrogen input into soils occurs mainly through fertilization or microorganisms, which are able to convert nitrogen gas (N_2) into ammonium (NH_4^+; nitrogen fixation). Although ammonium is considered as the major form to which rice is especially adapted (Wang et al. 1993), it has been suggested that rice plants may also adsorb significant amounts of nitrate (NO_3^-) formed by nitrification of ammonium (Kronzucker et al. 2000). In rice cultivation the yield-limiting factor is the availability of nitrogen due to large losses in flooded soils trough denitrification or leaching (De Datta et al. 1989, Cassman et al. 1998). Nitrogen limitation of plant productivity in salt marshes has also been demonstrated (Bagwell et al. 2000). Generally, nitrogen fertilizer efficiency in flooded rice fields is poor. Flooded rice crops typically use only 20-40% (Vlek et al. 1986), whereas upland crops frequently use 40-60% of the applied nitrogen.

2.2. Inorganic nitrogen cycling in flooded soils

The biochemical nitrogen cycle in wetland ecosystems features a combination of chemical transformations and transport processes not shared by many other ecosystems (Mitsch and Gosselink 2007). This is mainly in consequence of the compartmentalization which develops due to the limitation of oxygen (see chapter 1.2.). As the organic nitrogen cycle was not investigated in this thesis, the following descriptions focus on the main processes of the inorganic nitrogen cycle.
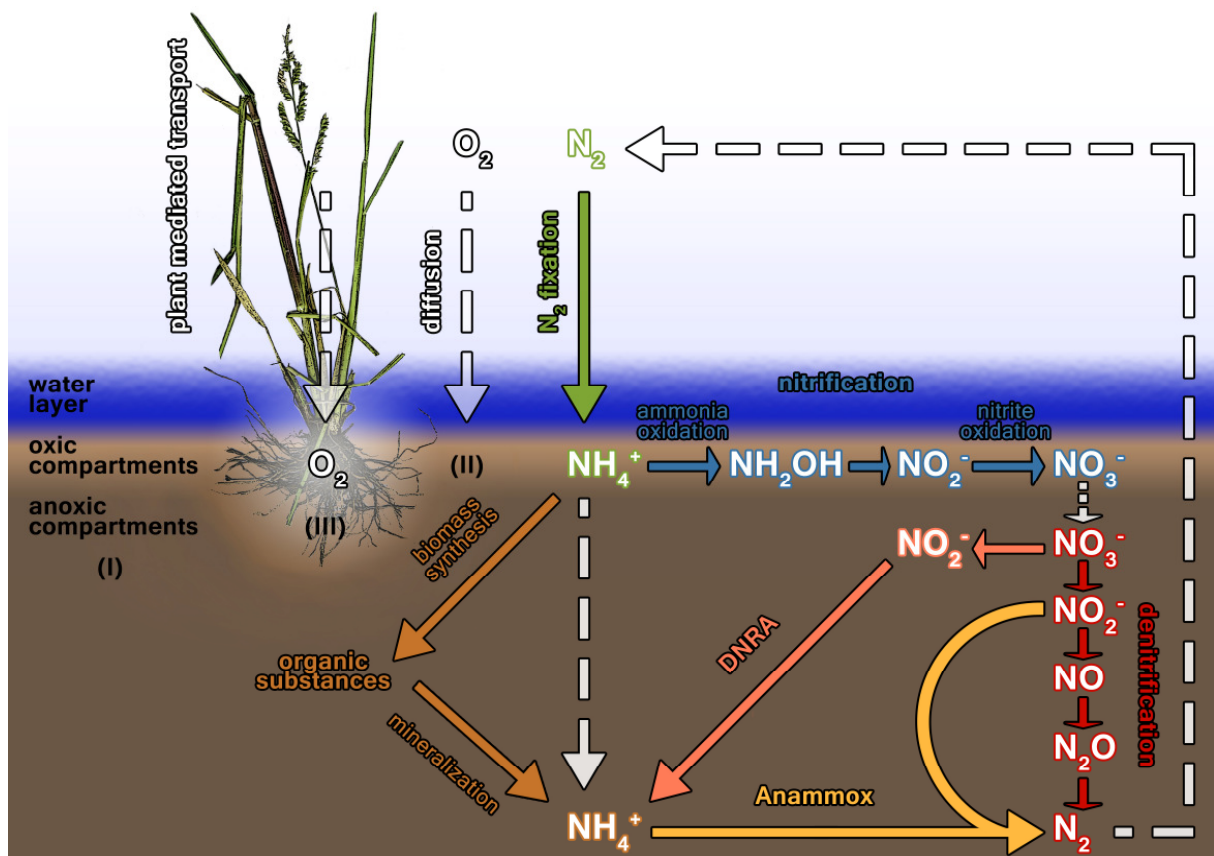
Figure 3: **Simplified scheme of the nitrogen cycle in a paddy soil with focus on the inorganic part of the cycle, representing the interaction between aerobic (II, III) and anaerobic (I) zones. Modified from Francis et al. (2007), Conrad et al. (2007) and Conrad and Frenzel (2002). Graphical design by Stephan Winklmeier.**

2.2.1. Nitrogen fixation

The biological reaction counterbalancing the loss of N from flooded soils is biological nitrogen fixation, the enzymatic transformation of nitrogen gas (N_2) to ammonia (NH_3) (Roger et al. 1992, Ladha et al. 2003). This process is unique to certain groups within the domains *Bacteria* and *Archaea* (diazotrophs) (Young 1992). Rice can meet a notable proportion of its nitrogen requirement from biological nitrogen fixation (Roger and Ladha 1992, Roger 1995, Ladha and Reddy 2003) and paddy fields are habitats for numerous groups of diazotrophs (Engelhard et al. 2000, Kennedy et al. 2004, Ariosa et al. 2005). However, the majority of nitrogenase gene fragments retrieved from rice roots cannot be assigned to cultivated diazotrophs (Engelhard et al. 2000, Hurek et al. 2005). Root environments of flooded plants

may be particularly favorable for nitrogen fixation, because they probably constitute a suitable low-O_2 environment (Hurek and Reinhold-Hurek 2005). Rice crops belong to the *Poaceae*, which do not naturally form specialized symbiotic structures such as root nodules (Knauth et al. 2005). But internal tissues of rice have been shown to be colonized by endophytes and the nitrogenase may be expressed in the aerenchyma of the roots (Hurek et al. 1994, Hurek et al. 1997, Egener et al. 1999, Engelhard et al. 2000). Also in salt marshes nitrogen fixation is carried out through plant-associated diazotrophs. Surprisingly, the diversity of nitrogen-fixing microbes there is high in comparison to the relatively small group of known diazotrophic bacteria and archaea (MCClung et al. 1983, Young 1992). In natural ecosystems like salt marshes, diazotrophy is the most important source of nitrogen as no fertilization takes place (Hanson 1983).

Four different types of nitrogenases are known up to now. Closely related are (I) the molybdenum-based (Mo-), (II) the vanadium based (V-) and (III) the iron-based (Fe) nitrogenase which have all three a common ancestor (Newton 2007). The fourth type of nitrogenase invented independently and was so far only found in the thermophilic streptomycete *Streptomyces thermoautotrophicus* (Ribbe et al. 1997).

All nitrogen-fixing microbes contain the molybdenum-based nitrogenase, which is the most efficient catalyst for N_2 reduction, while the others occur randomly as additional enzyme. It is composed of two metallo-proteins: (I) the MoFe-protein, also called dinitrogenase, encoded by *nifD* and *nifK* genes and (II) the Fe-protein dinitrogenase reductase, encoded by the *nifH* gene (Burris 1991). The *nifH* gene is a commonly used marker for studying the diazotrophic assemblage as it is highly conserved among all diazotrophs (Young 1992, Zehr et al. 2003, Raymond et al. 2004). Biological nitrogen fixation requires large amounts of energy, which is at least 16 molecules of ATP to fix one molecule N_2 (Zehr et al. 2003).

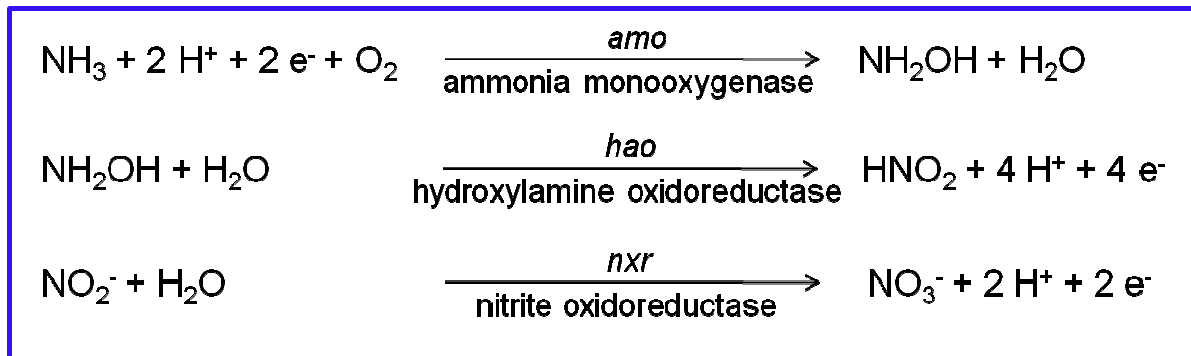$$N_2 + 8\,H^+ + 8\,e^- \xrightarrow[\text{nitrogenase}]{\text{nif}} 2\,NH_3 + H_2$$
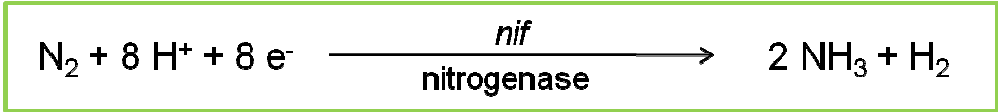
Equation 1: Reaction equation of biological N_2 fixation. The indication below the arrow describes the responsible enzyme; the indication above the arrow describes the corresponding gene.

2.2.2. Nitrification

The presence of oxic/anoxic interfaces in flooded soils provides a suitable environment for the coupling of nitrification and denitrification leading both to N loss from soils (Arth et al. 1998, Arth et al. 2000, Nicolaisen et al. 2004). Nitrification occurs in aerobic zones and may be supported by oxygen release from rice roots (Arth et al. 1998, Brune et al. 2000). Some reports indicate high rates in the rhizosphere (Reddy et al. 1989), but even higher rates of nitrification should be expected at the soil surface of recently fertilized soils (Liesack et al. 2000).

Nitrification is a two-step process of enzymatic oxidation: (I) ammonia-oxidation to nitrite and (II) nitrite oxidation to nitrate (Bothe et al. 2000, Prosser et al. 2002, Regan et al. 2002).

$$NH_3 + 2\ H^+ + 2\ e^- + O_2 \xrightarrow[\text{ammonia monooxygenase}]{amo} NH_2OH + H_2O$$

$$NH_2OH + H_2O \xrightarrow[\text{hydroxylamine oxidoreductase}]{hao} HNO_2 + 4\ H^+ + 4\ e^-$$

$$NO_2^- + H_2O \xrightarrow[\text{nitrite oxidoreductase}]{nxr} NO_3^- + 2\ H^+ + 2\ e^-$$

Equation 2: Reaction equation of nitrification. Indications below the arrows describe responsible enzymes; indications above the arrows describe corresponding genes.

Neither aerobic oxidation of NH_4^+ nor of NO_2^- produces sufficient reducing power to reduce $NAD(P)^+$ to $NAD(P)H$. Therefore reversed electron transport was postulated (Ferguson et al. 2007).

Ammonia oxidation is the first and rate-limiting step of nitrification and can be carried out by archaea and bacteria (Schleper et al. 2005, Francis et al. 2007). For a long time it was thought that autotrophic ammonia-oxidizing bacteria (AOB) are the major contributors to ammonia oxidation in soils. Substantial discoveries of the last years indicate that archaea belonging to the Thaumarchaeota linage can also perform ammonia-oxidation (ammonia-oxidizing archaea: AOA) (Bothe et al. 2000, Brochier-Armanet et al. 2008, Spang et al. 2010). Both archaeal and bacterial ammonia oxidizers are found in the majority of terrestrial

ecosystems including agricultural, grassland, forest and alpine soils. For both the key functional enzyme is the ammonia monooxygenase oxidizing ammonia to hydroxylamine (Equation 2). The quantification of respective *amoA* genes indicates greater abundance of ammonia-oxidizing archaea in many soils, including paddy soils (Leininger et al. 2006, Boyle-Yarwood et al. 2008, Chen et al. 2008, Nicol et al. 2008).

The AOB community in rice soils consists of three major groups, i.e. *Nitrosomonas communis* cluster, *Nitrosospira* cluster 3a and 3b (Wang et al. 2008). Different dominating groups were found in different studies as well as a dependence of diversity composition of AOB on the rice variety cultivated (Rotthauwe et al. 1997, Horz et al. 2000, Nicolaisen et al. 2004, Bowatte et al. 2007, Wang et al. 2008).

Nitrification processes link decomposition of organic matter, releasing NH_4^+ and denitrification, for which they provide the electron acceptor. Much of the nitrate-nitrite formed by nitrification may subsequently be denitrified, as most NO_x^- will diffuse into the anoxic soil instead of being taken up by the roots (Reddy et al. 1989, Jensen et al. 1993, van Cleemput et al. 2007).
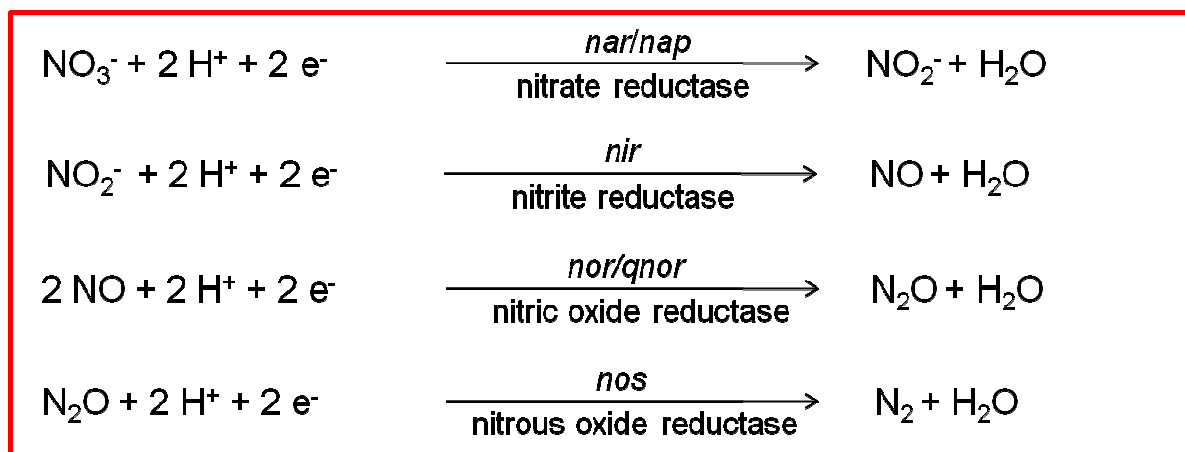

2.2.3. Anaerobic ammonia oxidation (Anammox)

Ammonia oxidation cannot only occur in aerobic compartments of wetlands through nitrification, but also in anaerobic ones (compartment II and III in Figure 3). The process of anaerobic ammonia oxidation, shortly Anammox, was firstly found in wastewater treatment systems (Mulder et al. 1995). Little is known about the ecological relevance in wetland systems (Zhu et al. 2010). Anammox has not been addressed in this thesis, as there are only limited molecular tools available for investigation. But it is briefly described in the following, because it might play a role in wetland ecosystems. Anammox bacteria are chemolithoautotrophic or mixotrophic (Kartal et al. 2008) and form a monophyletic group, the *Brocadiales*, within the *Planctomycetes*. They use nitrite or nitrate instead of oxygen as electron acceptor to oxidize ammonium to dinitrogen gas (Strous et al. 1999, Kartal et al. 2008, Kuenen 2008). The biochemistry of Anammox includes some specialties only found in

this process so far: (I) the use of hydrazine (N_2H_4) as free catabolic intermediate, (II) the biosynthesis of ladderane lipids and (III) the presence of an intracytoplasmatic compartment called anammoxosome (Francis et al. 2007). In marine sediments the process was found to count to a really high value of N loss (up to 50%) (Thamdrup et al. 2002, Kuypers et al. 2005, Kuypers et al. 2006, Hamersley et al. 2007). Annamox has been detected in various aquatic ecosystems, particularly in oceanic oxygen-minimum zones and marine surface sediments (Thamdrup and Dalsgaard 2002, Trimmer et al. 2003, Risgaard-Petersen et al. 2004, Rysgaard et al. 2004, Engstrom et al. 2005, Rich et al. 2008), but also in freshwater lakes (Schubert et al. 2006). The occurrence of Anammox in such a broad range of aquatic environments suggests that it occurs ubiquitously. Furthermore, as the process requires the concomitant presence of oxidized and reduced inorganic nitrogen compounds under anoxic conditions, it is likely that oxic/anoxic interfaces, as they are present in paddy soils or tidal wetlands, may be possible habitats for Anammox bacteria (Jetten et al. 1998, Jetten et al. 2001, Francis et al. 2007, Humbert et al. 2010). Humbert et al. (2010) gained with 16S rRNA primer for Anammox bacteria specific products for 60 out of 112 terrestrial soil samples, including paddy soils and marsh sediments, indicating that Anammox bacteria are also present in terrestrial environments. But it has to be taken into account, that only a part of the clone sequences were closely related to known Anammox bacterial genera, while the rest, including paddy soil sequences, were related to *Planctomycetes* 16S rRNA sequences branching outside the "Anammox bacterial cluster". It is questionable if they belong to a new cluster of so far uncultivated Anammox bacteria. Zhu et al. (2010) reported a relative high biodiversity of Anammox bacteria in various freshwater wetlands. Cai et al. (2002) found clear hints for Anammox processes in paddy soils by ^{15}N tracer experiments. Summarizing, so far nearly nothing is known about the distribution, diversity and activity of Anammox bacteria in terrestrial ecosystems, including wetlands. Thus, the overall contribution of Anammox to N losses in paddy fields and marsh sediments remains unclear.

2.2.4. Denitrification

Denitrification is the step wise reduction of nitrogen oxides (NO_3^- and NO_2^-) to gaseous end products (NO, N_2O, N_2) (Zumft 1997) in an anaerobic respiration process associated with electron transport phosphorylation (van Spanning et al. 2007). Nitrogen oxides are used as an alternative electron acceptor for energy production when oxygen is limiting (Heylen et al. 2006). The process is catalyzed by four enzymatic reaction steps: (I) nitrate to nitrite (nitrate reductase), (II) nitrite to nitric oxide (nitrite reductase), (III) nitric oxide to nitrous oxide (nitric oxide reductase), (IV) nitrous oxide to dinitrogen (nitrous oxide reductase) (Equation 3) (Heylen et al. 2006, van Spanning et al. 2007). All enzymes are complex multisite metalloproteins (Berks 1995). They are produced only under (close to) anaerobic conditions, indicating that denitrifiying organisms are mostly facultative anaerobes (Zumft 1997). The more energy-exploiting respiration of O_2 usually occurs in preference to the use of N-oxides (van Spanning et al. 2007). Denitrification ability is widespread among a variety of microorganisms, including over 40 genera of bacteria, halophilic and thermophilic archaea, fungi, and foraminifera (Zumft 1997, Bothe et al. 2000, Liesack et al. 2000, Philippot et al. 2007, van Spanning et al. 2007, Hayatsu et al. 2008).

$$NO_3^- + 2\,H^+ + 2\,e^- \xrightarrow[\text{nitrate reductase}]{nar/nap} NO_2^- + H_2O$$

$$NO_2^- + 2\,H^+ + 2\,e^- \xrightarrow[\text{nitrite reductase}]{nir} NO + H_2O$$

$$2\,NO + 2\,H^+ + 2\,e^- \xrightarrow[\text{nitric oxide reductase}]{nor/qnor} N_2O + H_2O$$

$$N_2O + 2\,H^+ + 2\,e^- \xrightarrow[\text{nitrous oxide reductase}]{nos} N_2 + H_2O$$

Equation 3: Reaction equation of denitrification. Indications below the arrows describe responsible enzymes; indications above the arrows describe corresponding genes.

The reduction of nitrite to nitric oxide by the nitrite reductase is the first step distinguishing denitrifiers from nitrate-respiring organisms. It is carried out by two functionally and physiologically equivalent enzymes (Glockner et al. 1993, Zumft 1997, Braker et al. 2000,

van Cleemput et al. 2007): containing either (I) copper (encoded by *nirK* gene) or (II) cytochrome cd_1 (encoded by *nirS* gene). Only one of these enzyme types is found per cell, although the Nir type may differ within the same genus and even within the same species (Coyne et al. 1989, Heylen et al. 2006). Together with the nitrous oxide reductase (encoded by *nosZ* gene) they are the main targets for investigation of denitrifying bacteria (van Cleemput et al. 2007). Yoshida et al. (2009) found in their study a large diversity of *nirS* and *nirK* clones from paddy fields with many *nirS* clones being closely related to *Burkholderiales*, *Rhodocyclales* and *Rhodobacteriales*, which supported results by Saito et al. (2008) with SIP and diverse other studies (Ishii et al. 2009, Yoshida et al. 2010). *NirK* clones were related to *Rhizobiales* (Yoshida et al. 2009), similar to previous studies (Bremer et al. 2007, Saito et al. 2008). But this does not necessarily mean that the NirK-harboring denitrifiers belong to the *Rhizobiales*, as *nirK* phylogeny was found to be incompatible with that of 16S rRNA (Heylen et al. 2006). Strains belonging to the genus *Pseudogulbenkiania* were found to be dominant in different paddy soils (with and without crop rotation) by Tago et al. (2011) suggesting that they may be ubiquitous in various rice paddy soils.

Paddy soils have a strong denitrifying activity (Yoshida et al. 2009). Denitrification is therefore one of the major pathways of N loss (Reddy and Patrick 1986, Freney et al. 1990), beside ammonia volatilization and anaerobic ammonia oxidation (Anammox) (Lam et al. 2011), as NO_3^- leaching plays in general only a minor role in wetland rice systems (Zhu et al. 2002). Next to N_2, denitrification can also lead to the emission of N_2O and NO to the atmosphere, which may contribute to global warming and destruction of the ozone layer (Hofstra et al. 2005, Philippot et al. 2007, van Cleemput et al. 2007).

However, during the growing of rice under continuously submerged conditions the emission of N_2O and NO are lower than in upland crop fields due to sufficiently reducing conditions and the large availability of organic substances leading to a complete reduction of nitrate and nitrite to N_2 (Hou et al. 2000, Liesack et al. 2000, Nishimura et al. 2005, Akiyama et al. 2006). Changes in land use from paddy rice to upland crops resulted in 4.0 to 5.3-fold increasing N_2O emissions (Nishimura et al. 2005).

2.2.5. Dissimilatory nitrate reduction to ammonia (DNRA)

Next to denitrification another process of nitrate reduction may play a role in paddy soils: dissimilatory nitrate reduction to ammonia (DNRA) (Chen et al. 1995a, Chen et al. 1995b, Yin et al. 2002, Rütting et al. 2011). As, like for Anammox, no functional molecular tools are established for DNRA so far, the process was not investigated in this thesis, but will be described briefly. It is carried out in two steps, whereas (I) the first step is NO_3^- reduction to NO_2^-, like for denitrification and nitrate assimilation, followed by (II) NO_2^- reduction to NH_4^+ (Mohan et al. 2007). Because ammonia is less mobile than nitrate, the process of DNRA may conserve nitrogen in the ecosystem (Buresh et al. 1978, Tiedje 1988). DNRA occurs under low oxygen concentrations like denitrification. Therefore, portioning of nitrate between both processes occurs (Tiedje et al. 1983). In contrast to denitrification, nitrate is not used respiratorily as terminal electron acceptor, but fermentatively as electron sink in DNRA (Cole 1990). Under NO_3^- limiting and strongly reducing conditions, as they may occur in paddy soils, there is a shortage of electron acceptors. DNRA consumes 3 electrons more per NO_3^- molecule than denitrification and might therefore be favored under high C/NO_3^- ratios when the electron acceptor (NO_3^-) becomes limiting (Tiedje et al. 1983). The ability of performing DNRA is widespread to many soil bacteria and fungi (Rütting et al. 2011).

3. Investigation of soil microbial communities linking function and diversity

The meaningfulness of studies about diversity and functional composition of microbial communities relies on the methodological tools used (Liesack et al. 2000). Consequently, they have to be chosen carefully.

Before molecular techniques were introduced into soil microbiology, the characterization of microbial community diversity and function depended on cultivation. However, this allows only the identification of a very small part of microorganisms. Estimations speak about less than 1% (Amann et al. 1995). But the diversity in soil habitats is enormous, containing an estimated up to 10^6 different species in a single gram (Dykhuizen 1998). Most communities

are dominated by only a small number of species, whereas the vast majority of populations are quite uncommon. The application of PCR since the early 1990s (Giovannoni et al. 1990) in combination with the extraction of nucleic acids (DNA and RNA) from environmental samples has been central to the development of culture independent approaches in microbial ecology. Combination of PCR amplification of taxonomic (rRNA) or functional gene markers (encoding for key enzymes of certain processes) with fingerprinting- or sequencing-based analyses makes a description of the so far uncharacterized majority of environmental organisms possible (Head et al. 1998).

The **DNA extraction** from soil samples as the first step of molecular analysis is crucial (Martin-Laurent et al. 2001) and may be hindered by low yields of nucleic acids, difficulties in reproducibility (McIlroy et al. 2009) or contamination of nucleic acid extracts with humic substances, which might inhibit following PCR amplification (Rajendhran et al. 2008). In principal, two different strategies are possible to extract nucleic acids from environmental samples: (I) recovery of cells from the soil matrix prior to cell lysis or (II) direct lysis within the soil matrix (Holben et al. 1988). According to literature, direct lysis techniques yield more DNA (Holben et al. 1988, Steffan et al. 1988, Leff et al. 1995) and might therefore be less biased to study microbial community diversity and function. In both cases, the methods used often include various combinations of bead beating, detergents, enzymatic lysis, and solvent extraction (Krsek et al. 1999).

The ability to perform inorganic nitrogen processing may be widely spread within and between different genera, like it is the case, e.g., for denitrification. Thus, molecular markers for these communities are based on functional genes (Philippot et al. 2006). The choice of a representative gene(s) for a certain process is crucial. The denitrification pathway, e.g., contains four sequential steps, which are catalyzed by at least seven different enzymes (see Chapter 2.2.4). It has to be considered that a single gene normally gives not the information about a complete pathway (Philippot and Hallin 2006, Hallin et al. 2007).

The abundance of functional genes can be measured with **quantitative PCR (q-PCR)**. This method records the amplification of a PCR product via a corresponding increase in the

fluorescent signal during each cycle. We used SYBR® Green I as a fluorescent dye, which binds non-specifically to double-stranded DNA. As there is a direct proportionality between the intensity of the fluorescent signal during the exponential phase of PCR reaction and initial amount of target DNA, the copy number of the latter can be determined using a standard curve with target DNA of a known concentration (Hallin et al. 2007, Smith et al. 2009). The initial concentration of the target gene is then calculated by determining the cycle at which fluorescence for the first time significantly exceeds background accumulation (Heid et al. 1996). The big advantage of this method is that the quantification of the target gene is made during the exponential phase of PCR amplification avoiding "end point" PCR associated biases. End-point PCR is less sensitive and the proportions of numerically dominant amplicons do not necessarily reflect the abundance of sequences present within the environmental sample (Reysenbach et al. 1992, Suzuki et al. 1996, Lueders et al. 2004). Compared to sequence probes, the use of SYBR® Green I is simple and inexpensive, as it can be used for any reaction without sequence information. However, this leads in turn to the disadvantage that no discrimination between gene sequences, primer-dimer artefacts or amplification errors is made (Zipper et al. 2004). Thus, reliable primers are a prerequisite. The number of sequences in the database is increasing exponentially and with them sequence variation. Sometimes it may be necessary to use actual sequence information to design more broad range primers (Philippot et al. 2005, Hallin et al. 2007). A post-PCR dissociation curve was carried out to confirm that the fluorescence signal is generated only from target genes.

Terminal restriction fragment length polymorphism analysis (T-RFLP) is a fingerprinting method to investigate microbial diversity. This method is based on the difference in size of terminal restriction fragments from PCR-labelled amplicons. It is a high-throughput fingerprinting technique used to monitor changes in the structure and composition of microbial communities (Schütte et al. 2008). During PCR the forward primer is marked with a 5' fluorescent dye to amplify a fluorescence-labelled fragment, which is subsequently digested with one or several enzymes, typically using 4-base cutters (Clement et al. 1998).

Not surprisingly, the choice of restriction enzyme(s) is crucial. Sizes and fluorescence intensities of labelled DNA fragments are quantified by capillary gel electrophoresis comparing peaks to an internal size standard. The polymorphism is solely based on fragment length. Enzymes giving the best resolution of different T-RFs for sequences from the database are chosen *in silico*. Restriction efficiency should be tested by including the amplified product of a well characterized individual sequence in the restriction step as a control. Otherwise, incomplete or non-specific restriction may lead to an overestimation of diversity by increasing the number of fragments (Marsh et al. 2000, Nocker et al. 2007). A significant and non-linear discrepancy (up to 11 bp) is frequently observed between T-RFs determined experimentally and *in silico*, due to differences in GC-content and sequence length of the target gene (Kaplan et al. 2003). Depending on the choice of restriction enzyme, T-RFs rather represent operational taxonomic units, than specific sequence cluster or phylogenetic groups of organisms (Hallin et al. 2007). T-RFLP is a semi-quantitative method, as it comprises a quantitative reflection of the PCR product pool being different from the original community due to inherent biases of the end-point PCR methodology (Nocker et al. 2007).

But number and relative abundance of T-RFs give suitable means to determine microbial community diversity (Rich et al. 2004, Hannig et al. 2006, Hallin et al. 2007, Nocker et al. 2007). From T-RFLP results no direct sequence information is gained, as it is possible for other fingerprinting methods, e.g., DGGE (denaturing gradient gel electrophoresis). But a comparison of T-RFLP and DGGE detecting ribotypes in different soil samples showed T-RFLP to be at least five times more sensitive (Tiedje et al. 1999). Furthermore, a better comparison of fingerprints from different runs is given for T-RFLP, as one difficulty of DGGE is the variation in the denaturing gradient of hand-cast gels (Nocker et al. 2007).

4. Aims and hypotheses

Soil microbial characteristics may serve as potential indicators for soil quality, although the latter depends on a complex of soil physical, chemical and biological properties (Kennedy et al. 1995). The rationale to use microbial characteristics as soil quality indicators is their central role in cycling of carbon and nitrogen (Nannipieri et al. 1990).

Thus, due to the importance of soil microbes for nutrient turnover and consequently soil health, the influence of different types of land use changes has been studied with growing interest. This includes, e.g., conversion of grassland to forest (Berthrong et al. 2009), conversion of forests or grasslands to agricultural land (Postma-Blaauw et al. 2010), changes in tillage management (Cheneby et al. 2009, Vargas Gil et al. 2009, Attard et al. 2010) and shifts in the intensity of land use in agricultural (Dell et al. 2008) or forest soils (Fraterrigo et al. 2006, Azul et al. 2010). Also paddy soils have been investigated in particular, examining effects of temporary upland conversion (Chu et al. 2009). All these studies have shown significant shifts in microbial community structure and function as a consequence of land-use changes. But most of them focused on a very short time scale (review of effects of land-use changes on nitrogen transforming communities in **Publication VI**). The changes in microbial diversity and function observed might reflect a response of microbial communities towards the disturbance of a balanced ecosystem and not towards the type of management. Furthermore, nearly nothing is known about the effect of long-term cultivation after land-use changes from natural ecosystems to agricultural land, including an accumulation of agricultural management practices.

The Yangtze River Delta in China provides the unique opportunity of a chronosequence approach for paddy soils with up to 2000 years of continuous agricultural management. Tidal wetlands are the parent material for land reclamation in this area and the consequences of tidal wetland conversion for soil microbial communities are so far poorly understood. Many soil physical and chemical parameters will alter in consequence to land-use changes: Saltwater conditions are turned into freshwater conditions. The frequent submergence and drainage due to crop rotation changes redox conditions. Moreover, the rice plants start to

affect the ecosystem through root exudation and litter. The ongoing soil development leads to a continuous input of carbon and nitrogen as well as accumulation effects of agricultural management practices, e.g., fertilizers (Figure 4).
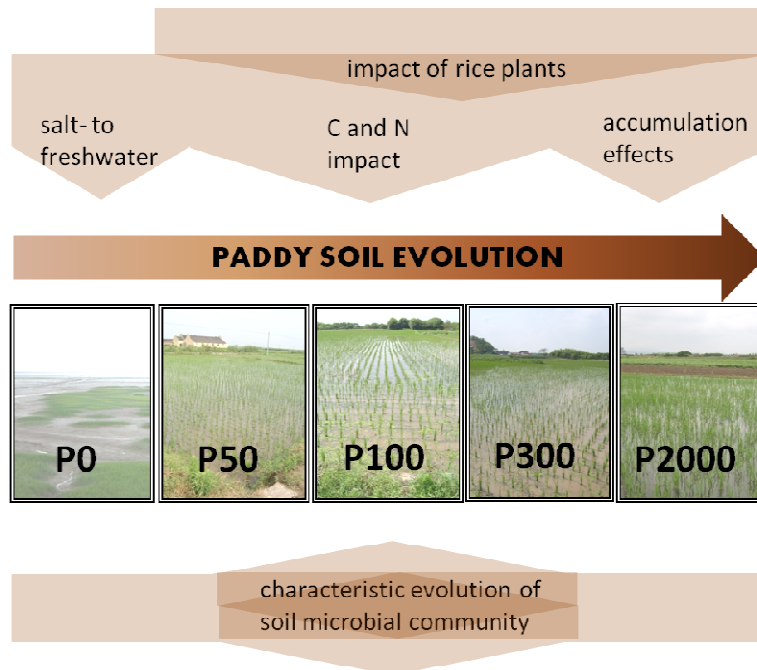

Figure 4: **The paddy soil chronosequence. Important physical, chemical and biological parameters acting during paddy soil development. An interaction between changing soil properties and soil microbial communities is hypothesized.**

Studying this unique chronosequence we focused on the inorganic nitrogen turnover as a very important nutrient cycle in agriculture which is especially true for rice cultivation, as paddy soils are known to be strongly nitrogen-deficient (Olk et al. 1996). We examined the nitrogen transforming soil microbial community in a tidal wetland and four paddy soils cultivated for 50, 100, 300 and 2000 years, respectively, and linked these results to changing physical and chemical conditions during paddy soil evolution. We postulated in general that the altering soil properties influence the nitrogen-transforming microbial communities and also that microorganisms adapt to the changing conditions and shape their environment. Thus, the main hypotheses of this thesis were (*Hypothesis I to III*):

(I)   The overall structural and functional diversity will change soon after conversion of natural tidal wetlands into cultivated paddy soils.

(II)   With ongoing agricultural management and paddy soil formation further population shifts are expected due to accumulation effects and ongoing changes in soil physical and chemical properties.

(III)   Microorganisms will adapt over time to the altered conditions.

Results of the molecular analysis of the nitrogen transforming microbial communities are described in **Publication I** (whole nitrogen cycle) and **Publication II** (nitrification in particular). **Publication III** is dealing with the microbial contribution to organic nitrogen accumulation; **Publication IV** with the heterogeneity of stable and fast cycling biochemical properties. And **Publication V** describes method development for simultaneous DNA and RNA extraction.

Most studies published so far concerning the effect of land-use changes on soil microbial communities focused on alterations related to phylogenetic groups based on 16S rRNA sequences or on one functional marker. But 16S rRNA analysis gives little insight into the functional role of each phylogenetic group (Torsvik et al. 2002a). Therefore, we investigated the main processes of inorganic nitrogen cycling: nitrogen fixation, nitrification and denitrification by q-PCR and T-RFLP of functional genes. Quantified genes were encoding for subunits of the nitrogenase (*nifH*), the bacterial and archaeal ammonia monooxygenase (*amoA*), the nitrite reductase (*nirK*, *nirS*) and the nitrous oxide reductase (*nosZ*). As *nifH*, archaeal *amoA* and *nosZ* genes turned out to be very abundant and may therefore highly contribute to the respective turnover processes, these genes were chosen for diversity analysis with T-RFLP (**Publication I**).

In the last years, archaea have been identified as key players in the global nitrogen cycle, especially in nitrification (Venter et al. 2004, Schleper et al. 2005). As recently postulated ammonia-oxidizing archaea belong to the new archaeal phylum Thaumarchaeota, for which the glycerol dialcyl glycerol tetraether lipid crenarchaeol might be a good biomarker (Spang et al. 2010). The measurement of membrane lipids offers a phenotypic criterion which requires no amplification of target sequences and is therefore not bonded by end-point PCR

biases (Ramsey et al. 2006) and may amend gene-based results. Leininger et al. (2006) calculated a ratio of *amoA* AOA copy numbers to the phenotypic biomarker crenarchaeol and found it to be very constant for different upland soils. However, so far no data existed concerning the ratio of *amoA* AOA gene copy numbers and crenarchaeol from flooded soils. We hypothesized the ratio for the paddy soil chronosequence to be constant and similar to that found for upland soils (*Hypothesis IV*, **Publication II**).

To estimate the overall microbial biomass we measured microbial biomass C and N by the chloroform fumigation extraction method (Jörgensen et al. 1991). These data were used in combination with measurements of amino sugar residues to study the contribution of microorganisms to long-term nitrogen accumulation processes (**Publication III**).

As in any field study, our chronosequence analysis is dealing with a lot of different parameters. Although crop rotation, agricultural management and water regime were very comparable between all sites, it cannot be excluded that between site differences, in addition to soil age related parameters, have an influence on the microbial community.

Therefore, statistical analysis was performed, to reveal weather intra-site specific differences of stable and labile biochemical soil properties were really lower than inter-site specific ones (*Hypothesis V*). Additionally, to get a better understanding of nitrogen cycling in the field, a greenhouse experiments under defined conditions was necessary. In this context, it would be interesting to measure gene expression (RNA) in addition to the genetic potential (DNA). If a direct comparison between genetic potential and transcript rates should be made, there is a need for co-extraction of DNA and RNA due to different types of bias, which are linked to each individual nucleic acid extraction protocol. Therefore, we developed an improved protocol for simultaneous extraction of DNA and RNA from soil, using phenol-chloroform with subsequent column-based separation of DNA and RNA. A comparison was made with the well established protocol published by Griffith et al. (2000) and two commercial kits for DNA or RNA extraction. Molecular analysis of nucleic acid extracts was carried out according to the methods used for our chronosequence approach, targeting the *nosZ* gene as an example

of a functional gene of the nitrogen cycle via q-PCR and T-RFLP. Critical points were further

yield and purity of nucleic acids (**Publication V**).

Discussion

The present study was part of the FOR 995 "Biogeochemistry of paddy soil evolution" and has been funded by the Deutsche Forschungsgemeinschaft. The aim of the project was to investigate the consequences of land-use changes from tidal wetlands to paddy soils and long-term paddy soil development up to 2000 years. In the Yangtze River Delta in China it is a common practice to perform land reclamation by sea dyke building. As the points in time of sea dyke construction are well documented, the region of Cixi, Zhejiang Province is ideally suited for a chronosequence approach. The focus of this Ph.D. thesis was to investigate the respective soil microbial communities involved in nitrogen cycling, as the macronutrient nitrogen is important for rice growth, especially in flooded soils, which are known to be strongly nitrogen-deficient. Changes in physical and chemical soil properties during paddy soil evolution might be accompanied by a characteristic development of the soil microbial community structure and function. To study the interplay of the different functional groups involved in nitrogen turnover, effects on nitrogen fixation, nitrification and denitrification were investigated.

1. The three phases of paddy soil development

Summarizing the results of the molecular analysis of microbial communities in the chronosequence samples (**Publication I**), we are able to outline three phases of paddy soil development. This is indicated by changes in abundance pattern and genotype richness of microbes involved in the three processes of interest, occurring at different points in time of soil cultivation (Figure 5). A development in different phases could be confirmed by changes in soil physical and chemical parameters. However, it has to be considered that no exact points in time, at which alterations between the different phases take place, can be assumed from a chronosequence with only five different sites.
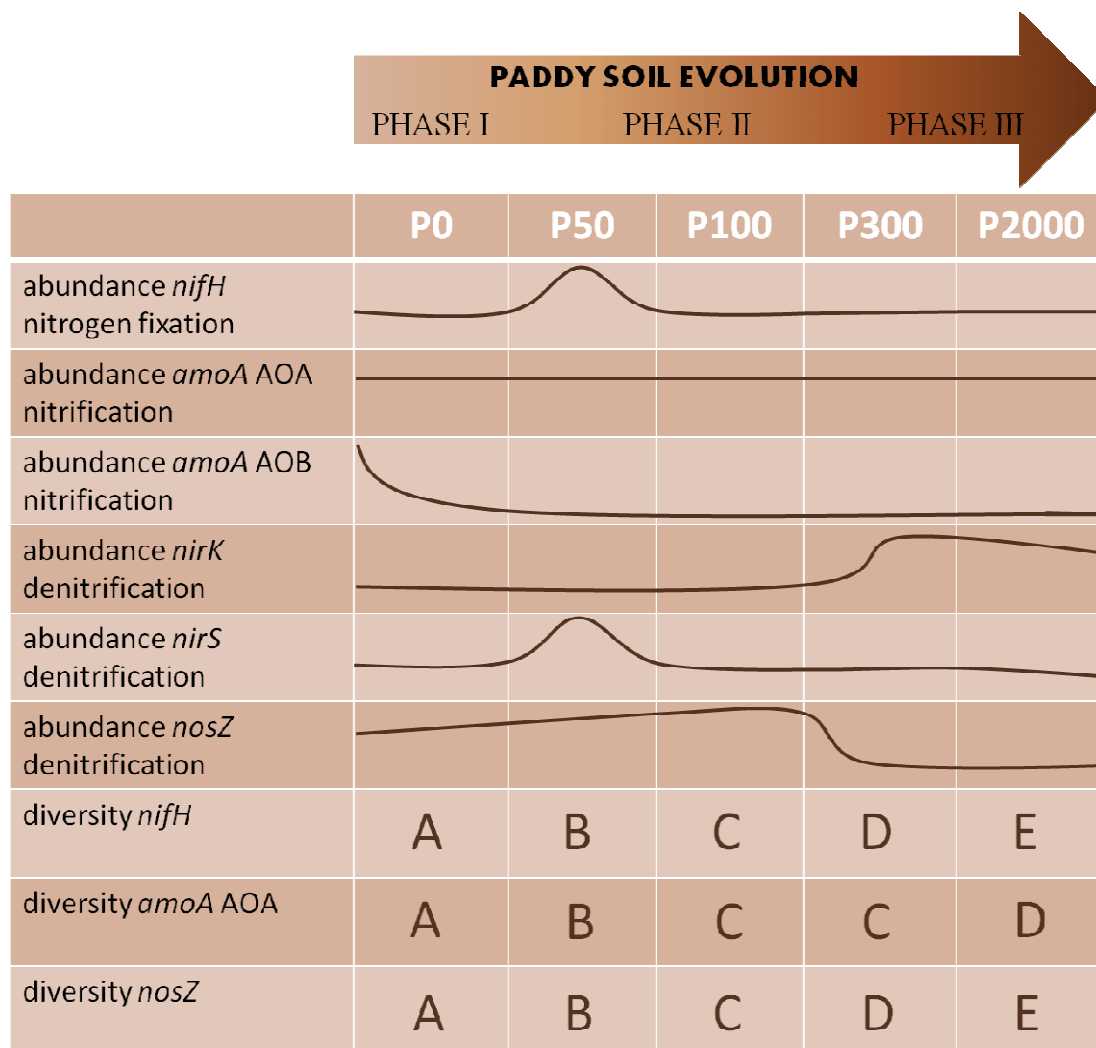
	P0	P50	P100	P300	P2000
abundance *nifH* nitrogen fixation					
abundance *amoA* AOA nitrification					
abundance *amoA* AOB nitrification					
abundance *nirK* denitrification					
abundance *nirS* denitrification					
abundance *nosZ* denitrification					
diversity *nifH*	A	B	C	D	E
diversity *amoA* AOA	A	B	C	C	D
diversity *nosZ*	A	B	C	D	E

Figure 5: **Main phases of paddy soil development, derived from marine sediments. Alterations of microbial community function and structure. Different letters indicate significant differences between paddy soil ages.**

Linear regression analysis with logarithmized age showed that microbial biomass C contents increased significantly with cultivation time of soils throughout the chronosequence and paralleled increasing amounts of extracted DNA. Therefore, we normalized gene copy numbers to the amount of extracted DNA.

The **first phase** (0-50 years) represents the conversion of tidal wetlands into paddy soils and the beginning of agricultural management. This phase is characterized by the change from saltwater to freshwater influenced habitats. While the tidal wetland has a electrical conductivity ($EC_{1:2.5}$) of 4.2 mS cm^{-1}, this drops down to 0.4 mS cm^{-1} in all other sites (Jahn et al. 2011). In the initial paddy soils the impact of rice plants through rhizosphere and litter effects starts. Another important factor influencing soil microbial communities are the

beginning cyclic changes of redox conditions due to regular flooding (anoxic conditions during rice growing period) and subsequent drainage (oxic conditions during/after rice harvesting). Jahn et al. (2011) could also measure that redox potentials showed high spatial variability after flooding, presumably due to oxic microsites within one horizon. P0 is characterized by low contents of organic nitrogen and microbial biomass. Pronounced are changes in the abundance of nitrogen-fixing microbes. *NifH* gene copy numbers were significantly higher in P50 compared to P0 and the other paddy soils. A possible explanation is the influence of the rice plants in the young paddy soils, whereas the impact of plants in the tidal wetland is very low, as there are only a few grasses. Rice plants are competitors for ammonia and nitrate and at the same time donator for bioavailable carbon. This finding is in line with the results of Brankatschk et al. (2010) investigating a soil chronosequence of the Damma glacier forefield. The authors found *nifH* gene abundance reaching its maximum with the first occurrence of plant patches (after 50 years) and not at the very initial sites (after 10 years) with spare vegetation. The lower relative abundance of nitrogen-fixing microbes in P100, P300 and P2000 compared to P50 might be due to ongoing fertilization and associated accumulation effects.

According to the lipid distribution pattern (measured by Cornelia Müller-Niggemann – University Kiel), changes in microbial community structure occurred rapidly over a period of less than 50 years. This was confirmed by T-RFLP profiles, which were significantly different between P0 and the paddy soils for all genes investigated (*nifH*, *amoA* AOA and *nosZ*).

While gene abundances of ammonia-oxidizing archaea (*amoA* AOA) remained unchanged in all phases of paddy soil evolution, *amoA* copy numbers of their bacterial counterparts (*amoA* AOB) showed a significant decrease with the beginning of paddy soil formation and stayed at a constantly low level along the chronosequence. Evidence that root exudates of rice can reduce nitrification rates in soil has been reported recently (Tanaka et al. 2010). Ammonia-oxidizing archaea might be able to respond very fast to the changing environmental conditions (Leininger et al. 2006) and are not inhibited in their abundance by the plants.

Within the **intermediate phase** (100-300 years) changes in diversity pattern (observed from T-RFLP results) were less pronounced. There was, e.g., no significant difference between P100 and P300 for archaeal *amoA* genes, suggesting that the changes of soil microbial structure are slower at this stage. In general, the total number of T-RFs declined along the chronosequence, with exception of *nosZ* genes. In other studies it was also found that managed ecosystems may have less diverse microbial communities compared to their natural counterparts (Torsvik et al. 2002b, Yao et al. 2006). Most of the soil development processes at this phase are related to the dissolution of carbonates. A statistically significant carbonate loss in the topsoils was already observed after 100 years and a complete decalcification of all topsoil horizons within the first 300 years. The complete decalcification of the total soil profile requires almost 700 years of rice cultivation. Due to frequent flooding and drainage this process is significantly accelerated compared to soil development under non-paddy agricultural management. In non-paddy soils only a decalcification of the upper 20 cm was observed after 700 years (Wissing et al. 2011). Decalcification leads to a decrease of pH values. As long as carbonates are present, the $pH_{(KCl)}$ value remains above 7. Without carbonates most paddy topsoils are in the range of 5-6. An influence of pH values was, e.g., described for ammonia oxidizers: It appears in general that AOA ecotypes in the topsoils are more tolerant to low pH values than AOB ecotypes (Erguder et al. 2009). This fits to the significant increase in AOA:AOB ratios (gene abundances) between P50 (10) and P2000 (23).

Within the intermediate phase, there is still an increase of organic carbon in the topsoils (up to 300 years of paddy soil cultivation). Afterwards, oscillating organic carbon concentrations were found between 700 and 2000 years of paddy soil management (Mueller-Niggemann et al. 2011, Wissing et al. 2011). Paddy soils are known to accumulate organic matter. This might be due to the periodic short-term redox cycles induced by changes from flooded to non-flooded conditions, leading to decelerated organic carbon decomposition (Kögel-Knabner et al. 2010, Wu 2011). The increasing organic carbon contents with cultivation time

might be driver for the increasing microbial biomass during paddy soil development (Powlson et al. 1987, Anderson et al. 1989).

Significant changes in gene abundance pattern of denitrification (*nirK* and *nosZ)* occurred only in the **final phase** (300-2000 years) of paddy soil evolution, indicating that it takes at least 300 years to reach typical pattern for these genes. While *nirK* gene copy numbers increased significantly after this time, *nosZ* gene abundances decreased. This might be due to accumulation effects of agricultural management practices. A high availability of nitrate and nitrite favors the more energetic nitrite reduction compared to the reduction of N_2O (Blackmer et al. 1978, Firestone et al. 1979). T-RFLP pattern showed significant shifts for all genes under investigation between P2000 and the other paddy soils. This indicates alterations in the microbial community structure for the main nitrogen-transforming processes and hints that accumulation effects over such a long time period of 2000 years have a great impact on soil microbial community structure. Different soil chemical processes have reached a saturation state in the final phase of paddy soil development like, e.g., organic nitrogen accumulation (**Publication III**).

Soil texture was very similar between all age groups and was strongly dominated by silt-sized particles. The small decrease in clay content (from 16 to 10%) is due to a displacement to deeper soil horizons caused by repeated flooding (Jahn et al. 2011). This shows that all paddy soils of the chronosequence developed from comparable parent materials (tidal wetlands) and under the same ecological conditions. This assumption is supported by the very similar mineral assemblage with comparable total contents of, e.g., Al, Si, Ti and Zr (Jahn et al. 2011). The distribution of n-alkanes, n-fatty acids and n-alcohols of higher plants and algae also showed that the parent material of all investigated sites comes only from Holocene marine and brackish/lagoonal to limnic deposits (Jahn et al. 2011). Thus, although it has been described that soil texture can have a great impact on the diversity of microorganisms (Schutter et al. 2001, Garbeva et al. 2004), an influence of different soil

textures on soil microbial community structure can be largely excluded in our chronosequence.

To sum up, our main hypotheses could be confirmed. We found differences in nitrogen transforming microbial community structure and function along the chronosequence, following changes in soil physical and chemical properties. Thus, soil characteristics related to paddy soil pedogenesis are likely to be important drivers for the observed changes in gene abundance and diversity pattern of soil microorganisms. In different studies investigating glacier forefields, composition and function of microbial communities were also found to be dynamic over hundreds and thousands of years of soil and ecosystem development (Ohtonen et al. 1999, Sigler et al. 2004, Tscherko et al. 2004, Nicol et al. 2005, Brankatschk et al. 2010). Most pronounced were alterations between the tidal wetland and the paddy soils (*Hypothesis I*), but certain functional groups changed only after at least 300 years of paddy soil management, e.g., abundance pattern of the denitrification genes *nirK* and *nosZ* (*Hypothesis II*). During ongoing paddy soil cultivation microbial communities adapt to the changes in soil structure and organic matter quality and specific phylotypes of selected functional groups become dominant. To give an example: *amoA* AOA gene T-RF-163, which only accounted for 14% in the tidal wetland, amounted to three quarters of the total *amoA* community (72%) in the 2000 years cultivated paddy soil. As a continuous development of soil microbial function and diversity was observed, it can be concluded, that changes are caused by the type of management and are not response towards the disturbance of a balanced ecosystem (*Hypothesis III*).

2. Nitrification in the paddy soil chronosequence: ammonia-oxidizing archaea versus ammonia-oxidizing bacteria

For more than 100 years, it was believed that bacteria were the only group responsible for the oxidation of ammonia. In 2004 a unique ammonia monooxygenase gene was detected on an archaeal-associated scaffold from samples of the Sargasso Sea (Venter et al. 2004). However, in the paddy soil chronosequence as well as in several other studies investigating

paddy soils (Briones et al. 2002, Chen et al. 2008, Chen et al. 2011) a higher abundance of ammonia-oxidizing archaea compared to their bacterial counterparts was found. *AmoA* AOA gene abundances in the chronosequence ranged between 1.7×10^3 and 5.5×10^3 copies ng^{-1} DNA and showed no significant differences between the tidal wetland and the paddy soils and also within the four paddy soils cultivated for different time periods. Abundance of ammonia-oxidizing bacteria was in general at least one order of magnitude lower than their archaeal counterparts in the paddy soils (9.9×10^1 – 5.1×10^2 copies ng^{-1} DNA). In contrast, in the tidal wetland gene copy numbers of AOA and AOB were comparable (**Publication I**). This indicates that archaea are able to adapt better to changing environmental conditions. A more versatile metabolism for ammonia-oxidizing archaea was also suggested by Leininger et al. (2006). AOA in soils might have the property to live mixotrophic in contrast to AOB, meaning that they are also able to use organic energy sources (Tourna et al. 2011). Furthermore, it was recently hypothesized that AOA have a lower oxygen demand than AOB (Schleper et al. 2010), which would be beneficial in oxygen depleted paddy soils. Based on the genome analysis of *Candidatus* Nitrosopumilus maritimus and the fact that known AOA do not contain a homolog of the bacterial hydroxylamine oxidoreductase, it is thought that the mechanism of ammonia oxidation in AOA may be different from that in AOB. Possibly, ammonia is not oxidized via hydroxylamine such as in AOB, but via nitroxyl to nitrite (Walker et al. 2010). Another possible explanation for the higher abundance of AOA compared to AOB in the paddy soil chronosequence is the relatively low content of bio-available ammonium (between 0.42 and 27 µg N g^{-1} dw). This indicates a rapid immobilization of N_{min} to organic and volatile N forms in flooded soils (Olk et al. 1996, Pande et al. 2003, Pan et al. 2009). Adaptation of AOA to low ammonium concentrations has been reported (Hatzenpichler et al. 2008, Erguder et al. 2009). In contrast, minimum total ammonia concentrations required for growth of cultured AOB are 100-fold higher than for AOA (Martens-Habbena et al. 2009, Koper et al. 2010).

Although *amoA* AOA gene abundance pattern remained constant along the whole paddy soil chronosequence, T-RFLP profiles showed drastic changes in the community composition.

Comparing dominant T-RFs of P50 and P2000: only 3 T-RFs can be found in both paddy soils (T-RF-74; T-RF-163 and T-RF-254) – in completely different percentages of the total community. Thus, there might be functional redundancy. This means, that different species perform the same functional role in ecosystems, here ammonia oxidation, and thus, changes in species diversity do not affect ecosystem functioning (Loreau 2004).

It was recently postulated that ammonia-oxidizing archaea belong to the deep-branching new archaeal phylum Thaumarchaeota and not to the Crenarchaeota as thought before (Brochier-Armanet et al. 2008, Spang et al. 2010). So far, it is not clear if all Thaumarchaeota have the capability to perform ammonia oxidation (Pester et al. 2011).

There are indications that Thaumarchaeota might contain a specific membrane lipid: the glycerol dialkyl glycerol tetraether lipid crenarchaeol. It was recently identified as a component of the lipid membranes of three cultivated AOAs: *Candidatus* Nitrosopumilus maritimus (Schouten et al. 2008), *Candidatus* Nitrosocaldus yellowstonii (De La Torre et al. 2008) and *Candidatus* Nitrososphaera gargensis (Pitcher et al. 2010). So far no crenarchaeote or any other archaeon or bacterium different from Thaumarchaeota has been shown to harbor crenarchaeol (Spang et al. 2010).

The assumption that crenarchaeol might be a good biomarker for AOA was supported by the study of Leininger et al. (2006): The authors could proof a constant ratio between gene copy numbers of archaeal ammonia monooxygenase genes (*amoA*) and crenarchaeol for many different upland soils. Surprisingly, calculated ratios for the chronosequence varied. They were significantly higher in the paddy soils compared to the tidal wetland and in general higher as in upland soils (**Publication II**). Thus, our *Hypothesis IV* had to be discarded. The results lead to the assumption that archeal ammonia oxidizers different from crenarchaeol-containing Thaumarchaeota may play an important role in paddy soils. This might be due to (I) Thaumarchaeota exhibiting no crenarcheaol or (II) another archaeal phylum different from Thaumarchaeota containing the *amoA* AOA gene. But the question remains if the higher ratio of archaeal *amoA* copy numbers to crenarchaeol in the paddy soils compared to the tidal wetland is really due to a different community structure with more ammonia-oxidizing

archaea containing no crenarcheaol in the paddy soils. As lipids are phenotypic marker, their synthesis depends on the current environmental conditions. Thus, another possible explanation would be that there is a lower expression of crenarchaeol in the paddy soils due to so far unclear reasons. It could also be that there is a better conservation of fossil lipids in P0 and a lower input in the paddy soils. Only further molecular studies, including metagenomic tools could give a better insight which organisms are involved in archaeal ammonia oxidation in flooded soils.

3. Microbial community analysis in flooded soils: snapshot versus time-integrated markers

The characterization of microbial communities can be based on genotypic and phenotypic criteria. In this Ph.D. thesis we compared results of our nucleic acid-based approaches, detecting copy numbers of the archaeal *amoA* gene with phenotypic fingerprinting methods, measuring the glycerol dialkyl glycerol tetraether lipid crenarchaeol (**Publication II**). Only genetic methods require the amplification of target sequences, resulting in the typical "end point" PCR biases (see chapter 3 of the Introduction part). But it is possible to get a high taxonomic resolution with these methods in comparison to phenotypic approaches (Ramsey et al. 2006). Therefore, it seems likely that it is favorable to use both methods in combination. But the interpretation of a comparison of data is not trivial, as the stability of the different markers in flooded soils might be very different. The analysis of core glycerol dialkyl glycerol tetraether lipids like crenarchaeol provides a time-integrated view, as these markers can be stable from decades to millennia (Kuypers et al. 2001). In contrast, it has been reported that nucleases rapidly hydrolyze free DNA added to soil (Romanowski et al. 1992, Widmer et al. 1996). Thus, DNA extraction and subsequent molecular analysis rather reflect the recent microbial community at the point in time of sampling. However, soils are chemically complex and contain surface-reactive particles (clay, sand, silt and humic substances) which can adsorb nucleic acids (Ranjard et al. 2001, Pietramellara et al. 2009). It has been estimated that between 0.03 µg (Selenska et al. 1992) to 1 µg (Ogram et al. 1987) of DNA per gram of

soil is present in an extracellular form. But this is only a small part, as most of the extracellular DNA released after cell lysis is degraded within hours to weeks (Nielsen et al. 2007).

Another phenotypic marker was examined in **Publication III**: amino sugar residues. There was no comparison made with genetic markers, but with microbial biomass N (N_{mic}), measured by chloroform fumigation extraction. This method detects only living cells (Jörgensen and Brookes 1991). So it represents a snapshot of the current microbial community. It has further been reported that N_{mic} responds quickly to short term environmental changes (Chantigny et al. 1996, Bai et al. 2000). Thus, the ratio of both markers could give an insight in stability and accumulation of amino sugar residues. These markers have been shown to survive their producers (Glaser et al. 2006) and to have a turn-over time in the range of a few years, at least for well aerated soils (Derrien et al. 2011). While amino sugar-N was initially in the same order of magnitude as N_{mic}, with ongoing paddy soil cultivation the amino sugar-N contents exceeded those of N_{mic}. The calculated maximum of the ratio was reached after around 190 years of paddy management, however, with great variability (Figure 4 in **Publication III**). This suggests that at a certain size parts of the microbial residue pool have become bioavailable and have been metabolized. Other studies also showed that the contents of steady-state amino sugar-N did usually not exceed the amount of microbial biomass N by more than a factor of 3 (Kandeler et al. 2000, Joergensen et al. 2010).

To summarize: Concerning the comparison of different biomarkers for microbial communities, not only the different taxonomic resolutions have to be taken into account but also the different persistences in soils need to be considered. The latter might be dependent on different factors like, e.g., soil type or management practices. Even a differential degradation of free lipids within one soil, depending on the fraction size has been reported (Quenea et al. 2004). Further, anaerobic conditions in flooded soils might lead to a better conservation of free biomarkers compared to aerated soils.

4. Methodological challenges and future perspectives

The biggest challenge in the chronosequence approach was the comparability of the different fields, concerning between-site differences that are not related to soil age like, e.g., fertilization, crop rotation or water regimes. Although a similar management has been performed on all sites with the same rice cultivars and one upland crop per year in winter, results are difficult to interpret. High variability due to fluctuating conditions and the complexity of natural soil ecosystems are a common problem of field studies (Eller et al. 2005). A statistical comparison of intra- versus inter-site specific spatial variability for different biochemical factors was conducted (**Publication IV**). Five field replicates in a distance of 100 m^2 were sampled, each composited of seven subsamples. Stable parameters investigated were, e.g., total organic carbon and total nitrogen; fast cycling parameters investigated were microbial biomass C and N, nitrate, ammonium, dissolved organic C and N. Although the variation of CV (coefficient of variation) values observed for labile parameters as microbial biomass C and N was with 20-40% higher than CV values for more stable parameters (10-20%), the intrinsic heterogeneity per field was smaller than between study sites for all parameters (tested with the Kruskal-Wallis test, cluster analysis and principal component analysis). This confirms our *Hypothesis V* and indicates that it is possible to discuss long-term evolutionary trends of organic and minerogenic components along the investigated chronosequence. The results further implicate that the five field replicates sampled for the statistical analysis as well as the molecular analysis of the chronosequence might be sufficient also for fast cycling parameters. In general, a higher sampling frequency for labile compared to more stable parameters is needed. However, it is not clear if these results can be transferred to molecular data characterizing microbial communities, as functional groups might behave different from the total microbial biomass.

To get a better understanding of differences in nitrogen fluxes observed in the field, a greenhouse experiment under defined conditions was carried out, comparing the 50 and the 2000 years cultivated paddy soil (collaboration with Adrian Ho and Peter Frenzel, MPI Marburg). Abundance pattern of functional groups of microbes involved in nitrogen cycling

will be analyzed using high throughput sequencing. As it was planned to measure gene expression (mRNA) in addition to the genetic potential (DNA), we developed an improved protocol for simultaneous extraction of DNA and RNA from soil using phenol-chloroform with subsequent column-based separation of DNA and RNA. This was compared to the well established protocol published by Griffith et al. (2000) (**Publication V**). The Griffith protocol resulted in high standard deviations for RNA yield and post-extraction analysis measuring the transcript copy numbers of *nosZ*. In contrast, our new developed protocol revealed better reproducible results and seems therefore more suitable comparing the 50 and 2000 years paddy soils, which contain very different amounts of nucleic acids (140 and 1100 µg g^{-1} dw, respectively). The final comparison of results from the chronosequence approach in the field and the greenhouse study will give a better insight in nitrogen-transforming processes in paddy soils and a closer link to activity and turnover rates.

However, our results cannot be generalized to different types of land-use changes like, e.g., from upland soils into paddy soils, which is a frequent practice in many regions of Asia different from the Yangtze River Delta. These soils undergo further terrestrial soil formation, before paddy cultivation starts, while in the case of tidal wetland conversion point zero of paddy soil formation (besides desalinization) is identical with point zero of terrestrial soil formation. The conversion of tidal wetlands has some special characteristics: (I) it is a conversion of an already flooded soil into a paddy, thus microbial communities already tolerate anaerobic conditions and (II) tidal wetlands are characterized by relatively high salt contents. Both parameters might have influenced the obtained results about diversity and abundance of functional groups involved in nitrogen turnover. It is further unclear if results can be transferred to other microbial groups. For nitrogen turnover, investigated in this thesis, we studied mainly the metabolization of ammonia and nitrate, which are easy degradable (Myrold et al. 1986, Davidson et al. 1992, Stark et al. 1997). Dynamics of microbial communities involved in degradation of more persistent polymeric substances (Fewson 1988), e.g., cellulose degradation and protein mineralization could be completely different. The degradation potential for cellulose and proteins is correlated to the formation of

stable organic matter (Paul 1984). So, in contrast to nitrifiers and denittrifiers, which respond very soon to land-use changes, alterations of microbial communities involved in degradation of polymers might be observed only later in paddy soil development. Therefore, it would be interesting to compare results from tidal wetland conversion to paddy soil development from upland soils as well as development of nitrogen transforming microorganisms to development of microbial communities involved in polymer degradation.

Conclusions

Paddy soil chronosequences derived from tidal wetlands through successive sea dyke building are a good model to study effects of land-use conversion and long-time effects of agricultural management on soil microbial community structure and function. Nitrogen is an interesting study object, as paddy fields are usually nitrogen deficient. Using q-PCR and T-RFLP as molecular techniques, it was possible to follow changes in abundance and diversity of functional groups. These results could be linked to pedological data along the chronosequence, showing that developing microbial communities reflected the different chemical and physical properties of the soils, which changed in terms of soil development. As alterations in microbial community structure and function occurred at very different points in time along the chronosequence, it was possible to outline three phases of paddy soil development:

(I)   the change from tidal wetlands to paddy soils: Young paddy soils were characterized by high abundances of *nifH* genes and declining abundances of bacterial ammonia oxidizers, whereas denitrification gene abundances remained constant. Diversity pattern of all genes investigated changed significantly.

(II)   Ongoing paddy soil cultivation showed a slower alteration of the soil microbial communities, while

(III)   long-term cultivation led to again significant differences in diversity pattern (for all genes investigated) and changes in abundances of *nirK* and *nosZ* genes (denitrification). This is possibly due to accumulation effects (**Publication I**).

We could also observe that gene copy numbers of ammonia-oxidizing archaea, which dominated over their bacterial counterparts in the paddy soils, remained constant along the whole chronosequence. In contrast, the lipid marker for Thaumarchaeota, to which ammonia-oxidizing archaea belong, declined. Surprisingly, this indicated that ammonia-oxidizing archaea different from crenarchaeol-containing Thaumarchaeota might play an important role in paddy soils (**Publication II**).

Summarizing diversity analyses: During ongoing paddy soil cultivation specific phylotypes of selected functional groups became dominant leading to the conclusion that they are able to adapt to the changes in soil structure and organic matter quality. The continuous development of soil microbial function and diversity indicates that these changes are rather caused by the type of management and are not response towards the disturbance of a balanced ecosystem.

Furthermore, analysis of the high throughput sequencing approach of samples from our greenhouse experiment will help to get a better understanding of nitrogen transformations in the field.

However, our data are only related to land-use changes from an already flooded, natural ecosystem (tidal wetland) to cultivated paddy soils and hence cannot be generalized to, e.g., conversion of upland soils to flooded rice fields, which is performed in many other regions of Asia. For future experiments it would be interesting to check if our data are transferable to other forms of land-use change.

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List of abbreviations

a	year
Al	aluminum
amoA	gene encoding the α-subunit of the ammonia monooxygenase
Anammox	anaerobic ammonia oxidation
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
ATP	adenosine triphosphate
C	carbon
CH_4	methane
C_{mic}	microbial carbon
CO_2	carbon dioxide
Ct	threshold cycle
CV	coefficient of variance
DGGE	denaturing gradient gel electrophoresis
DNA	desoxyribonucelic acid
DNRA	dissimilatory nitrate reduction to ammonia
dw	dry weight
E_0	standard reduction potential
EC	electrical conductivity
et al.	et alii
e.g.	for example, *exempli gratia*
KCl	potassium chloride
mRNA	messenger ribonucleic acid
N	nitrogen
N_2	dinitrogen
$NAD(P)^+/NAD(P)H$	nicotinamide adenine dinucleotide (phosphate)
NH_3	ammonia
NH_4^+	ammonium
nifH	gene encoding the dinitrogenase reductase subunit of the nitrogenase
nirK	gene encoding the copper containing nitrite reductase
nirS	gene encoding the cytochrome cd_1-nitrite reductase

N$_{mic}$	microbial nitrogen
NO	nitric oxide
N$_2$O	nitrous oxide
NO$_3^-$	nitrate
NO$_2^-$	nitrite
nosZ	gene encoding the nitrous oxide reductase
O$_2$	dioxygen
P0	tidal wetland, day 0 of paddy soil chronosequence
P50	50 years cultivated paddy soil
P100	100 years cultivated paddy soil
P300	300 years cultivated paddy soil
P2000	2000 years cultivated paddy soil
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
Si	silicium
SO$_4^{2-}$	sulfate
Ti	titanium
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
Zr	zirconium

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Publication I

Changes in Diversity and Functional Gene Abundances of Microbial Communities Involved in Nitrogen Fixation, Nitrification, and Denitrification in a Tidal Wetland versus Paddy Soils Cultivated for Different Time Periods[▽]

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In many areas of China, tidal wetlands have been converted into agricultural land for rice cultivation. However, the consequences of land use changes for soil microbial communities are poorly understood. Therefore, we investigated bacterial and archaeal communities involved in inorganic nitrogen turnover (nitrogen fixation, nitrification, and denitrification) based on abundances and relative species richness of the corresponding functional genes along a soil chronosequence ranging between 50 and 2,000 years of paddy soil management compared to findings for a tidal wetland. Changes in abundance and diversity of the functional groups could be observed, reflecting the different chemical and physical properties of the soils, which changed in terms of soil development. The tidal wetland was characterized by a low microbial biomass and relatively high abundances of ammonia-oxidizing microbes. Conversion of the tidal wetlands into paddy soils was followed by a significant increase in microbial biomass. Fifty years of paddy management resulted in a higher abundance of nitrogen-fixing microbes than was found in the tidal wetland, whereas dominant genes of nitrification and denitrification in the paddy soils showed no differences. With ongoing rice cultivation, copy numbers of archaeal ammonia oxidizers did not change, while that of their bacterial counterparts declined. The *nirK* gene, coding for nitrite reductase, increased with rice cultivation time and dominated its functionally redundant counterpart, *nirS*, at all sites under investigation. Relative species richness showed significant differences between all soils with the exception of the archaeal ammonia oxidizers in the paddy soils cultivated for 100 and 300 years. In general, changes in diversity patterns were more pronounced than those in functional gene abundances.

The influence of different types of changes in land use on microbial communities has been studied with growing interest in recent years due to the importance of soil microbes in geochemical nutrient turnover and soil health. This includes studies of the conversion of grassland to forest (6), changes in tillage management (4, 11, 38), and shifts in the intensity of land use in both agricultural (17) and forest (5, 19) ecosystems. Some articles have also addressed paddy ecosystems in particular and effects of temporary upland conversion (14). All these studies have shown significant shifts in the microbial community structure and function as a consequence of land use changes. However, since in most cases only effects shortly after land use changes were studied, it is not clear if the related changes were caused by the type of management or rather

reflected a response of microbial communities toward the disturbance of a balanced ecosystem. To address this question, we compared four paddy soils that have been cultivated with rice for different time periods (50 to 2,000 years) with a tidal wetland, which typically represents the parent material for agricultural land reclamation in China (39).

Most studies published so far concerning the effects of land use changes on soil microbial communities have focused on alterations related to phylogenetic groups based on 16S rRNA sequences or on one functional marker. In modern rice cultivation, however, the yield-limiting factor is the availability of nitrogen (N) due to large losses in flooded soils (9, 18). Therefore, the objective of this study was to reconstruct functional microbial communities that are involved in key processes of the inorganic nitrogen cycle and to link these results to abiotic and biotic properties of the different soils of the chronosequence. We investigated functional gene abundances of bacteria and archaea performing nitrogen fixation, nitrification, and denitrification by quantifying genes encoding subunits of the

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TABLE 1. Characterization of the 5 examined soils[a]

Soil parameter	Value(s) for soil				
	P0	P50	P100	P300	P2000
Soil texture (% sand, silt, clay)	7.4, 80.4, 12.2	0.4, 83.6, 16.0	2.0, 81.2, 16.8	3.4, 81.2, 15.4	4.0, 85.1, 10.9
pH	8.1 (0.13) a	7.6 (0.08) b	7.6 (0.13) b	7.5 (0.08) b	7.3 (0.10) c
TOC (%)	0.58 (0.17) a	1.7 (0.14) b	1.7 (0.16) b	2.5 (0.16) c	3.1 (0.11) d
TN (%)	0.060 (0,012) a	0.17 (0.014) b	0.19 (0.015) c	0.27 (0.020) d	0.36 (0.019) e
WEOC (μg g^{-1} dw)	9.5 (2.8) a	17 (5.4) b	18 (11) ab	21 (8.3) ab	14 (5.3) ab
WEON (μg g^{-1} dw)	0.43 (0.19) a	0.46 (0.77) a	5.5 (6.6) a	BDL[b]	0.36 (0.60) a
Nitrate (μg N g^{-1} dw)	2.1 (0.70) a	12 (1.2) ab	8.3 (2.9) ab	16 (6.5) b	2.2 (1.7) a
Ammonium (μg N g^{-1} dw)	0.42 (0.12) a	6.0 (3.5) a	25 (19) a	27 (30) a	22 (11) a
Cmic (μg g^{-1} dw)	150 (58) a	720 (120) b	1,000 (330) b	1,800 (780) b	5,100 (1,300) c
Nmic (μg g^{-1} dry wt)	39 (6.8) a	28 (18) a	110 (55) ab	92 (36) ab	150 (30) b
DNA content (μg g^{-1} dry wt)	140 (35) a	760 (24) bc	630 (120) b	810 (220) bc	1,100 (140) c

[a] Soils (tidal wetland and paddy soils cultivated for 50, 100, 300, and 2,000 years) at 0- to 20-cm depth were analyzed using different parameters: soil texture, pH value (CaCl$_2$), total organic carbon (TOC), and total nitrogen (TN), water extractable organic carbon (WEOC) and nitrogen (WEON), nitrate and ammonium concentrations, microbial biomass C and N, and DNA content. Standard deviations are given in parentheses ($n = 5$). Significant differences are indicated by different letters.
[b] Below detection limit of 0.2 μg g^{-1} soil dry weight.

nitrogenase (nifH), ammonia monooxygenase (amoA), nitrite reductase (nirK and nirS), and nitrous oxide reductase (nosZ). Since nifH, archaeal amoA, and nosZ genes turned out to be highly abundant and may therefore greatly contribute to the respective turnover processes, these genes were chosen for diversity analysis using terminal restriction fragment length polymorphism analysis (T-RFLP). For sampling, the beginning of the vegetation period was chosen to make the paddy soils more comparable with the tidal wetland, since at this time point agricultural activities like fertilizer or pesticide application, as well as the rhizosphere effect of rice plants, have been considered low.

During paddy soil evolution, different chemical and physical soil properties may influence soil microbial communities involved in nitrogen turnover. Directly after conversion of tidal wetlands into paddy soils, microbial communities might respond mainly to the leaching of marine salts by rainfall and the beginning of agricultural management. With ongoing paddy soil cultivation and continuous input of carbon and nitrogen to the system, microbes may adapt to the new conditions and become key players in certain functional traits. Long-term cultivation of rice with repeated tillage management fertilization and pesticide application, as well as effects by the plants (rhizosphere and litter), may strongly influence soil microbial communities and lead to a general increase in abundance and activity due to accumulation effects. Thus, we postulate that differences in microbial community structure and function along the chronosequence will be found.

MATERIALS AND METHODS

Site description and soil sampling. The study sites are located in the area of Cixi, Zhejiang Province, People's Republic of China, a subtropical monsoon area with a mean annual temperature of 16.3°C and precipitation of 1,325 mm (40). We sampled a chronosequence of four paddy soils that have been under rice cultivation for approximately 50, 100, 300, and 2,000 years (P50, P100, P300, and P2000), as well as a tidal wetland (P0), which represents the parent material of land reclamation for agricultural use by sea dike building. The coordinates of the sampled sites are as follows: P0, 30°19′N, 121°09′E; P50, 30°11′N, 121°22′E; P100, 30°09′N, 121°21′E; P300, 30°06′N, 121°31′E; P2000, 30°05′N, 121°27′E. All five sites under investigation are located within a 40-km area.

The duration of rice cultivation at the respective sites was estimated according to well-documented points in time of sea dike construction (Cixi County Annals, abstracted information in Chinese, available at www.cixi.gov.cn), summarized by

Cheng et al. (12). Thus, all paddy soils developed from comparable parent materials (tidal wetlands) and under the same ecological conditions. This is supported by the very similar textures among all age groups, which are strongly dominated by silt-sized particles (Table 1) and by a very similar mineral assemblage with comparable total contents, e.g., Al, Si, Ti, and Zr (data not shown). The small decrease in clay content (from 16 to 10%) is due to a displacement to deeper soil horizons caused by repeated flooding.

Since all fields are located in the same region and the agricultural management has been centrally controlled in China since 1949 by instructions of the Technical Service Bureau, comparable management has been performed for all sites. At all fields, rice was cultivated one time per year in summer using the same rice cultivars and an upland crop was cultivated one time per year in winter. Types, rates, and methods of application of fertilizers and pesticides were similar between the fields. The freshwater for flooding was not completely salt free but originated from the same river for all investigated sites, with comparable flooding regimes among the paddy soils driven by the interest for optimal crop yields (Zhihong Cao, Chinese Academy of Sciences, Nanjing, personal communication). All paddy fields are at least 10 km away from the sea; thus, direct flooding with marine water cannot occur. The topsoils are not influenced by groundwater due to the morphology of the soils. Only in the first years of reclamation might there have been an influence of salt being washed into freshwaterways, but this is part of the soil development and the shift from saltwater to freshwater conditions.

Soils were sampled at the beginning of July 2009, shortly after flooding. Rice plants on all paddy fields were at similar and early development stages (visual observation). Five independent field replicates in an area of 120 m^2 were taken at each site with a soil auger from a 0- to 20-cm depth, each replicate being composed of seven individual soil cores (taken in a distance of 1 m^2), which were pooled and homogenized to reduce heterogeneity. Aliquots (5 g) for DNA extraction were shock frozen in liquid nitrogen directly after sampling and stored at −80°C, whereas the remaining soil was stored at 4°C and analyzed in the following 2 weeks.

Soil physical and chemical properties. Soils were extracted with 0.01 M CaCl$_2$ at a soil-to-liquid ratio of 1:3. For determination of water extractable organic carbon (WEOC) and nitrogen (WEON), a total organic carbon analyzer, the Dimatoc 100 (Dimatec Analysentechnik GmbH, Germany), was used. Determination of ammonium (NH$_4$$^+$-N) and nitrate (NO$_3$$^-$-N) concentrations was done by continuous flow analysis with a photometric autoanalyzer (CFA-SAN Plus; Skalar Analytik, Germany). For the determination of microbial biomass carbon (Cmic) and nitrogen (Nmic), aliquots of the soils were fumigated with chloroform for 24 h prior to CaCl$_2$ extraction (23). To measure total organic C (TOC) and N (TN), soil samples were frozen at −25°C and then lyophilized (FinnAqua Lyovac GT2; AMSCO Finn-Aqua GmbH, Germany). Homogenized samples were subjected to elemental analysis using an Elementar Vario EL III instrument in combustion mode. Carbonate carbon was determined using a total inorganic bound carbon (TIC) module attached to the Elementar analyzer, whereby carbonate was released from soils in a reaction chamber after addition of HCl and purged into the machine by a helium stream.

DNA extraction. DNA of each of the 25 samples (5 sites × 5 replicates) was extracted in three subsamples from 0.5 g of soil with the FastDNA Spin kit for

TABLE 2. Thermal profiles and primers used for real-time PCR quantification of different functional genes[a]

Target gene	Source of standard (reference)	Thermal profile	No. of cycles	Primers (reference)	Primer concn (10 μM)	DMSO[c]
nifH	*Sinorhizobium meliloti*	95°C, 45 s; 55°C, 45 s; 72°C, 45 s	40	nifHF (30), nifHR (30)	0.3	
amoA AOA	Fosmid clone 54d9 (37)	94°C, 45 s; 55°C, 45 s; 72°C, 45 s	40	amo19F (25), CrenamoA616r48x (32)	0.5	
amoA AOB	*Nitrosomonas* sp.	94°C, 45 s; 58°C, 45 s; 72°C, 45 s	40	amoA1F (31), amoA2R (31)	0.75	
nirK	*Azospirillum irakense*	95°C, 15 s; 63-58°C, 30 s; 72°C, 30 s	5[b]	nirK876 (20), nirK5R (7)	0.5	0.625
		95°C, 15 s; 58°C, 30 s; 72°C, 30 s	40			
nirS	*Pseudomonas stutzeri*	95°C, 45 s; 57°C, 45 s; 72°C, 45 s	40	cd3aF (26), R3cd (36)	0.5	0.625
nosZ	*Pseudomonas fluorescens*	95°C, 30 s; 65-60°C, 30 s; 72°C, 30 s	5[b]	nosZ2F (21), nosZ2R (21)	0.5	
		95°C, 15 s; 60°C, 15 s; 72°C, 30 s	40			

[a] PCR mixtures consisted of Power Sybr green master mix (12.5 μl), BSA (3%, 0.5 μl), and template (2 μl, 0.5 to 2.6 ng μl^{-1}), as well as primer and DMSO, as referenced in the table (in μl).
[b] Touchdown: -1°C cycle^{-1}.
[c] DMSO, dimethyl sulfoxide.

soil (MP Biomedicals) according to the protocol of the manufacturer. Quality and quantity of the DNA extracts were checked with a spectrophotometer (Nanodrop; PeqLab, Germany). Quantities of extracted DNA were very similar for all subsamples from one replicate, with an average variation of 15.6%. They were pooled and stored at -20°C until use.

Real-time PCR assay. Quantitative real-time PCR was carried out on a 7300 real-time PCR system (Applied Biosystems, Germany) using SYBR green as a fluorescent dye. The respective reaction mixtures were composed as shown in Table 2.

Dilution series of the different DNA extracts were tested in a preexperiment with all soils to avoid inhibition of PCR, e.g., by coextracted humic substances. DNA extract dilutions of 1:128 turned out to be best suited (data not shown) and were used in three subsamples for the experiment. As standards, serial plasmid dilutions of the respective functional genes ranging from 10^1 to 10^7 gene copies μl^{-1} were used (sources of standards are shown in Table 2).

All PCR runs started with an initial enzyme activation step performed at 95°C for 10 min. The subsequent thermal profile was different for each gene, as shown in Table 2, followed by a melting curve, consisting of 95°C for 15 s, 60°C for 30 s, and a subsequent temperature increase until 95°C with a ramp rate of 0.03°C s^{-1}.

Specificity of the amplified products was checked by the observation of a single melting peak and the presence of a unique band of the expected size in a 2% agarose gel stained with ethidium bromide. PCR efficiencies (Eff) were calculated from the standard curve by the formula Eff $= [10^{(-1/\text{slope})} - 1] \times 100\%$ and accounted for 91.8 to 93.6% for *nifH* genes, 94.1 to 98.1% for archaeal *amoA* genes, 83.1 to 83.5% for bacterial *amoA* genes, 90.1 to 90.8% for *nirK* genes, 90.1 to 98.2% for *nirS* genes, and 93.7 to 97.2% for *nosZ* genes.

T-RFLP. Diversity analysis by terminal restriction fragment length polymorphism analysis (T-RFLP) was performed with one functional gene of each examined process, nitrogen fixation (*nifH*), nitrification (*amoA* from ammonia-oxidizing archaea [AOA]), and denitrification (*nosZ*), using the primer pairs described for quantitative real-time PCR (Table 2). The forward primer was labeled with 5′-carboxyfluorescein. PCR amplifications were carried out with each of the 25 DNA samples in triplicate. PCR profiles were as follows: for *nifH*, according to real-time PCR (Table 1); for *amoA* AOA, 30 cycles, 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C; for *nosZ*, touchdown PCR with 5 cycles consisting of 95°C for 15 s, 65°C for 30 s, and 72°C for 30 s, followed by 30 cycles at a 60°C annealing temperature.

The restriction enzymes AatII (*nifH*), MwoI (*amoA* AOA), and HpyCH4V (*nosZ*) were selected based on *in silico* T-RFLPs using the program REPK (restriction endonuclease picker) (15). Digestion was performed according to the manufacturer's protocol.

For T-RFLP migration, 15 ng of digested amplicons were mixed in triplicate with 0.25 μl Genome Lab DNA size standard 600 and 26.75 μl of Genome Lab sample loading solution (Beckman Coulter, Germany) and separated on a capillary electrophoresis sequencer (CEQ 2000 genetic analyzer; Beckman Coulter,

Germany) run with the following program: denaturation for 2 min at 90°C, injection for 30 s at 2,000 V, and separation for 60 min at 4,800 V.

Chromatograms were analyzed with the CEQ 8000 genetic analysis system software (Beckman Coulter) using the quartic model as a size-calling algorithm (according to the Genome Lab fragment analysis protocol), with a slope threshold of 1, a relative peak height threshold of 10% (relative to the second-highest peak), and a confidence level of 95% to identify peaks. Amplified fragment length polymorphism analysis was done with a binning range of 1 bp. T-RFLP profiles of the three technical replicates for each sample were highly similar.

The data set was normalized to the percentage of the total peak height of a sample (2), excluding peaks smaller than 0.5%.

Statistical analysis. Real-time PCR data and soil chemical parameters were subjected to analysis of variance (ANOVA) using the statistical software program SPSS 13.0. Normal distribution of the variables and homogeneity of variances were checked by the Kolmogorov-Smirnov test, box plot analysis, and the Levene test.

To analyze the terminal restriction fragment data, we used between-group analysis (BGA) (16) based on correspondence analysis.

For a global test of any difference between the groups, the between-groups inertia percentage was used as a test statistic. Based on 999 permutations, a P value was calculated. In case of a significant result ($P < 0.05$), we performed tests comparing all pairs of groups; the P values were adjusted for multiple comparisons by the method of Hommel.

All analyses were undertaken with the ADE4 package (13) within the R software environment (www.R-project.org).

RESULTS

Soil chemical properties. TOC increased significantly with cultivation time of soils, from 0.58% in P0 up to 3.1% in P2000. The same trend, with significant differences between all soils, was found for TN (Table 1).

WEOC concentrations were higher in P50 than in P0. For WEON, no significant differences between the soil samples of all sites under investigation were found, with the exception of P300, for which it was below the detection limit. Also, ammonium and nitrate concentrations of the soil samples showed no significant differences between all five sites measured, with the exception of the nitrate concentration in the P300 soil, which was significantly higher than that in P0 and P2000 (Table 1).
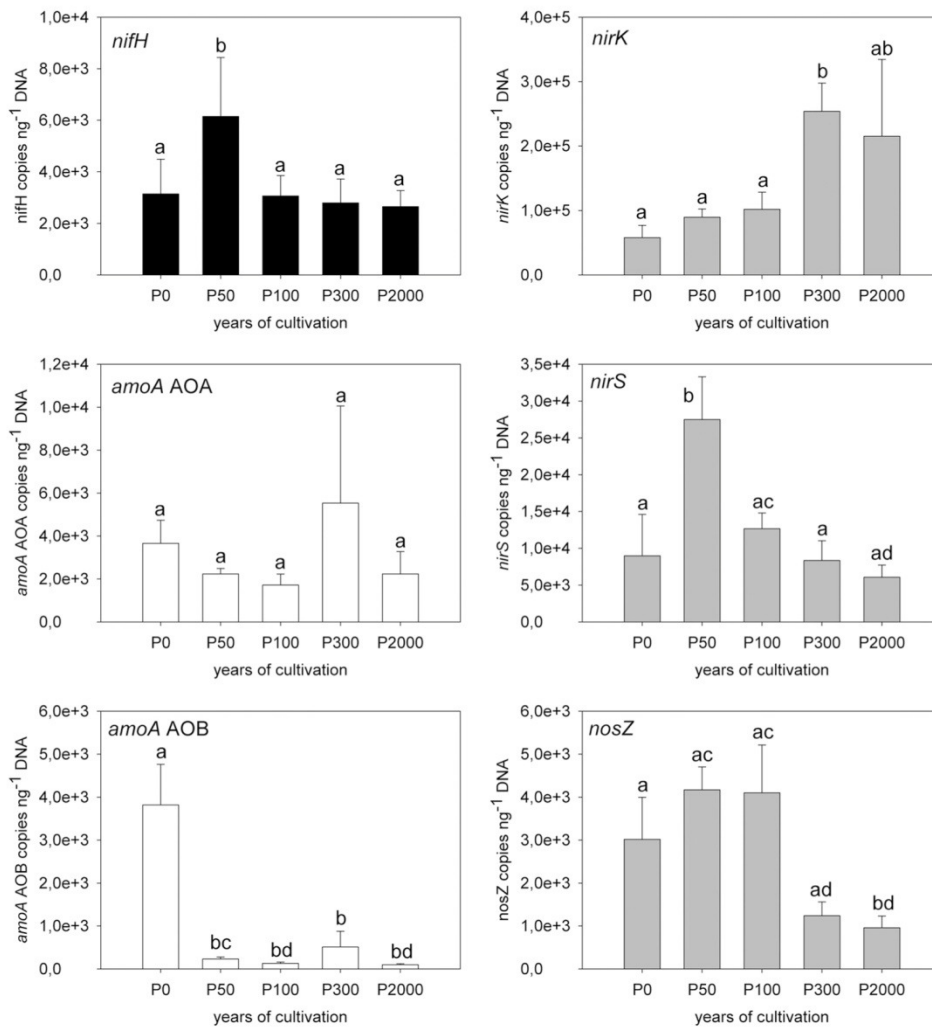
FIG. 1. Copy numbers of the *nifH*, *amoA* AOA and AOB, *nirK*, *nirS*, and *nosZ* genes in the tidal wetland (P0) and the 50-, 100-, 300-, and 2,000-years-cultivated paddy soils ($n = 5$; error bars represent standard deviations). Significant differences are indicated by different letters.

Linear regression analysis with logarithmized age showed that microbial biomass C contents increased highly significantly with cultivation time of soils and paralleled increasing amounts of extracted DNA. The same trend was observed for microbial biomass N, but the difference was significant only between P0 (39 μg g^{-1} soil) and P2000 (150 μg g^{-1} soil) (Table 1).

Quantification of functional genes. Due to the significant increase in microbial biomass from P0 to P2000 (Table 1), we normalized gene copy numbers to the amount of extracted DNA. Gene copy numbers of *nifH* were comparable between all five soils and ranged between 2.7×10^3 and 1.6×10^4 copies ng^{-1} extracted DNA. For soil samples from P50 only, a significantly higher copy number was measured than with the other four soils.

Gene copy numbers of ammonia-oxidizing archaea (*amoA*

AOA) were higher in all samples than those of the bacterial counterpart (ammonia-oxidizing bacteria, *amoA* AOB), with the exception of P0, where values were comparable (for AOA, 3.7×10^3 copies ng^{-1} DNA; for AOB, 3.8×10^3 copies ng^{-1} DNA). AOA/AOB ratios increased significantly from P0 (1) to P2000 (23). For archaeal *amoA* gene copy numbers, no significant difference for all soils investigated was found ($1.7 \times 10^3 - 5.5 \times 10^3$ copies ng^{-1} DNA). In contrast, bacterial *amoA* gene copy numbers in the paddy soils were 9.9×10^1 (P2000) to 5.1×10^2 (P300) copies ng^{-1} DNA and, therefore, significantly lower than those for P0 (Fig. 1).

In general, *nirK* gene copy numbers dominated over *nirS* genes by a factor of 3 to 35 in all soils investigated. *nirK* copy numbers in P300 were significantly higher than those in P0 (2.5×10^5, compared to 5.8×10^4 copies ng^{-1} DNA in P0). The *nirS* gene copy numbers were significantly higher in P50
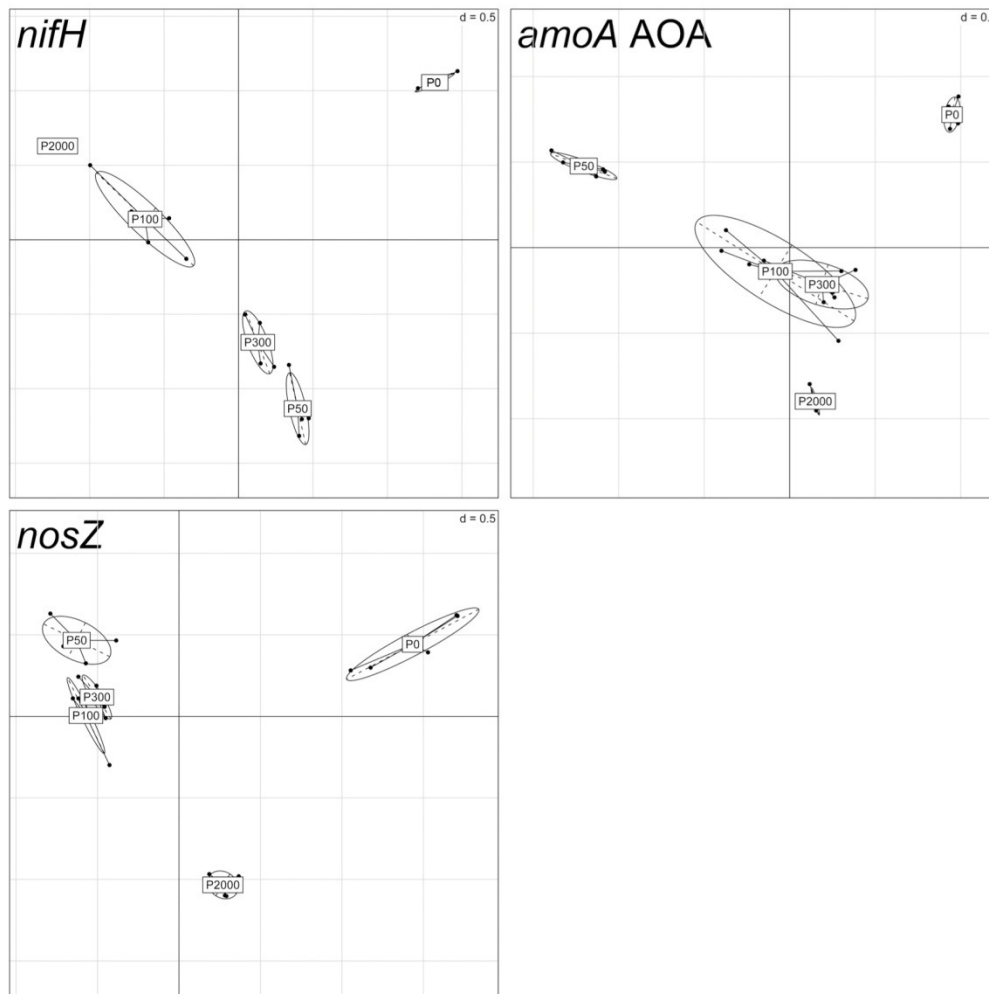
FIG. 2. Between-group analysis based on correspondence analysis of the T-RFLP data set for *nifH*, *amoA* AOA, and *nosZ* gene fragments. The first two axes explain 66.8 to 80.0% of variance. Symbols illustrate the five field replicates for each soil (P0, P50, P100, P300, and P2000). Ellipses surround the 5 replicates for each soil, showing that they cluster together.

than in P0, but there was no significant difference between P0 and the paddy soils different from P50. *nosZ* gene copy numbers were significantly lower in soil samples of P2000 than in those of P0, P50, and P100. Copy numbers for P300 were in between and showed neither a significant difference from those for P2000 nor from those for the other soils. In general, *nosZ* gene copy numbers were in the range of 10^3 copies ng^{-1} extracted DNA. The gene abundance results are summarized in Fig. 1.

Diversity analysis of functional genes. Statistical evaluation of genotype numbers for *nifH*, *nosZ*, and archaeal *amoA* genes by between-group analysis (BGA) showed a clustering according to the different soils for all genes under investigation, with the exception of P50, P100, and P300 for *nosZ*, all of which clustered together (Fig. 2). Also, no significant differences were found between P100 and P300 for *amoA* AOA.

For *nifH*, the numbers of different terminal restriction fragments (T-RFs) in each soil were comparable and ranged between 21 and 23, with the exception of P2000, where only 15 T-RFs were detected and 1 very dominant T-RF was found, accounting for 40% of the total relative community richness (T-RF-171). This T-RF was also found in P100, with 21% of the total relative community richness. Dominant T-RFs in the other soils were different from each other. Statistical analysis comparing pairs of groups revealed significant differences between all five soils (Fig. 3 and Table 3).

T-RFLP analysis of the archaeal *amoA* genes showed a significant decrease in the genotype number from P0 (10) to P2000 (6). Dominant T-RFs in P0 were T-RF-197, -254, and -163, whereas T-RF-197 was unique for this soil. T-RF-163 became more dominant with cultivation time of soils and contributed to 72% of the total *amoA* AOA community in P2000.
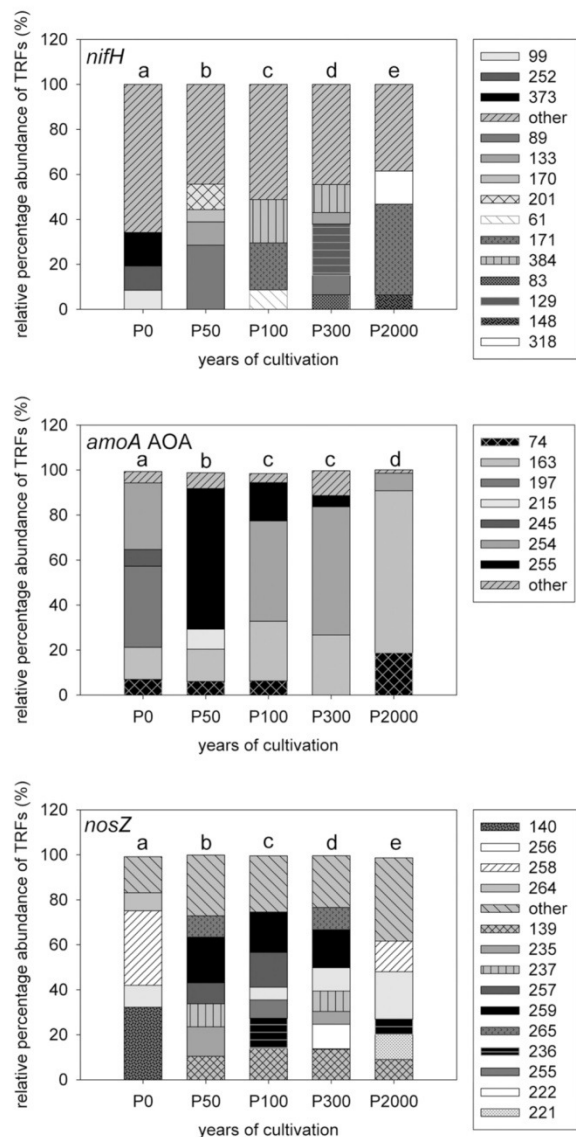
67

FIG. 3. Contributions of major T-RFs to total *nifH*, *amoA* AOA, and *nosZ* gene fragment diversity in the tidal wetland (P0) or 50-, 100-, 300-, or 2,000-year-cultivated paddy soils. T-RFs which contributed to less than 5% were summarized as "other." Significant differences are indicated by different letters.

Other T-RFs, like T-RF-255, were present only in the paddy soils.

For *nosZ*, the total number of T-RFs in all paddy soils ranged between 19 and 21 but was lower in P0 (13). One dominant T-RF, T-RF-259, was found only in P50, P100, and P300. T-RF-139 was detected in all paddy soils but not in P0. The dominant T-RFs in P0 (T-RF-140 and -258; 32 and 33%, respectively) were not detected or were detected only in very small amounts in the paddy soils (1 to 14%) (Fig. 3).

DISCUSSION

Different chemical and physical properties could prove that the soils investigated developed in a chronological way. A significant loss of carbonates during paddy soil formation was found in paddy topsoils by considering the inorganic carbon concentrations (data not shown). A strong decalcification of the upper 20 cm was already observed after 50 years of rice cultivation (from 0.5% to 0.03%), whereas the complete decalcification of the total soil profile required almost 700 years of rice cultivation. In contrast, examination of neighboring upland fields of similar ages after 700 years of (nonpaddy) agricultural management showed only a decalcification of the upper 20 cm (L. Wissing et al., unpublished data). P2000 was completely free of carbonates, which may be a driver for the pH value being significantly lower than those of the other paddy fields (Table 1). These observations emphasize alteration of paddy soils due to the continuous rice paddy cultivation, with carbonates being leached by the periodical flooding and drainage.

The evolution of the paddy soils according to our age estimations was also confirmed by the continuously increasing TOC accumulation in the topsoils with cultivation time, from 1.7% in P50 to 3.1% in P2000, which is a possible driver for the increasing microbial biomass during paddy soil development (3, 29). This increasing TOC content may be caused by paddy soil management and especially by flooding of the fields during rice growth, since under waterlogged conditions, soil organic matter decomposition proceeds at lower rates than in well-drained, aerobic soils (27). Also, total nitrogen content of the soils increased chronologically with time in cultivation (Table 1).

Gene copy numbers and relative genotype richness of functional microbial communities involved in nitrogen transformation showed differences between the five soils investigated (P0,

TABLE 3. *P* values of pairwise comparisons for T-RFLP profiles of the functional genes *nifH*, *amoA* AOA, and *nosZ*, adjusted for multiple comparisons by the method of Hommel[a]

Gene and soil	*P* value for T-RFLP profile comparison				
	P0	P50	P100	P300	P2000
nifH					
P0	1.000	0.008*	0.008*	0.008*	0.008*
P50		1.000	0.008*	0.008*	0.008*
P100			1.000	0.008*	0.008*
P300				1.000	0.008*
P2000					1.000
amoA AOA					
P0	1.000	0.015*	0.014*	0.015*	0.015*
P50		1.000	0.015*	0.015*	0.012*
P100			1.000	0.192	0.015*
P300				1.000	0.012*
P2000					1.000
nosZ					
P0	1.000	0.008*	0.008*	0.008*	0.008*
P50		1.000	0.008*	0.008*	0.008*
P100			1.000	0.008*	0.008*
P300				1.000	0.008*
P2000					1.000

[a] Significant impacts are marked by asterisks ($P < 0.05$).

P50, P100, P300, and P2000). Low competition for free ammonia between plants and microbes in P0 might be a possible explanation for the high abundance of ammonia-oxidizing microbes in relation to the overall microbial biomass at this stage (significantly higher relative copy numbers of *amoA* AOB than were found in all paddy soils under investigation and comparable amounts for *amoA* AOA). Competition between plants and microbes for free ammonia has been shown in different studies (24, 33). Tanaka et al. (35) recently reported evidence that root exudates of rice can reduce nitrification rates in soil.

Gene copy numbers of archaeal ammonia oxidizers were constant at all phases of paddy soil development, indicating that they are able to adapt to changing environmental conditions (25). Another possible explanation, namely, that AOA just do not respond to these changes, can be excluded from the significantly changing T-RFLP pattern. A dominance of archaeal ammonia oxidizers over their bacterial counterparts in paddy soils has also been found in previous studies (8, 10).

Concerning alterations in the diversity pattern during paddy soil development, clear shifts in relative genotype richness for all genes under investigation (*nifH*, *amoA* AOA, and *nosZ*) were observed between P0 and the paddy soils. It could be assumed that microorganisms respond to the development from saltwater- to freshwater-influenced habitats at this first phase of paddy soil evolution. An influence of salinity on archaeal *amoA* community composition, for example, was suggested by Abell et al. (1).

After the conversion of the tidal wetland into paddy soils and the beginning of agricultural management, the high proportion of nitrogen-fixing microbes in P50 was very pronounced. In contrast, relative abundances of the dominant genes of nitrification (*amoA* AOA) and denitrification (*nirK*) in the paddy soils showed no significant differences between P0 and P50, indicating the important role of the plants as drivers for the nitrogen cycle, since they are competitors for ammonia and nitrate, as well as being donators of bioavailable carbon through exudation (34).

With ongoing rice cultivation, microbial biomass increased constantly (although the differences between P50, P100, and P300 were not significant) and reached its maximum in P2000. The relative abundance of nitrogen-fixing microbes at P100, P300, and P2000 was significantly lower than that for P50, probably due to ongoing fertilization. This hypothesis is confirmed by the relatively high abundance of nitrite reducers with the highest *nirK* copy numbers in P2000. These long-term fertilization effects have been also described by other authors (e.g., see reference 28). In general, *nirK* copy numbers were higher than *nirS* copy numbers in all soils investigated. A dominance of *nirK* over *nirS* in different soils was also found in previous studies (e.g., see reference 22).

Overall, our study indicated evidence of alterations in microbial communities involved in inorganic nitrogen cycling among the different soils investigated. We could clearly prove that (i) microbial communities between tidal wetland and paddy soils differ significantly and (ii) that during ongoing paddy soil cultivation, microbial communities adapt to the changes in soil structure and organic matter quality and specific phylotypes of selected functional groups become dominant. This addresses our introduced question, showing that changes are caused by the type of management and are not a response to the disturbance of a balanced ecosystem. In general, changes in the diversity pattern were more pronounced than those in functional gene copy numbers. The named soil properties changing along the chronosequence as a result of paddy soil evolution may be drivers for the observed shifts in microbial community function and diversity. However, it cannot be excluded that other between-site differences provide an explanation for the observed results, although agricultural management and water regimes were comparable between the different sites. This is a typical problem of field studies in general. To clarify, an additional experiment in the greenhouse under controlled conditions will be necessary. A closer link to activity and turnover rates would be possible by analysis of transcripts.

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Publication II

SHORT COMMUNICATION

Comparison of lipid biomarker and gene abundance characterizing the archaeal ammonia-oxidizing community in flooded soils

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Lorenz Schwark · Michael Schloter

Abstract In the last years, archaea have been identified as key players in global N cycling, especially in nitrification. Ammonia-oxidizing archaea (AOA) are postulated to belong to the new phylum Thaumarchaeota for which the lipid crenarchaeol should be specific. The ratios between two independent markers for AOA, the ammonia mono-oxygenase gene and crenarchaeol have been studied in different aerated soils, but so far not in flooded soils. This study investigated ammonia-oxidizing archaea in four paddy soils and a tidal wetland. Ratios were significantly higher in the paddy soils compared to the tidal wetland and in general higher as in upland soils, leading to the assumption that archaeal ammonia oxidizers different from crenarchaeol-containing Thaumarchaeota may play an important role in paddy soils.

Keywords Ammonia-oxidizing archaea (*amoA* gene) ·
Paddy soil · Tidal wetland · Isoprenoidal GDGT ·
Crenarchaeol · Caldarchaeol

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Introduction

Archaea represent a considerable fraction of microorganisms in terrestrial ecosystems. They play an important role in the global nutrient cycles mainly of C and N (Gattinger et al. 2004; Leininger et al. 2006). In the N cycle, archaea have been found to be particularly involved in nitrification (Schleper et al. 2005; Venter et al. 2004). This process results in the formation of nitrate, which is a substrate for denitrification that leads to N losses from soil (Wrage et al. 2001).

As recently postulated, ammonia-oxidizing archaea belong to the new archaeal phylum Thaumarchaeota (Spang et al. 2010) for which the glycerol dialkyl glycerol tetraether lipid crenarchaeol is a good biomarker. Leininger et al. (2006) could prove for many different upland soils a relative constant ratio between gene copy numbers of archaeal ammonium monooxygenase genes (*amoA*) and crenarchaeol being in the range of 15–60 ($\times 10^7$ gene copies g^{-1} dry soil / $\mu g\ g^{-1}$ dry soil), which supports assumption of crenarchaeol as biomarker for AOA (Spang et al. 2010). However, so far no data exist on the ratio of *amoA* gene copy numbers and crenarchaeol from flooded soils, e.g., natural wetlands or paddy soils; hence, it is unclear if archaeal nitrification is also exclusively performed by microbes of the phylum Thaumarchaeota in these particular soils. As a reference parameter estimating the total archaeal

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inhibition of PCR, e.g., by co-extracted humic substances. DNA extract dilution of 1:128 turned out to be best suited (data not shown). PCR efficiencies, calculated from the formula $Eff=[10^{(-1/slope)}-1]\times100\%$, were 94.1–98.1% for archaeal *amoA* genes and 83.1–83.5% for bacterial ones.

Glycerol dialkyl glycerol tetraether lipids were recovered from lyophilized soil via pressurized liquid extraction (DIONEX ASE 200) using a mixture of dichloromethane/methanol (3:1; *v*/*v*) at 100°C and 7×10^6 Pa. Extracts were cleaned by Al_2O_3-solid phase extraction and filtered through 0.45-mm polytetrafluoroethylene filters. Glycerol dialkyl glycerol tetraether fractions were analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry on a cyanopropyl column and protonated molecular ions were recorded in selected ion monitoring as described previously (Reigstad et al. 2008).

Data were subjected to analysis of variance using the statistic program SPSS 13.0. Normal distribution of the variables was checked by Kolmogorov–Smirnov test and boxplot analysis, and homogeneity of variances by Levene test.

Results and discussion

Soil pH values significantly decreased with cultivation time (from 8.1 in TW to 7.3 in P2000), most probably as consequence of a continuing decalcification due to flooding (Zou et al. 2011). In contrast, total organic C contents significantly increased with cultivation time from 0.58% in TW up to 3.1% in P2000. The same trend with significant differences between all soils was found for total N concentrations (Table 1). This may be caused by the agricultural management and again especially by the flooding of the paddy fields during rice growth, because under waterlogged conditions, soil organic matter decomposition proceeds at slower rates than in well-drained, aerobic soils (Neue et al. 1997).

Based on the amount of caldarchaeol, the highest archaeal biomass values were found in the paddy soils which have a long history of rice cultivation (P300 and P2000). In contrast in the tidal wetland the lowest amounts of caldarchaeol were detected (Fig. 1a). As the total amount of extracted DNA increased significantly from 140 μg g^{-1} dw in TW to 1,100 μg g^{-1} dw in P2000 (Table 1), the relative abundance of archaea based on ng of DNA decreased from TW to P2000 (data not shown).

The highest total and relative amounts of crenarchaeol were observed in TW (25.7 ng g^{-1} dw), whereas values in the paddy soils ranged between 8.1 and 22.1 ng g^{-1} dw. Compared to the amounts of crenarchaeol measured by Leininger et al. (2006) for upland soils, lower values of crenarchaeol were assessed in flooded soils. A shift in the ratio of caldarchaeol vs. crenarchaeol from 1.6 in TW up to 7.7 in the paddy soils might reflect an increasing contribution

Fig. 1 Total copy numbers of *amoA* AOA genes (**a**) and values of isoprenoidal glycerol dialkyl glycerol tetraether lipids (caldarchaeol and crenarachaeol) (**b**) as well as ratios of total *amoA* AOA copy numbers to amounts of caldarchaeol (**c**) and crenarchaeol (**d**), respectively ($\times10^7$ copies g^{-1} dw / ng g^{-1} dw), in the tidal wetland (TW), the 50, 100, 300, and the 2,000 years cultivated paddy soils (*n*=5, *error bars* represent standard deviations). Significant differences are indicated by different *letters*
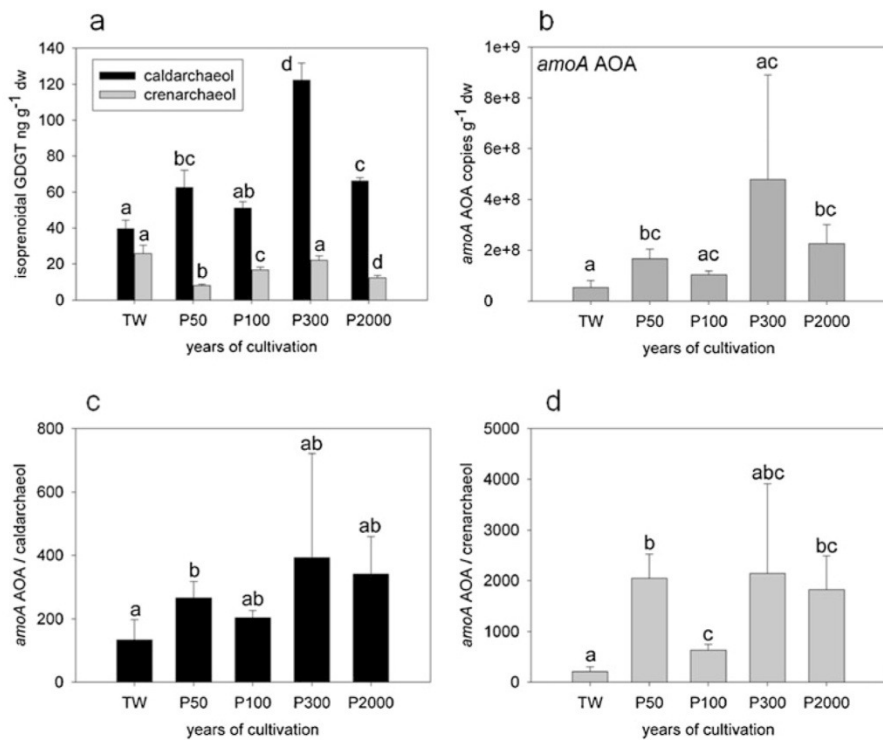
of other archaea like methanogens in the soils under rice cultivation (data not shown).

With exception of the TW, the absolute gene copy numbers of *amoA* AOA determined in the four paddy sites (related to g^{-1} dw) were higher compared to values measured by Leininger et al. (2006). Lowest archaeal *amoA* copy numbers g^{-1} dw were measured in the TW compared to the four paddy soils (Fig. 1b). A reason could be the significantly higher pH value in TW because decreasing gene copy numbers of archaeal *amoA* genes with increasing soil pH values were described in several studies (Erguder et al. 2009; Gubry-Rangin et al. 2010; Nicol et al. 2008). Furthermore, *amoA* gene copy numbers of the ammonia-oxidizing archaea followed the increasing microbial (Cmic, DNA) and archaeal (caldarchaeol) biomass, respectively. Relative abundances (normalized on total amount of extracted DNA) showed no significant difference between all sites and ranged between 1.7×10^3 and 5.5×10^3 copies ng^{-1} DNA (data not shown). One reason may be that archaeal ammonia oxidizers are able to adapt to changing environmental conditions as suggested by Leininger et al. (2006).

Concerning ammonia-oxidizing bacteria, absolute and relative abundances were lower in the paddy soils than in TW (relative amounts between 9.9×10^1 and 5.1×10^2 copies ng^{-1} DNA) and in general at least one order of magnitude lower than AOA expect in TW (data not shown). A dominance of ammonia-oxidizing archaea over their bacterial counterparts in paddy soils has been shown previously (Chen et al. 2008).

Calculating the ratios of archaeal *amoA* copy numbers to caldarchaeol ($\times 10^7$ copies g^{-1} dw / µg g^{-1} dw) showed no significant difference between all paddy soils (Fig. 1c). This indicates that the share of ammonia-oxidizing archaea on the archaeal community remained relatively constant with cultivation time. In contrast, a trend of higher ratios in the paddy soils compared to TW could be found, which was only significant for P50, supporting the hypothesis of archaeal ammonia oxidizers adapting well to the conditions in a paddy soil.

Ratios of archaeal *amoA* copy numbers to crenarchaeol ($\times 10^7$ copies g^{-1} dw / µg g^{-1} dw) were (a) higher in the paddy soils (between 610 and 2,200) compared to TW (210) and (b) in general significantly higher compared to values observed by Leininger et al. (2006) which ranged between 15 and 60. This may lead to the assumption that archaeal ammonia oxidizers different from crenarchaeol-containing Thaumarchaeota could play an important role in flooded soils.

However, the multidisciplinary approach in assessing microbial processes in soils and sediments by molecular genetics and lipid analysis requires a specification of the compatibility of the methods. DNA extraction and subsequent molecular analysis rather reflect the composition of the recent microbial community at this time point of sampling (snapshot), whereas extraction and analysis of core glycerol dialkyl glycerol tetraether lipids provides a time-integrated (decades to millennia) view of the preservable microbial input into soils and sediments (Kuypers et al. 2001). Comparing the data of this study with data measured in aerated soils, the reduced turnover rates in the paddy soils, which were shown by constantly increasing total organic C and total N values with cultivation time, should be taken into account that may also lead to higher amounts of extracellular DNA and lipids in soil (Lindahl 1993; Poinar et al. 1996; Willerslev et al. 2004; Pietramellara et al. 2009; Harvey and Macko 1997). However, based on the observation that *amoA* AOB gene copy numbers decreased from TW to P2000, while *amoA* AOA showed the opposite tendency higher enrichment rates of extracellular DNA in paddy soils compared to tidal wetlands in our study are unlikely.

Thus, the question remains if the higher ratios of archaeal *amoA* copy numbers to crenarchaeol in the paddy soils compared to TW are (a) due to a different community structure with more ammonia-oxidizing archaea containing no crenarchaeol in the paddy soils or (b) due to a better conservation of fossil lipids in TW and a lower input in the paddy soils.

To get a better insight which organisms are involved in archaeal ammonia oxidation in flooded soils, further molecular studies are necessary, e.g., by using metagenomic tools.

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Publication III

Accumulation of nitrogen and microbial residues during 2000 years of rice paddy and non-paddy soil development in the Yangtze River Delta, China

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Abstract

Lowland rice paddy soils may accumulate significant amounts of organic matter. Our aim was to investigate the role of prolonged paddy management on the nitrogen (N) status of the soils, and to elucidate the contribution of bacteria and fungi to long-term N accumulation processes. For this purpose, we sampled a chronosequence of 0–2000 years of rice cropping with adjacent non-paddy systems in the Bay of Hangzhou, China. The samples were analyzed for bulk density, total, mineral and microbial N (N_{mic}), and amino sugars as markers for microbial residues. The results showed that during the first 100 years of land embankment, both paddy and non-paddy soils accumulated N at a rate of up to 61 and 77 kg ha^{-1} per annum, reaching steady-state conditions after 110–172 years, respectively. Final N stocks in paddy fields exceeded those of the non-paddies by a factor of 1.3. The contribution of amino sugars to total N increased to a maximum of 34 g N kg^{-1} N in both land-use systems, highlighting a significant accumulation of N in microbial residues of the surface soils. Correspondingly, the ratio of N_{mic} to microbial residue-N decreased to a constant value. In the paddy subsoils, we found that bacterial residues particularly contributed to the pool of microbial residue-N. Nevertheless, the absolute contents of amino sugars in paddy subsoils decreased during the last 1700 years of the chronosequence. We conclude that under paddy cultivation, soil microorganisms may accumulate parts of this N in their residues despite low overall N availability. However, this N accumulation is limited to initial stages of paddy soil development and restricted to the surface horizons, thus challenging its sustainability with future land-use changes.

Keywords: amino sugars, cultivation chronosequence, microbial biomass, nitrogen accumulation, paddy soil

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Introduction

Rice is one of the world's three major crops. Overall, more than 50% of the world's population feeds on rice. At present, about 29.9 million ha of land are used for lowland rice cropping, which accounts for about 40% of the total world rice grain (FAOSTAT, 2010). These paddy systems are known to accumulate organic matter (Cao *et al.*, 1984; Cai, 1996; Pan *et al.*, 2004, 2009), i.e. there may be a potential that these soils accumulate nitrogen (N) in organic forms despite the demand for

this nutrient as a result of intensive cropping (Olk *et al.*, 1996). Generally, more than 90% of the N found in soil is bound organically (Stevenson, 1982; Knicker & Kögel-Knabner, 1998). Nevertheless, in paddy soils, this soil organic N (SON) is not made available to plants at prolonged cropping. Olk *et al.* (1996) reported that the bioavailability of soil-N decreased over time, and attributed this to an increasing fixation of ammonium by lignins. Although this may be a valid mechanism in rice monocultures, lignin may be rapidly oxidized under aerobic conditions. Hence, when paddy management is combined with aerobic crop sequences, microorganisms may rapidly re-utilize this N and store it temporarily in their cell biomass. In Nepal, for instance, this mechanism is used to improve N balances of paddy

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systems by straw additions (Pande & Becker, 2003). However, little is known on the extent and sustainability of microbial N accumulation with prolonged paddy management.

The exact amount of microbial cell wall-N is difficult to assess, because many of the involved N structures also occur in plants. Amino sugars, however, are known to preferentially derive from microbial cell wall residues, because plants do not synthesize amino sugars in significant amounts (Parsons, 1981). Amino sugars may contribute up to 10% to the total N pool (Stevenson, 1982). As amino sugar turnover time is in the range of a few years (Derrien & Amelung, 2011), different amino sugars represent the accumulated residues of dead cells of bacteria and fungi (Guggenberger *et al.*, 1999; Amelung *et al.*, 2002). For paddy soils, high absolute amino sugar contents were previously found and attributed to elevated organic matter input from crop residues and organic fertilizer (Zong *et al.*, 2008; Hao *et al.*, 2010; Huang *et al.*, 2010). However, comprehensive studies on microbial residues as affected by prolonged paddy cultivation in comparison with non-paddy soils are still missing.

The objective of this study was to evaluate to what degree and how fast prolonged paddy management may result in an accumulation of N and how much of this N is stored within the residues of bacteria and fungi. For this, a chronosequence of paddy soils (up to 2000 years of rice cultivation) in the Bay of Hangzhou, China, was identified (Cheng *et al.*, 2009), where cultivation is characterized by a cropping sequence of rice followed by an upland crop. To relate accumulation rates directly to paddy management, adjacent non-paddy cropping systems formed on similar substrates were sampled for comparison. We hypothesized that paddy soils are able to accumulate N in organic forms and that this process continues for several hundred years. We furthermore hypothesized that this sequestration is accompanied by an immobilization of N in microbial residues, which is more pronounced in paddy soils than in non-paddy soils.

Materials and methods

Study site

Soil samples were taken from a land reclamation area in the Bay of Hangzhou near the City of Cixi, Zhejiang Province. Successive dyke building, which started 1000 years ago, created a peninsula of about 433 km^2 mainly consisting of Yangtze River sediment on which paddy and non-paddy soils of the chronosequence were formed. Annual precipitation averages 1325 mm with a maximum between April and October. The mean annual temperature is 16.3 °C (Cheng *et al.*, 2009).

Soil samples

Site selection was based on a previous survey on soils and historic record (Cheng *et al.*, 2009). A sample of the marine Yangtze sediment was taken in the Bay of Hangzhou as substrate reference and was denominated tidal wetland (age = 0 years). As a starting point of terrestrial soil formation, a non-cropped salt marsh (shrub vegetation) was sampled behind the youngest dike (build in 1977). In late spring 2008, shortly after the harvest of the upland crops, at the time of rice transplanting, a chronosequence of six soils being 50, 100, 300, 700, 1000 and 2000 years (P50–P2000) under paddy cultivation, and four adjacent soils under non-paddy cultivation for 50, 100, 300 and 700 years (NP50–NP700) were sampled by horizon to a depth of >100 cm (Table 1). Except for the 300 year-old paddy field where water melons were grown and the 2000 year-old site (already transplanted rice), fertilizer had not been applied at the date of sampling. The age of the sites was reconstructed from the historic records of the time of dyke construction bordering these areas (Cheng *et al.*, 2009). The traditional paddy management practice in this region in China is a crop rotation of rice in the wet season followed by wheat or other upland crops in the dry season (Fan *et al.*, 2005). The non-paddy sites did not show any evidence of significant soil mottling, thus supporting the assumption that they had never been used for paddy cropping.

At all sites, three field replicates were sampled for analyses. Soil profiles were dug within one representative main site and two subsites located at different fields of the same site. Each soil pit was sampled by pedogenetic horizon. Bulk density was recorded for all soil horizons. Their texture ranged from silty loam to silty clay-loam (Table 1). The paddy profiles were generally characterized by a puddled layer, low in bulk density (about 1 g cm^{-3}) and an underlying dense (1.4–1.6 g cm^{-3}) plough pan. Below, horizons were differentiated by increasing mottling with iron and manganese concretions. Due to a lack of vesicular pores and sorted soil aggregates in the puddled layer as well as a platy structure in the plough pan – both prerequisites for an anthraquic horizon – all paddy soils were classified as Cambisols or Stagnosols (IUSS Working Group WRB, 2006). At paddy sites P100, P700 and P1000, two plough pans were formed due to a change in management depth. At sites P700, P1000, and P2000, dark layers were found in the subsoils, which were classified as buried topsoil horizons (2Ahgb, 3Ahgb; Table S1; supporting information). Most non-paddy soils showed two A horizons influenced by ploughing (Table S1; supporting information); however, the second one (Ap2) was not dense enough to be designated a plough pan. The redoximorphic subsoil was homogeneous in color and structure.

All bulk soil samples were air-dried, sieved to a size <2 mm prior to transport and ground before chemical analyses. Fresh topsoil (0–20 cm) samples for analyses of microbial biomass and mineral N were cooled to 4 °C during transport and processed within 2 weeks after sampling.

Carbon and nitrogen analyses

The contents of total carbon and nitrogen were determined in duplicate after dry combustion at 950 °C with a Vario EL ele-

Table 1 Geographic location, pH, texture, and denomination of sample sites

Years of embankment	Site	Soil type	Latitude, longitude	Actual crop	Texture*	pH$_{KCl}$ topsoil
Tidal flat						
0	TW	Salic, tidalic Fluvisol (calcaric, sodic, siltic)	N: 30°19.557', E: 121°08.691'	–	SiL	8.2
Salt marsh						
30	SM	Gleyic, salic Fluvisol (calcaric, sodic, siltic)	N: 30°18.819', E: 121°10.111'	Shrubland	SiL	7.8
Paddy (P)						
50	P50	Stagnic gleyic Cambisol (calcaric, siltic)	N: 30°11.031', E: 121°21.366'	Fallow	SiL	7.3
100	P100	Gleyic Cambisol (eutric, siltic)	N: 30°09.827', E: 121°20.971'	Rice, harvested (burned)	SiL–SiCL	5.1
300	P300	Gleyic Cambisol (eutric, siltic)	N: 30°06.437', E: 121°30.280'	Water melon	SiL	5.5
700	P700	Endogleyic Stagnosol (albic, eutric, siltic, thaptomollic)	N: 30°10.408', E: 121°09.180'	Rice, harvested	SiL	6.8
1000	P1000	Endogleyic Stagnosol (albic, ruptic, eutric, siltic)	N: 30°09.763', E: 121°06.957'	Fallow	SiL	5.5
2000	P2000	Endogleyic Stagnosol (albic, ruptic, eutric, siltic)	N: 30°05.425', E: 121°26.775'	Rice, transplanted	L–SiCL	5.1
Non-paddy (NP)						
50	NP50	Endogleyic, hyposalic, endofluvic Cambisol (calcaric, siltic)	N: 30°13.152', E: 121°21.382'	Cotton	SiL	7.1
100	NP100	Endogleyic Cambisol (calcaric, siltic)	N: 30°11.884', E: 121°21.196'	Water melon	SiL	7.5
300	NP300	Haplic Cambisol (eutric, siltic)	N: 30°06.932', E: 121°30.646'	Fallow	SiCL	6.7
700	NP700	Haplic Cambisol (eutric, siltic)	N: 30°10.967', E: 121°08.706'	Cotton	SiL	6.0

*Texture as recorded in the field following World Reference Base for Soil Resources (WRB): SiL, silty loam; SiCL, silty clay-loam; L, loam.

mental analyzer (Elementar Analysensysteme, Hanau, Germany). Total inorganic carbon was determined by dissolution of carbonates with 42% phosphoric acid and subsequent infrared detection of the evolving CO_2 (C-MAT 550; Ströhlein GmbH, Viersen, Germany), and soil organic carbon (SOC) was calculated by subtracting inorganic carbon from the total carbon content.

Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were analyzed using the chloroform fumigation extraction method (Brookes et al., 1985) for the top 20 cm of paddy soils. In brief, samples were fumigated for 24 h in chloroform prior to 0.01 M $CaCl_2$ extraction and subsequent C and N analysis using a DIMATOC 100 total organic carbon analyzer (DIMATEC Analysentechnik GmbH, Essen, Germany). Microbial biomass C and N were calculated as difference between fumigated and non-fumigated samples (Brookes et al., 1985; Jörgensen & Brookes, 1991). Nitrate and ammonium concentrations were determined in $CaCl_2$ extracts by a colorimetric method using the commercial kits NANOCOLOR Nitrat50 (Macherey-Nagel, Düren, Germany) (detection limit: 0.3 mg N kg^{-1}) and Ammonium3 (detection limit: 0.04 mg N kg^{-1}). In the top 20 cm of paddy soils, the sum of nitrogen found in nitrate and ammonium did not exceed 1.5% of total nitrogen (Table 2), whereas in the subsoils mineral N fractions contributed up to 4% to total N.

The fraction of non-hydrolysable N was determined using dry combustion after 12 h of hydrolysis in 6 M HCl at 105 °C and subsequent rinsing and drying. On average, 70% of total N in our samples belonged to the hydrolyzable N fraction.

Amino sugar analysis

Among the different amino sugars, glucosamine (Glu), galactosamine (Gal), muramic acid (MurA), and mannosamine were employed as markers for microbial N accumulation. The amino sugar determination followed a modified method of Zhang & Amelung (1996). In a preliminary step, high interfering iron loads were lowered by washing ground samples with 0.5 M trifluoro-acetic acid. Then samples were subjected to hot hydrochloric acid (8 h at 105 °C), filtered (glass fiber filters, GF6; Whatman GmbH, Dassel, Germany) and dried via rotary evaporation (45 °C, Turbo–Vap Multiroti; Büchi Synchore Line; Büchi Labortechnik GmbH, Essen, Germany). Extracts were purified by two centrifugation steps, one after pH adjustment (pH 6.6–6.8) and another after dissolution of the previously freeze dried supernatant in methanol. Derivatization was carried out using hydroxylamine hydrochloride and dimethylaminopyridine (in pyridine–methanol: 4 : 1; 80 °C for 30 min) followed by addition of acetic anhydride (80 °C for 20 min). The four amino sugar monomers were then separated with a gas chromatograph (Agilent 6890; Agilent Technologies GmbH, Böblingen, Germany) using a capillary column (Optima-5; length 30 m; inner diameter 0.25 mm; Macherey-Nagel) and a split ratio of 30 : 1, and analyzed using flame ionization detection. Myo-inositol was used as first internal standard and β-endosulfan as second internal standard to assess the recovery of myo-inositol for quality control. The β-endosulfan replaced methylglucamine, the recovery of which failed,

Table 2 Absolute contents (average ± SD) of total nitrogen (N$_{tot}$; $n = 3$), mineral N (N$_{min}$), and N within living (N$_{mic}$) and dead (amino sugar-N; $n = 3$) microbial biomass in the top 20 cm of paddy soils (P) and tidal wetland (TW)

Site	Duration of management (years)	N$_{tot}$ (mg kg^{-1} soil)	N$_{min}$* (mg kg^{-1} soil)	N$_{mic}$† (mg kg^{-1} soil)	Amino sugar-N (mg kg^{-1} soil)	Stored N‡ (t ha^{-1})
TW	0	310	4.4 ± 2.3	27.4 ± 5.6	5.3	0.0
P50	50	1133	3.7 ± 2.0	33.4 ± 8.5	30.3	2.5
P100	100	1697	7.7 ± 3.9	37.6 ± 3.3	63.7	3.7
P300§	300	2302	28.0 ± 12.0	4.5 ± 0.7	82.0	4.8
P700	700	1883	7.2 ± 2.8	32.6 ± 2.8	61.8	4.9
P1000	1000	1189	6.5 ± 0.8	37.7 ± 3.8	43.9	4.9
P2000	2000	3192	21.7 ± 5.0	36.6 ± 9.7	96.2	4.9

*Sum of NH$_4^+$ and NO$_3^-$ extracted with CaCl$_2$ ($n = 5$).
†Microbial nitrogen after chloroform fumigation extraction ($n = 5$).
‡Calculated from sequestration rate function (see Eqn 2; Table 3); TW used as reference.
§Field cropped with water melons at high pesticide loads at time of sampling.

possibly due to interferences with still remaining iron. Recoveries of myo-inositol averaged 83% for the topsoil and 65% for the subsoil samples. Analysis was repeated when undercutting a recovery of 60%. Amino sugars were analyzed in all surface soil samples taken, including puddled layers and plough pans of paddy soils as well as two A horizons of the non-paddy soils. We analyzed all three field replicates per site, each sample was processed in duplicate. The amino sugar analysis of subsoil samples was difficult, most probably due to enhanced and variable iron loads. Hence, analyses of subsoils were restricted to one main profile per management time, but then performed in triplicate.

The different amino sugars are markers for a different origin of the pool of microbial residue-N. Glucosamine (Glu) is common in fungal chitin (Parsons, 1981), but is also found in the peptidoglycan cell wall of bacteria (Parsons, 1981; Chantigny *et al.*, 1997). MurA uniquely derives from bacteria (Parsons, 1981; Guggenberger *et al.*, 1999; Amelung *et al.*, 2008). Galactosamine (Gal) frequently occurs in capsular and extracellular polysaccharides of bacteria (Parsons, 1981), but it may also be part of fungal cell walls (Parsons, 1981; Glaser *et al.*, 2004; Engelking *et al.*, 2007). The microbial origin of mannosamine is unclear (Amelung *et al.*, 2008).

Statistical evaluation

The data were subjected to analyses of variance (ANOVA) using STATISTICA (8.0 for Windows; Statsoft Europe GmbH, Hamburg, Germany). The Tukey's test was performed to determine the significance of differences recorded among horizons in paddy and non-paddy soil profiles. The impact of management, cultivation age, and field heterogeneity on total amino sugar variability was assessed using multivariate analysis of variance (MANOVA). For correlations, the Pearson approach was used. Regression functions were calculated using SIGMAPLOT (10.0 for Windows; Systat Software Inc., Chicago, IL, USA). To estimate accumulation rates of total N and amino sugars, a mono-exponential regression model was used:

$$X_t = (X_e - X_0) \times (1 - e^{-kt}) + X_0, \qquad (1)$$

where X_t is the parameter of issue at cultivation time t (years), X_e is the parameter concentration at absolute equilibrium, X_0 is the initial parameter concentration in the tidal wetland ($t = 0$), and k is a rate constant. Accumulation rates were determined using the first derivative of (1) as

$$f'(t) = (X_e - X_0) \times k \times e^{-kt}, \qquad (2)$$

where $f'(t)$ is the annual accumulation rate in the year t.

Results

Nitrogen accumulation in paddy and non-paddy soils

The content of total nitrogen varied from 0.8 to 3.6 g kg^{-1} soil in the paddy and non-paddy topsoils (Table S1; supporting information), which is equivalent to 2.3–6.5 t N ha^{-1} in the top 20 cm and to 7–12 t N ha^{-1} in the 100 cm soil profile (Figs 1a and c; 2a and b). The tidal wetland and the 30-years embanked salt marsh contained lower N contents, adding up to stocks of 2.5 and 3 t ha^{-1} in 100 cm, respectively (Fig. 2c and d). Hence, the arable soils stored more N, on the average, than did the tidal wetland and the marsh. In general, the N stocks in paddy topsoils were 80% higher than in non-paddy surface horizons ($P < 0.0001$; Fig. 2).

The topsoils of both paddy and non-paddy soils showed up to four times higher average N contents than did the subsoils (paddy soils: 2.2 ± 0.4 g kg^{-1} soil vs. 0.4 ± 0.1 g kg^{-1} soil; non-paddy soils: 1.2 ± 0.3 g kg^{-1} soil vs. 0.4 ± 0.3 g kg^{-1} soil; Fig. 1a and c). As soil depth increased, C/N ratios generally increased and N content decreased at all sites (Fig. 1). Although in the paddy soils, the decrease in N content started only
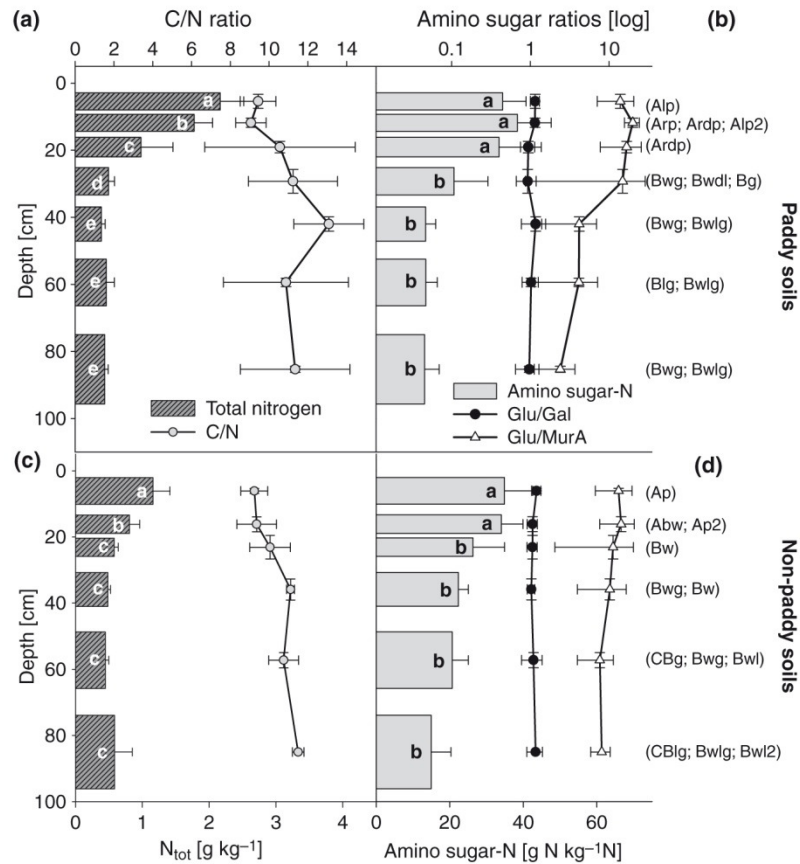
Fig. 1 Depth distribution of (a, c) total soil nitrogen (N_{tot}) and carbon to nitrogen ratio (C/N) as well as (b, d) the amino sugar concentration and composition in paddy (top) and non-paddy soil profiles (bottom). Shown are arithmetic means for 50, 100, 300, and 700 year-old sites (error bars = SD; $n = 8$); bars with same letters show no significant difference (ANOVA, Tukey's test, $P < 0.05$). Glu/Gal, glucosamine-to-galactosamine ratio; Glu/MurA, glucosamine-to-muramic acid ratio; the denomination of diagnostic horizons after World Reference Base of Soil Resources (WRB) is shown in parentheses.

below the Ardp horizon, the non-paddy soils had already lost N at 20 cm soil depth, lacking this plough pan (Fig. 1). In general, the contribution of the subsoil to total N stocks was greater in the non-paddy (65%) than in the paddy fields (50%; Fig. 2c and d). The buried topsoil material in P700, P1000, and P2000 was not characterized by peaking N contents (Table S1; supporting information), i.e. it did not disturb the evaluation of N storage in these paddy profiles.

With increased duration of management after land embankment, soil formation had changed tidalic and salic Fluvisols in the wetland and the salt marsh to Cambisols under non-paddy cropping and in the paddies of young to medium age (P50, P100, P300, P700). Stagnosols with endogleyic features developed in the older paddy soils (P1000, P2000; Table 1). Along with this land reclamation, the N content in A horizons doubled within the first 30 years of land embankment (difference between tidal wetland and salt marsh), whereas

the C/N ratio remained high at 16 ± 1. As soon as agricultural management was established, the C/N values dropped to 10 ± 1, and N accumulation continued until a maximum was reached in the 300 year-old site under paddy-wheat rotation and after 100 years under continuous non-paddy cropping (Fig. 2). Thereafter, only the 2000 year-old paddy showed a significantly elevated N content ($P < 0.05$). The 1000 year-old paddy showed lower absolute contents of N in the topsoil than the younger and older paddy fields (Fig. 2). Possibly, this is due to the removal of topsoil for dyke maintenance diluting topsoil with subsoil material (personal communication with Cao Z. H.).

With increasing N content, also the respective stocks increased, rising to a maximum of 10.3 t N ha^{-1} in 100 cm paddy soils and to 7.5 t N ha^{-1} in non-paddy soils (Fig. 2c and d; Table 3). The accumulation of N could be described with mono-exponential growth curves to a constant maximum (Fig. 2). Accordingly,
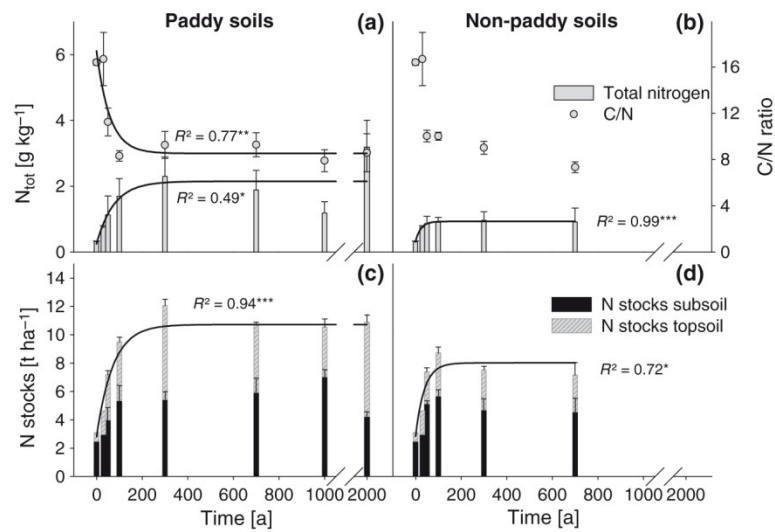
Fig. 2 Impact of land-use duration on (a, b) the contents of total nitrogen (N_{tot}) and the carbon-to-nitrogen ratio (C/N) (weighted means of upper 20 cm, $n = 6$; error bars = SD) as well as (c, d) the N stocks in top- and subsoil ($n = 3$; error bars = SD) of paddy (left) and non-paddy soils (right). The asterisks indicate level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; mono-exponential regression; 0 years, tidal wetland; 30 years, salt marsh.

Table 3 Kinetic parameters of the mono-exponential model (Eqn 1: $X_t = (X_e - X_0) \times (1 - e^{-kt}) + X_0$) calculated for different nitrogen (N) pools (see also curve fits in Figs 2, 3 and 4)

Parameter	Unit	$k \pm SE$	X_0	X_e	Accumulation rate$_{80}$[†] (1 yr^{-1})	Time to steady-state[‡] (years)	R^{2}[§]
Paddy							
Amino sugar content (topsoil)	[g kg^{-1}]	0.014 ± 0.008	0.0	1.0	0.007	198	0.67*
N_{tot} content (topsoil)	[g kg^{-1}]	0.013 ± 0.011	0.3	2.2	0.013	193	0.49*
Amino sugar-N (topsoil)	[g N kg^{-1} N]	0.021 ± 0.010	15.8	34.4	0.247	119	0.75*
Microbial biomass-N (topsoil)[¶]	[g N kg^{-1} N]	0.039 ± 0.020	88.5	20.3	−0.886	122	0.91*
Amino sugar-N/N_{mic} (topsoil)		0.014 ± 0.013	0.2	2.1	−	190	0.40
Amino sugar-N stocks (100 cm depth)	[t ha^{-1}]	0.016 ± 0.009	0.0	0.3	0.001	185	0.68*
N stocks (100 cm depth)	[t ha^{-1}]	0.014 ± 0.003	2.8	10.7	0.061	172	0.94***
Non-paddy							
Amino sugar content (topsoil)	[g kg^{-1}]	0.017 ± 0.007	0.1	0.5	0.003	163	0.84*
N_{tot} content (topsoil)	[g kg^{-1}]	0.043 ± 0.005	0.3	0.9	0.015	78	0.99***
Amino sugar-N (topsoil)	[g N kg^{-1} N]	0.019 ± 0.006	16.7	34.9	0.136	123	0.90*
Amino sugar-N stocks (100 cm depth)	[t ha^{-1}]	0.027 ± 0.012	0.0	0.2	0.003	112	0.83*
N stocks (100 cm depth)	[t ha^{-1}]	0.026 ± 0.015	2.9	8.1	0.077	110	0.72*

k, rate constant; X_0, concentration at time point zero; X_e, equilibrium concentration; SE, standard error.

[†]Averaged for the cultivation period until 80% of X_e were reached using the first derivative of the curve fits from Figs 2, 3 and 4 $f'(t) = (X_e - X_0) \times k \times e^{-kt}$ (Eqn 2).

[‡]Defined as annual increase <0.1% of absolute value of the respective parameter.

[§]Coefficient of determination of the curve fits (see Figs 2, 3 and 4), asterisks indicate level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; mono-exponential regression.

[¶]N_{mic} proportions of N_{tot} decreased with cultivation time; fit equation used here: $X_t = X_e + (X_0 - X_e) \times e^{-kt}$.

we defined the time when the steady-state equilibrium was reached as the time where the annual increase in N contents was <0.1% of the total value of the respective parameter. The calculations showed that steady-state equilibria were reached after 110 years for the total N stocks in the non-paddy soils and after 172 years in the

respective paddy fields (Table 3). The time to steady-state is driven by two factors: (i) the rate constants k and the related initial accumulation rates (linearized relationship before reaching steady state; Table 3), which were 61 kg ha^{-1} per annum for the total N stocks in the paddies compared with 77 kg ha^{-1} per annum in the non-paddies, and (ii) the steady-state level (X_e) of total N content, which was 30% higher in paddy than in non-paddy soils (Table 3). These temporal trends were most evident for paddy topsoils, whereas the respective subsoils showed little if any cultivation effects on total N stocks (Fig. 2c and d). Obviously, most of the N storage under paddy management was restricted to the puddled layer.

Intriguingly, initial rates of N accumulation were similar in range to those found in different forest stands (Yang *et al.*, 2011). The same study also indicated that annual N accrual declined at later stand ages of forest development, until equilibrium of N stocks was reached.

Nitrogen pools

The inorganic N (N$_{min}$) fraction (ammonium and nitrate) contributed less than 0.5% to total N in paddy topsoils, independent from the time of rice cultivation. In the subsoils, it accounted for 1.4% of N in the tidal wetland and up to 3% in the paddy fields. Hence, inorganic N forms hardly contributed to the total N accumulation process, i.e. the majority of N was SON.

The fraction of living microbial biomass-N (N$_{mic}$) made up 0.2–3.1% of total N in paddy soils. The absolute contents of N$_{mic}$ slightly increased upon land reclamation (Table 2). When related to total N, the N$_{mic}$ proportion decreased from 88 g N kg^{-1} N in the tidal wetland to 22 g N kg^{-1} N in the P100 field. The 300 year-old paddy, being the only site planted with water melons at the time of sampling, showed extraordinary low N$_{mic}$ concentrations with 2 g N kg^{-1} N.

Amino sugars serve as markers for microbial residues. The contents of amino sugar-N represented up to 3.7% of total N (Table 2). This is only half of that typically found for other soils such as the native or cultivated US prairie (Amelung *et al.*, 1999; Zhang *et al.*, 1999). However, this amino sugar-N proportion was initially in the same order of magnitude than that of N$_{mic}$. Later, e.g. in the 2000-year old paddy fields, the amino sugar-N contents exceeded those of N$_{mic}$ up to a factor of 3.6 (Table 2), therewith approaching typical ratios of amino sugar-N to N$_{mic}$ between two and five as found for temperate soils (Jörgensen *et al.*, 1995; Kandeler *et al.*, 2000; Appuhn & Jörgensen, 2006). However, not only does all microbial residue-N consist of amino sugars but also of amino acid-N and nucleic acid-N, i.e.

the true contribution of microbial residue-N to total N will be higher than indicated by the mere marker analyses.

Similar to total N, the largest absolute contents of amino sugars were also found in the puddled layer of paddy soils. When related to total N (in the following equated with amino sugar concentrations), the amino sugar concentrations were not significantly different between both management systems ($P = 0.83$) again with highest contributions of 34 g amino sugar-N kg^{-1} N allocated in the top horizons (Fig. 1b and d). As soil depth increased, both systems showed a significant ($P < 0.05$) absolute and relative amino sugar depletion in the subsoil, with the paddy soils showing a more abrupt decrease below the plough pan (Fig. 1b and d). Hence, the contribution of the subsoils to total amino sugar storage was again larger for the non-paddies (50%) than for the paddies (30%). However, neither the paddy nor the non-paddy sites indicated a pronounced microbial enrichment of subsoil-N. Also, the fossil A horizons did not reveal any peculiar amino sugar concentration (see also Table S1; supporting information).

With increasing duration of land embankment, the concentrations of amino sugar-N paralleled the total N accumulation pattern. It increased from 17 g N kg^{-1} N in the tidal wetland to 36 g N kg^{-1} N in the 300 year-old paddy (Fig. 3a and b). Calculated steady-state equilibria were reached after 119 years in the paddy and after 123 years in the non-paddy topsoils (Table 3). The similar exponential increase, approaching a maximum of 0.2 t N ha^{-1} for the entire non-paddy profiles and 0.3 t N ha^{-1} for the paddy profiles, was true for the amino sugar-N stocks (Table 3, Fig. 3c and d). Here again, in the paddy topsoils, the steady-state levels were 20% higher and they were reached later compared with the non-paddy soils (Fig. 3, Table 3). In contrast, the paddy subsoils exhibited an increased loss of amino sugar-N stocks instead of a continued gain after 300 years of paddy management (Fig. 3c).

Intriguingly, with the establishment of a steady-state amino sugar contribution to total N, also the ratio of amino sugar-N to N$_{mic}$ approached a fairly constant level after 190 years of paddy land use (Fig. 4, Table 3), confirming that this level reflected a true steady-state and biologic equilibrium between build-up and death of the members of the soil microbial community.

Amino sugar composition

Along with the changes in amino sugar concentrations, we observed alterations in their composition. With lower contributions of amino sugar-N to total N, the composition of amino sugars as represented by Glu/
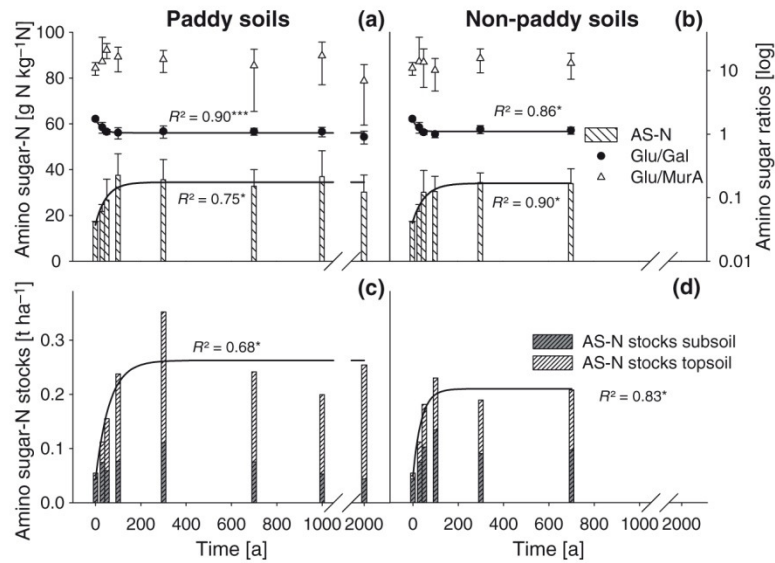
Fig. 3 Impact of land-use duration on (a, b) the concentration and composition of amino sugar-N (AS-N; weighted means of upper 20 cm, $n = 6$; error bars = SD), and (c, d) the AS-N stocks ($n = 1$) in top- and subsoil of paddy (left) and non-paddy soils (right). The asterisks indicate level of significance: $*P < 0.05$, $***P < 0.001$; mono-exponential regression. Glu/Gal, glucosamine-to-galactosamine ratio; Glu/MurA, glucosamine-to-muramic acid ratio; 0 years, tidal wetland; 30 years, salt marsh.
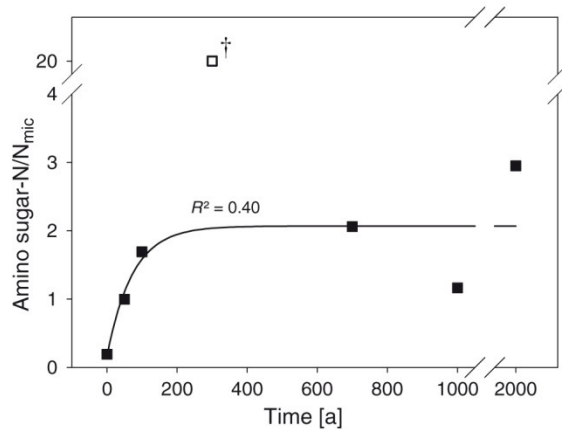
Fig. 4 Ratio of amino sugar-nitrogen (AS-N; weighted means of Alp, Ardp1 and Ardp2 horizons; $n = 3$) to nitrogen in microbial biomass (N_{mic}; obtained using chloroform fumigation extraction method; homogenized samples from 0 to 20 cm depth; $n = 5$). [†]The 300 year-old paddy was planted with water melons at the time of sampling, thus treated as an outlier for the assessment of living microbial biomass and excluded from regression.

Gal and Glu/MurA ratios also changed with depth. Although the Glu/Gal ratio remained rather constant for both management systems, the ratio of Glu/MurA was significantly ($P < 0.05$) lower in paddy subsoil than in paddy topsoil (Fig. 1b). In non-paddy soil profiles, the range at which this ratio varied was smaller,

averaging 12 at the top and eight in the subsoil (Fig. 1d).

Both the Glu/MurA and Glu/Gal ratios dropped after initial cultivation in paddy and non-paddy soils (Glu/Gal: from 1.6 to 1.2, Glu/MurA: from 30 to 10; Fig. 3a and b). No consistent changes in these ratios with prolonged cultivation were determined in the older sites. Hence, after reclaiming the tidal and salty wetlands and with increased duration of both paddy and non-paddy cropping, the steady-state equilibrium of microbial residue-N composition was approached after about 65–80 years.

Discussion

Nitrogen storage in paddy and non-paddy soils

In the Bay of Hangzhou, land reclamation and subsequent rice cropping have been practiced for millennia (Cheng *et al.*, 2009). Embankment and thus the conversion of the frequently turned over tidal marine sediment to a terrestrial system led to an initial accumulation of organic matter from the growing shrub vegetation (Kögel-Knabner, 2002) in a shallow Ah horizon (Figs 1a and c; 2a and b). As a result, also the N concentration increased, especially in the topsoil (Fig. 2a and b); the widened C/N ratio being an indicator of the terrestrial origin of this soil organic matter (SOM). As soon as the soil became aerated, processes

like desalination and decalcification also started, resulting in changes of microbial community structure and function toward a terrestrial one (Hassink *et al.*, 1991).

With the start of cultivation, the N stocks continued to increase exponentially (Fig. 2c and d) due to increased biomass input from the growing crops and manure application. The C/N ratio now decreased concomitantly with the microbial transformation of SON, continued input of green manure, and mineral fertilizers (Benbi & Senapati, 2010; Jörgensen *et al.*, 2010). After 50 years of fresh water irrigation and cropping, the topsoils were completely desalinated (unpublished data). Dense plough pans had developed below the puddled paddy surface horizons (Fig. 1; see also Table S1, supporting information for horizon denomination). These Ardp horizons had a platy structure and bulk densities approx. 0.1 g cm^{-3} higher than the respective non-paddy soil horizons. The incorporated crop biomass was stored in the tilled horizons of both land-use systems; however, the paddy Ardp horizons inhibited root penetration, water percolation, and thus vertical transport of any kind of organic matter, therewith initializing a decoupling of surface and subsurface N dynamics, at least during the rice growing season. For this reason, the N contents declined stronger with depth in the paddy than in the non-paddy fields (the latter exhibiting a rather gradual N depletion; Fig. 1a and c), and the paddy fields finally stored higher portions of N in the topsoil than in the subsoil relative to the non-paddy soil profiles.

As the duration of cropping proceeded, the submerged conditions favored the storage of SOM in the paddy topsoils (Kögel-Knabner *et al.*, 2010). Consequently, both the contents and stocks of N in the paddy topsoils finally reached a level almost twice as high as that of the non-paddy topsoils (Fig. 2). The reduced translocation of SOM through the plough pan into the paddy subsoils may be an additional reason that these land-use systems mainly accumulated C and N in their surface horizons.

The accumulation of N in both land-use systems followed exponential growth curves to a maximum (Fig. 2, Table 3). These maxima indicated a saturation of total N concentrations and stocks in both systems, i.e. a steady-state equilibrium in which additional N input was balanced by N loss. In the paddy soils, this establishment of steady-state equilibrium for the N stocks was reached about 60 years later than in the non-paddy soils. This was not only due to (i) the higher end level of N in the paddy soils but also to (ii) a faster N accumulation rate in the non-paddies (Table 3), which also gained subsoil-N more easily. Thus, the increase in paddy N stocks with time of cultivation was

mainly driven by the topsoil, and subsoils contributing less to total N stocks of a given soil profile than the non-paddy subsoils (Fig. 2c and d). Again, this was probably due to the restricted input of SOM into the subsoil by the dense paddy plough pan.

The results show that, in contrast to other agricultural systems, paddy soils do accumulate organic matter for quite some time (Cao *et al.*, 1984; Cai, 1996; Kögel-Knabner *et al.*, 2010). Similar initial rates of SOM accumulation have been found for successions of secondary grass and forest ecosystems (Preger *et al.*, 2010; Yang *et al.*, 2011). However, in all of these studies, the SON accumulation did not continue for more than 200 years.

Nitrogen pools

The contents of ammonium and nitrate as bioavailable intermediates and end-products of plant input and fertilization remained low, indicating a rapid immobilization of N_{min} to organic and volatile N forms (Olk & Cassman, 1996; Pande & Becker, 2003; Wang *et al.*, 2004; Fan *et al.*, 2006; Li *et al.*, 2009; Pan *et al.*, 2009). The mineral N fractions were also prone to seasonal dynamics, taking into account that elevated N_{min} concentrations were found in the 300 and the 2000 year-old paddy fields, which were planted at the time of sampling (Table 2). Nevertheless, the majority of stored N was again SON.

Microbial biomass-N (mainly proteins) also responds quickly to short-term environmental changes (Chantigny *et al.*, 1996; Bai *et al.*, 2000; Gattinger *et al.*, 2002). The contents of N_{mic} declined rapidly with start of cultivation (Table 2). Rapid microbial reactions were also reflected in the P300 soil, where, due to common herbicide application in water melons, absolute N_{mic} contents were reduced by a factor of 10 (Fig. 4, Table 2; see also Perucci *et al.*, 2000; Vischetti *et al.*, 2002). However, the rapid changes in the N_{mic} pool cannot explain the medium to long-term accumulation of N in the paddy soils.

Amino sugars have been shown to survive their producers (Guggenberger *et al.*, 1999; Amelung *et al.*, 2001a; Glaser *et al.*, 2006) and to have a turn-over time in the range of a few years, at least for well-aerated temperate grassland soils (Derrien & Amelung, 2011). Absolute amino sugar amounts of paddy and non-paddy management systems were well inside the range found in literature (Zhang & Amelung, 1996; Amelung *et al.*, 2002). Amino sugar contributions to total N in paddy and non-paddy A horizons were similar to those of forest A horizons (Liang *et al.*, 2007a, 2008), but by up to 19.5 g N kg^{-1} N lower than in grassland topsoils (Zhang & Amelung, 1996; Amelung *et al.*, 2002). In

cropped ecosystems of the prairies, 3–8% of total N have been attributed to amino sugar-N (Amelung *et al.*, 1999; Zhang *et al.*, 1999; Liang *et al.*, 2007a), which was also up to a factor of 3 higher than the proportions found here. Although a significant positive correlation between amino sugars and N was found in soils of this study ($\rho = 0.89$, $P < 0.01$; Pearson correlation) as well as in above mentioned grassland soils, different amino sugar contribution to total N suggests a notable management influence on production and storage of these microbial residues (Guggenberger *et al.*, 1999; Zhang *et al.*, 1999; Liang *et al.*, 2007c). Lower contributions of amino sugar-N to total N in the sites studied here could be due to less synthesis of microbial cell walls and bacterial gums, more efficient decomposition of the microbial residues or restricted amino sugar stabilization at different mineralogy, or a combination thereof. Besides, higher portions of unknown stable N (e.g. lignin-N; Schmidt-Rohr *et al.*, 2004) may dilute amino sugar-N proportions. The warm climate should hardly restrict the synthesis of amino sugars (Amelung *et al.*, 1999), and minerals were shown to have minor impact on amino sugar concentrations in an incubation study (Amelung *et al.*, 2001b). A depletion of amino sugar-N proportions of total N has generally been attributed to conditions of substrate limitations (Amelung *et al.*, 1999, 2001a). Such substrate limitations may be aggravated in the sites under study, because of limited N and C availability.

The distribution of amino sugars in the profiles mirrored that of total N, though amino sugar gradients with depth, were even steeper than for total N (Fig. 1b and d). Again, this was probably due to the hindered vertical transport of microbially altered organic matter or of substrates available for microbial growth and amino sugar synthesis and another indication for the above mentioned decoupling process. In line with a limited amino sugar synthesis, also, the increase of the C/N ratio below the plough pans can be interpreted as a sign of less intense microbial immobilization of N in the paddy subsoils (Fig. 1). This finding, however, also means that the storage of N in microbial residues of paddy subsoils is limited. In general, amino sugars constitute only a minor fraction of total cell wall-N. Bacterial peptidogylcan, for example, typically consists of only two units amino sugar-N, but 10 units amino acid-N, i.e. total contents of microbial cell wall-N in soil can easily be six times higher than indicated by amino sugar analyses (Schleifer & Kandler, 1972; Vollmer & Bertsche, 2008; Vollmer *et al.*, 2008), not accounting for other N in membrane proteins, proteoglycans etc. Hence, storage of N in microbial residues is much larger than indicated by the mere contents of the biomarkers. Nevertheless, even when adding about 30–50%

amino acid-N to the total N of the surface soils (own unpublished data), a large fraction of the remaining N is either not subject to hydrolysis (about 30% of total N; data not shown) or not even identified. This remaining N may be N-lignins (Olk *et al.*, 2006), charred or other heterocyclic N (Schulten & Schnitzer, 1998; Leinweber & Schulten, 2000; Knicker *et al.*, 2005). However, little is known on the residence time of these N compounds and their participation in the N cycle of soils (Stevenson, 1982; Olk *et al.*, 2006).

Long-term microbial N accumulation

The changes in amino sugar contents in the paddy and non-paddy surface soils almost paralleled those of the N stocks (Figs 2 and 3), suggesting that total N storage and the accumulation of N in microbial residues was intrinsically coupled. After 30 years of land embankment, the amino sugar contributions to N in the topsoil had already increased by 30% compared with the tidal sediment (Fig. 3a and b), indicating that initial amino sugar accumulation was even faster than total N build-up. Adaptation of the microbial community to the cropping system in a desalinated environment with less osmotic stress (Polonenko *et al.*, 1986) thus resulted in increasing microbial N storage in the form of cell wall residues until steady-state conditions were reached (Fig. 3, Table 3). This increased accumulation of N in microbial residues, however, did not coincide with a significant increase in the concentrations of N_{mic}, reflecting that the parameter is more prone to seasonal variation than amino sugar contents (Chantigny *et al.*, 1996; Bai *et al.*, 2000; Gattinger *et al.*, 2002). During laboratory incubations, steady-state conditions for amino sugars were already reached within a period <1 year (Amelung *et al.*, 2001b), whereas here, it took about 100 years until the contribution of amino sugars to N remained at a more or less constant level with increasing duration of soil use. In opposition to laboratory incubations, plant derived SOM is continuously supplied in the field. It could thus be increasingly incorporated into microbial residues during land reclamation and initial cultivation decades under both management systems.

Amino sugar steady-state concentrations were reached later in the paddy topsoil than in the topsoil of the non-paddy sites, i.e. the paddy soils accumulated at comparable rates, but to a higher end level (Table 3). Obviously, the better oxygen supply in the non-paddy soils enhanced the turnover of microbial biomass, resulting not only in faster incorporation of N into microbial residues but also increased re-decomposition of SOM and the N pools compared with the paddy sites. Besides, there may be elongated adaptation times

of microbes in the paddy fields because of the frequent change from paddy to upland cropping. Under steady-state conditions, total SOM content and thus the supply with additional C sources probably controlled the concentrations of microbially stabilized N in both management systems (Liang & Balser, 2008).

When there are saturation processes, there is also a limited stabilization capacity for newly formed microbial residue-N. Indeed, the ratio of amino sugar-N to N_{mic} also approached a maximum after 190 years of paddy management, however, with great variability (Fig. 4). Obviously, the contents of steady-state amino sugar-N usually did not exceed the amount of microbial biomass-N in paddy soils by more than a factor of 3 (Table 3) as found for other soils (Kandeler et al., 2000; Jörgensen et al., 2010). This confirms that at a certain size, parts of the microbial residue pool have become bio-accessible for being re-consumed. Short-term disturbances like the growing of water melons in the 300 year-old paddy soil, known to be intensively treated with herbicides (Terry et al., 1997; Monks & Schultheis, 1998), only reduced microbial biomass (see above; Fig. 4), but did not affect the total amino sugar pool (Table 2).

When considering the entire soil depth, it is obvious that amino sugar stocks in the paddy subsoils did not continue to increase. Instead, amino sugars were lost during the last 1700 years of the paddy management chronosequence (Fig. 3c and d). With the lack of vertically transported C sources, microbes were increasingly forced to re-degrade their residues in the subsoil. However, this effect was overcompensated by the high net gain of N and microbial residue-N in the surface soils. Nevertheless, the loss of subsoil-N was probably an additional reason for the smaller rate constants of total N and amino sugar-N accumulation in the entire paddy profiles relative to the non-paddy sites, as mentioned above (Table 3).

Microbial origin of the residues

Due to their specific occurrence in cell wall structures of fungi and bacteria, ratios of glucosamine to galactosamine (Glu/Gal) and of glucosamine to muramic acid (Glu/MurA) have been frequently used to estimate contributions of those microbial groups to SOM turnover in soils (Kögel & Bochter, 1985; Zhang & Amelung, 1996; Amelung et al., 1999; Liang et al., 2007a). In general, amino sugar ratios determined in this study were of similar magnitude as those observed for anaerobic horizons by Jörgensen & Richter (1992) and in other studies at aerobic sites (Amelung et al., 2002; Dai et al., 2002; Liang et al., 2007b). In the present study, the contributions of MurA to total amino sugar content increased as total amino sugar concentrations decreased with soil depth, suggesting that mainly bacteria were involved in subsoil-N cycles. Liang & Balser (2008) attributed higher MurA proportions in subsoils of a glacial-landscape toposequence to a predominance of gram positive bacteria with thicker cell walls (Vollmer et al., 2008), which withstood changing water potentials due to ground water table influence in these profiles (Morris et al., 2004). In contrast, fungal-derived glucosamine was more abundant in the surface soils, possibly reflecting a mycorrhizal symbiosis in rice and non-paddy crops in the well-rooted surface soil.

After land embankment, bacteria were obviously the first to adapt to the frequently disturbed paddy and non-paddy systems and, hence, contributed the greater share to the amino sugar pool and to SOM accumulation (Fig. 3a and b). Although in the non-paddy soils, amino sugar ratios remained fairly constant after 100 years, the portions of MurA increased with paddy cultivation time (Fig. 3a). In line with Bossio & Scow (1998) and Nakamura et al. (2003), this finding probably reflects a promotion of anaerobe gram negative bacteria (Liang et al., 2008) in submerged crops. However, these trends were not significant; indeed, differences between paddy and non-paddy amino sugar composition were surprisingly small. Only genetic information would help, now, to decide whether similar species accumulated that had also advantages in N cycling in the subsoils.

Conclusion

Land embankment and initial cultivation significantly promoted the accumulation of N in organic forms. However, the soils were no endless sink for SON. Steady-state equilibrium was reached within the first 200 years, with the paddy soils reaching a higher end level than their non-paddy counterparts. The contents of microbial biomass-N only increased at initial stages of soil formation, but did not consistently change with prolonged paddy management. Total amino sugar stocks, however, paralleled N accumulation functions, indicating that microbial cell wall residues contribute to long-term N storage. Only in the subsoil, there was a loss of mainly fungal microbial residues after about a period of 300 years of paddy use, reflecting that processes in the subsoil were at least in part decoupled from those in the paddy topsoil. We attributed this finding to the formation of a dense plough pan due to repeated puddling, which increasingly hindered the supply of the subsoil with organic materials from the top. Apparently, the longer the use of soils for paddy management, the stronger is this decoupling of soil-N biogeochemistry in surface and subsurface soil along a time scale of the last two millennia.

Acknowledgements

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Supplemental material

Table S1: Roth et al.

Amino sugars and their specific contribution to total nitrogen (N_{tot}) and soil organic carbon (SOC) in tidal wetland (TW), saltmarsh (SM), paddy (P) and non-paddy (NP) profiles of different cultivation ages ($n = 3$; average ± SD).

Site	Depth	Horizon	SOC	N_{tot}	Amino sugars	Glu	Gal	MurA	Amino sugar-C/SOC[†]	Amino sugar-N/N_{tot}[‡]
	cm		g kg⁻¹soil				mg kg⁻¹soil		%	
TW	2-30		5,1	0,3	69,9 ± 0,8	38,6 ± 0,4	22,2 ± 1,7	3,6 ± 0,8	0,6 ± 0,0	1,7 ± 0,0
SM	0-13	Ah	10,9	0,8	217,5 ± 23,9	119,8 ± 25,1	80,8 ± 7,1	9,9 ± 11,3	0,8 ± 0,1	2,2 ± 0,3
	13-30	Bwg	5,3	0,3	91,9 ± 9,0	40,1 ± 7,8	35,1 ± 1,6	12,0 ± 0,5	0,7 ± 0,1	2,3 ± 0,2
P50	0-7	Alp	17,8	1,8	775,9 ± 163,6	381,6 ± 89,9	323,1 ± 69,0	27,5 ± 16,1	1,7 ± 0,3	3,4 ± 0,7
	7-14	Arp	14,5	1,4	538,0 ± 157,1	264,3 ± 74,3	231,7 ± 61,5	13,1 ± 4,4	1,5 ± 0,5	3,0 ± 0,9
	14-23	Ardp	5,7	0,5	121,6 ± 18,0	54,2 ± 8,5	57,9 ± 10,2	1,9 ± 0,7	0,9 ± 0,1	2,0 ± 0,2
	23-38	Bwg1	5,1	0,4	63,3 ± 5,5	27,4 ± 0,4	22,2 ± 4,3	13,8 ± 0,7	0,5 ± 0,0	1,1 ± 0,1
	38-50	Bwg2	5,2	0,3	55,4 ± 3,1	28,4 ± 2,1	18,8 ± 1,2	8,2 ± 4,8	0,4 ± 0,0	1,2 ± 0,0
	50-70	Bwg3	5,1	0,4	76,9 ± 8,3	37,1 ± 7,1	30,0 ± 5,3	8,3 ± 5,0	0,6 ± 0,1	1,6 ± 0,2
	70-100	Blg	5,4	0,4	73,8 ± 3,7	34,0 ± 2,8	26,7 ± 0,6	12,2 ± 0,4	0,6 ± 0,0	1,4 ± 0,1
P100	0-9	Alp1	17,6	2,1	1055,8 ± 186,7	524,3 ± 92,4	445,6 ± 90,3	37,8 ± 14,9	2,3 ± 0,5	4,1 ± 0,5
	9-15	Alp2	15,3	1,8	987,9 ± 117,6	485,1 ± 75,9	424,4 ± 46,6	27,4 ± 10,2	2,6 ± 0,6	4,2 ± 0,9
	15-21	Ardp	6,6	0,9	384,4 ± 94,3	154,3 ± 47,8	199,4 ± 43,2	13,2 ± 5,6	2,2 ± 0,4	3,2 ± 0,6
	21-30	Bwg1	5,8	0,5	112,3 ± 1,9	39,0 ± 0,6	59,3 ± 0,7	14,0 ± 2,0	0,6 ± 0,1	1,7 ± 0,1
	30-50	Bwg2	4,7	0,4	74,0 ± 1,4	27,4 ± 2,0	35,1 ± 1,6	11,4 ± 2,1	0,6 ± 0,1	1,3 ± 0,2
	50-75	Bwlg1	4,7	0,4	69,4 ± 2,5	27,2 ± 0,5	31,6 ± 0,1	10,6 ± 2,9	0,6 ± 0,2	1,2 ± 0,1
	75-100	Bwlg2	5,2	0,4	80,0 ± 1,9	31,1 ± 0,3	35,5 ± 1,4	10,7 ± 0,7	0,6 ± 0,1	1,3 ± 0,3
P300	0-18	Alp	22,6	2,5	1075,0 ± 109,2	512,5 ± 107,4	474,4 ± 43,3	49,4 ± 20,8	1,8 ± 0,2	3,2 ± 0,2
	18-24	Ardp	14,9	1,6	964,1 ± 297,6	474,5 ± 162,1	427,5 ± 125,7	21,8 ± 7,5	2,6 ± 0,5	4,7 ± 0,6
	24-30	Bwdl	7,1	0,7	261,0 ± 72,3	106,7 ± 29,8	138,2 ± 38,2	3,3 ± 1,2	1,8 ± 0,2	3,4 ± 0,4
	30-50	Bwl	5,2	0,5	112,1 ± 2,7	44,2 ± 0,6	52,2 ± 4,7	11,3 ± 5,1	0,9 ± 0,0	1,8 ± 0,0
	50-70	Bwlg1	4,9	0,5	95,7 ± 11,8	34,6 ± 5,0	41,4 ± 9,6	17,1 ± 6,4	0,8 ± 0,1	1,4 ± 0,2
	70-100	Bwlg2	4,7	0,5	115,1 ± 28,1	29,8 ± 8,0	41,5 ± 10,2	42,1 ± 25,0	1,4 ± 1,0	1,6 ± 0,2
P700	0-10	Alp1	23,2	2,3	1019,9 ± 125,2	472,1 ± 95,7	410,8 ± 50,6	101,7 ± 72,8	1,9 ± 0,3	3,3 ± 0,6
	10-16	Alp2	18,0	2,0	950,1 ± 173,2	467,0 ± 98,8	400,4 ± 70,6	22,6 ± 4,4	2,0 ± 0,2	3,7 ± 0,3
	16-22	Ardp	8,6	1,1	427,1 ± 140,0	175,6 ± 77,6	180,6 ± 70,7	59,0 ± 46,6	2,0 ± 0,6	2,9 ± 1,0
	22-45	Bg	4,1	0,6	157,2 ± 15,4	75,7 ± 9,2	74,6 ± 6,1	3,5 ± 0,2	1,5 ± 0,2	2,2 ± 0,2
[§]	45-69	2Ahgb	5,3	0,7	81,0 ± 23,4	37,9 ± 11,1	36,1 ± 9,6	5,5 ± 0,6	0,8 ± 0,2	0,9 ± 0,3
	69-106	2Blg1	3,3	0,5	31,6 ± 0,0	14,6 ± 0,0	13,4 ± 0,0	3,6 ± 0,0	0,4 ± 0,3	0,4 ± 0,2
[§]	106-116	3Ahlgb	6,0	0,4	50,1 ± 20,3	29,0 ± 12,2	18,8 ± 8,5	2,3 ± 0,4	0,3 ± 0,1	0,9 ± 0,4
	116-130	3Blg	2,1	0,3	34,2 ± 0,0	17,8 ± 0,0	15,3 ± 0,0	n.d.	0,7 ± 0,1	0,9 ± 0,3
P1000	0-10	Alp	14,0	1,6	781,0 ± 184,2	368,7 ± 90,0	304,5 ± 68,7	86,0 ± 80,6	2,3 ± 0,6	3,6 ± 0,9
	10-16	Al(d)p1	11,0	1,3	944,3 ± 51,7	508,7 ± 34,4	375,4 ± 20,0	19,6 ± 1,4	3,1 ± 0,6	5,5 ± 0,5
	16-21	Aldp2	6,1	0,7	336,3 ± 119,7	157,1 ± 64,5	155,3 ± 46,5	7,9 ± 4,0	2,2 ± 0,4	3,2 ± 0,7
[§]	21-40	2Ahgb	4,6	0,6	74,3 ± 4,2	36,0 ± 1,3	31,6 ± 2,5	5,5 ± 1,3	0,6 ± 0,0	0,9 ± 0,1
	40-55	2Bg	2,8	0,5	39,9 ± 0,4	18,9 ± 0,1	16,4 ± 1,0	4,6 ± 1,3	0,6 ± 0,0	0,6 ± 0,0
	55-80	2Bl1	3,9	0,6	66,3 ± 0,3	30,9 ± 0,3	28,9 ± 0,1	5,7 ± 1,1	0,6 ± 0,0	0,8 ± 0,0
[§]	80-93	3Ahlb	15,4	1,4	89,3 ± 2,4	41,3 ± 10,0	32,2 ± 1,3	15,7 ± 1,7	0,2 ± 0,1	0,5 ± 0,3
	93-100	3Bl	3,5	0,5	47,8 ± 0,0	16,7 ± 4,0	21,1 ± 2,3	10,1 ± 1,4	0,6 ± 0,0	0,7 ± 0,1
P2000	0-15	Alp	30,0	3,6	1434,1 ± 347,5	590,0 ± 161,1	662,3 ± 185,8	144,8 ± 72,9	2,0 ± 0,4	3,0 ± 0,6
	15-20	Ar(d)p	20,5	2,1	875,2 ± 252,6	372,9 ± 116,1	439,4 ± 131,0	36,1 ± 18,2	1,8 ± 0,6	3,2 ± 0,9
	20-27	Bdg	5,5	0,6	164,3 ± 69,3	51,5 ± 21,7	76,0 ± 44,6	32,9 ± 14,3	1,3 ± 0,5	2,2 ± 1,7
[§]	27-35	2AhgB	3,5	0,4	75,0 ± 18,0	21,9 ± 7,0	24,7 ± 5,6	26,5 ± 16,5	0,9 ± 0,3	1,1 ± 0,2
	35-50	2Bg1	2,6	0,4	44,6 ± 0,6	23,0 ± 1,2	18,9 ± 0,8	2,7 ± 0,2	0,7 ± 0,1	0,9 ± 0,1
	50-70	2Bg2	1,6	0,3	33,1 ± 3,7	10,9 ± 6,9	8,7 ± 2,5	12,8 ± 10,7	0,9 ± 0,2	0,7 ± 0,1
	70-100	2Blg	1,4	0,3	19,1 ± 3,3	10,3 ± 5,5	6,7 ± 2,0	2,1 ± 15,3	0,5 ± 0,1	0,4 ± 0,1
NP50	0-9	Ap	10,6	1,1	533,8 ± 46,4	270,6 ± 24,4	233,0 ± 20,0	12,7 ± 2,0	2,0 ± 0,2	3,8 ± 0,6
	9-17	ABw	8,2	0,8	350,4 ± 89,2	164,8 ± 49,7	155,6 ± 38,7	14,7 ± 6,7	1,7 ± 0,3	3,1 ± 0,6
	17-24	Bw	6,4	0,6	154,1 ± 55,3	62,5 ± 24,7	64,6 ± 26,5	19,3 ± 7,8	1,0 ± 0,4	1,9 ± 0,7
	24-45	BCg	5,6	0,5	141,0 ± 22,5	61,7 ± 10,3	58,9 ± 12,3	11,4 ± 2,5	1,0 ± 0,2	2,0 ± 0,4
	45-70	CBg	4,8	0,5	165,1 ± 0,5	84,8 ± 5,7	56,1 ± 14,3	24,2 ± 19,5	1,5 ± 0,0	2,6 ± 0,2
	70-100	CBlg	4,7	0,4	82,4 ± 1,8	37,8 ± 4,9	34,2 ± 3,4	4,6 ± 0,0	0,7 ± 0,0	1,6 ± 0,0
NP100	0-14	Ap1	10,8	1,0	395,3 ± 126,4	186,9 ± 68,7	174,9 ± 57,1	22,1 ± 6,6	1,6 ± 0,4	3,0 ± 0,8
	14-25	Ap2	8,1	0,8	325,6 ± 50,9	143,6 ± 25,3	156,3 ± 30,3	16,0 ± 7,2	1,6 ± 0,3	3,1 ± 0,6
	25-30	Bw	7,0	0,7	237,5 ± 8,0	106,0 ± 2,9	107,7 ± 9,4	18,5 ± 0,7	1,3 ± 0,0	2,7 ± 0,1
	30-38	BCwg1	6,2	0,5	145,4 ± 4,5	60,7 ± 1,7	68,9 ± 3,1	12,0 ± 2,3	1,0 ± 0,0	2,1 ± 0,0
	38-70	BCwg2	5,5	0,5	156,5 ± 8,1	63,1 ± 4,6	76,5 ± 4,4	12,5 ± 0,6	1,1 ± 0,1	2,2 ± 0,1
	70-100	BCwlg	5,3	0,5	158,1 ± 0,7	65,1 ± 0,4	76,7 ± 1,0	11,9 ± 0,8	1,1 ± 0,0	2,2 ± 0,0
NP300	0-11	Ah	10,5	1,2	529,4 ± 64,8	278,6 ± 46,7	212,8 ± 24,1	22,8 ± 10,5	2,0 ± 0,2	3,3 ± 0,4
	11-22	Bw1	6,6	0,7	331,4 ± 52,3	159,9 ± 26,7	149,8 ± 25,5	11,3 ± 5,5	2,0 ± 0,4	3,6 ± 0,5
	22-32	Bw2	6,2	0,6	255,4 ± 33,0	122,3 ± 17,9	118,5 ± 13,1	5,2 ± 1,3	1,8 ± 0,2	3,4 ± 0,4
	32-50	Bwg1	5,3	0,5	136,7 ± 2,7	65,5 ± 2,5	62,9 ± 4,8	6,4 ± 0,6	1,1 ± 0,0	2,3 ± 0,1
	50-70	Bwg2	4,6	0,4	85,6 ± 10,7	39,2 ± 5,9	38,3 ± 1,7	5,2 ± 2,5	0,8 ± 0,1	1,6 ± 0,2
	70-100	Bwlg	4,4	0,4	69,6 ± 9,6	35,8 ± 2,0	28,0 ± 7,0	4,7 ± 1,0	0,6 ± 0,1	1,3 ± 0,2
NP700	0-12	Ap1	11,0	1,4	782,2 ± 298,3	401,5 ± 133,0	318,0 ± 137,0	41,0 ± 23,9	2,6 ± 0,5	4,1 ± 0,8
	12-17	Ap2	7,1	0,9	445,6 ± 146,6	227,7 ± 69,0	188,0 ± 66,7	17,0 ± 9,6	2,6 ± 0,4	3,9 ± 0,5
	17-23	Bw1	5,3	0,5	207,7 ± 47,9	112,4 ± 20,9	84,8 ± 21,5	6,1 ± 0,7	2,1 ± 0,5	3,3 ± 0,8
	23-45	Bw2	5,3	0,4	147,2 ± 2,8	73,0 ± 5,2	64,9 ± 1,9	5,4 ± 2,8	1,2 ± 0,0	2,6 ± 0,1
	45-70	Bwl1	5,2	0,4	97,6 ± 3,9	44,6 ± 0,4	45,9 ± 2,9	3,9 ± 0,9	0,8 ± 0,0	1,9 ± 0,1
	70-100	Bwl2	4,8	0,4	46,7 ± 3,9	25,5 ± 1,2	18,8 ± 2,9	2,4 ± 0,2	0,4 ± 0,0	0,9 ± 0,1

[†] C content of amino sugars: 40.2% for glucosamine, galactosamine and mannosamine and 43% for muramic acid

[‡] N content of amino sugars: 7.8% for glucosamine, galactosamine and mannosamine and 3.6% for muramic acid

[§] horizons classified as buried A horizons

Publication IV

**Biogeosciences
Discussions**

Intra-versus inter-site macroscale variation in biogeochemical properties along a paddy soil chronosequence

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Abstract

In order to assess the intrinsic heterogeneity of paddy soils, a set of biogeochemical soil parameters was investigated in five field replicates of seven paddy fields (50, 100, 300, 500, 700, 1000, and 2000 yr of wetland rice cultivation), one flooded paddy nursery, one tidal wetland (TW), and one freshwater site (FW) from a coastal area at Hangzhou Bay, Zhejiang Province, China. All soils evolved from a marine tidal flat substrate due to land reclamation. The biogeochemical parameters based on their properties were differentiated into (i) a group behaving conservatively (TC, TOC, TN, TS, magnetic susceptibility, soil lightness and colour parameters, δ^{13}C, δ^{15}N, lipids and n-alkanes) and (ii) one encompassing more labile properties or fast cycling components (N_{mic}, C_{mic}, nitrate, ammonium, DON and DOC). The macroscale heterogeneity in paddy soils was assessed by evaluating intra- versus inter-site spatial variability of biogeochemical properties using statistical data analysis (descriptive, explorative and non-parametric). Results show that the intrinsic heterogeneity of paddy soil organic and minerogenic components per field is smaller than between study sites. The coefficient of variation (CV) values of conservative parameters varied in a low range (10 % to 20 %), decreasing from younger towards older paddy soils. This indicates a declining variability of soil biogeochemical properties in longer used cropping sites according to progress in soil evolution. A generally higher variation of CV values (>20–40 %) observed for labile parameters implies a need for substantially higher sampling frequency when investigating these as compared to more conservative parameters. Since the representativeness of the sampling strategy could be sufficiently demonstrated, an investigation of long-term carbon accumulation/sequestration trends in topsoils of the 2000 year paddy chronosequence under wetland rice cultivation was conducted. The evolutionary trend showed that the biogeochemical signatures characteristic for paddy soils were fully developed in less than 300 yr since onset of wetland rice cultivation. A six-fold increase of topsoil TOC suggests a substantial gain in CO_2 sequestration potential when marine tidal wetland substrate developed to 2000 year old paddy soil.

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1 Introduction

On global scale rice (*Oryza sativa*) is the most important staple crop feeding more than 50 % of the World's population. Cultivation of rice thus affords large proportion of arable land, amounting to app. 156×10^6 hectare, of which >90 % is used for wetland or paddy rice cultivation in 2008 (IRRI, 2010; Jahn et al., 2011). A critical factor in paddy rice cropping is the periodic flooding of soils and the associated fluctuations in soil redox conditions, biogeochemical cycling of essential and trace elements, and microbial community structure. Rice paddy fields are assumed to contribute significantly to the emission of potent greenhouse gases CH_4 and N_2O (e.g. IPCC, 2007; Conrad, 2009) and to the loss of nitrate into the environment (Koegel-Knabner et al., 2010). Consequently, the investigation of biogeochemical processes in paddy soils is of critical importance in order to assess environmental impact and initiate reduction strategies.

A major problem in the design of biogeochemical studies of paddy fields is the intrinsic heterogeneity of paddy soils in the spatial as well as the temporal realm. Spatial variability may occur on the micro (nm–mm), meso (cm) and macro (m–tens of m) scale level. In paddy soils microscale variability has been described for soil aggregates and within the rhizosphere, whereas mesoscale variations occur within paddy soil profiles on cm or decimetre scale and can be related preferentially to changes in redox conditions (Koegel-Knabner et al., 2010). Macroscale heterogeneity in paddy soils occurs over distances of meters or tens of meters and is less well studied than micro or mesoscale variability. The focus on such heterogeneity investigations has been placed on soil fertility, crop yields and nutrient levels in paddy fields (Tatsuya et al., 2004; Wang et al., 2009; Wei et al., 2009; Yanai et al., 2001; Zhao et al., 2009). However the heterogeneity of bulk organic, molecular and isotopic biogeochemical parameters used to interpret paddy soil processes has not yet been investigated on the macroscale. The objectives of this study thus were, first to evaluate intra- and inter-site spatial variability of geochemical properties indicative for soil organic matter (SOM),

minerogenic substrate and nutrients in paddy fields. Hereby, a differentiation of parameters assumed to behave conservatively by reflecting time-integrated properties (averaging over years or decades) versus fast reacting or labile parameters (reflecting daily, weekly or seasonal changes) was performed. Biogeochemical properties assumed to behave conservatively comprised soil TOC, TN, $\delta^{13}C$, $\delta^{15}N$ and extractable lipid content and composition (reflecting time-averaged influx and composition of crop biomass and microbial re-mineralization), soil magnetic susceptibility and spectral soil colour (reflecting time-averaged soil mineralogy and redox conditions). As labile parameters microbial biomass carbon and nitrogen, nitrate, ammonium, dissolved organic N, and dissolved organic C were considered.

Factors influencing macroscale paddy soil heterogeneity can be either linked to natural variability of the substrate on which paddy soils evolved and/or management practices that locally affect influx and efflux of various components into soils, which in turn regulates the soil microbial community. Management practices can cause very localized and arbitrary enrichment (spots of 1–3 m diameter) of fertilizers, pestizide application, vegetation waste, or biomass combustion residues (heaps of burning rice straw) on paddy fields. Additionally, more systematic in-field variations in soil properties may result from flow pathways of irrigation water and its suspended materials load. Puddling of rice fields (ploughing under flooded conditions) is considered a key factor in the homogenization of locally constrained inputs and when repeated oftentimes may finally lead to the establishment of homogeneous distribution of conservative soil parameters, whereas the labile components may still exhibit severe spatial variability on the field scale.

Depending on the methodological approach applied, challenges to obtain representative paddy soil samples may vary considerably. This may lead to incompatible results, if e.g. microbial ecology conducted by genomics or proteomics targeting labile components is compared to lipidomics (analysis of phospholipid fatty acids or other microbial membrane lipids) employing conservative components.

Secondly, if it could be proven that inter-site variations exceed intra-site variability for specific parameters, the biogeochemical trends over up to 2000 yr of rice cultivation could be evaluated for a chronosequence from the Zhejiang Province, China. Here rice cultivation started in coastal regions following land reclamation after dyke building at well dated times (Cheng et al., 2009; Jahn et al., 2011), which allows for investigation of long-term evolutionary trends in rice paddy biogeochemistry. It is postulated that ongoing paddy soil evolution will continuously diminish the intrinsic heterogeneity of young paddy soils and ultimately establish homogeneous soil biogeochemical conditions. Verification of paddy soil homogeneity in this investigation will contribute to validating pedogenic and biogeochemical studies of the same chronosequence conducted previously (Cheng et al., 2009; Bannert et al., 2011a, b; Jahn et al. 2011; Roth et al., 2011; Wissing et al., 2011). All biogeochemical investigations were carried out using 5 field replicates that were treated statistically and allow assessing whether a composition in one field or a trend over several fields is robust and representative.

2 Material and methods

2.1 Study sites

The sites are located in the coastal Cixi area (Hangzhou Bay) in the north-east of the Zhejiang province, China, as shown in Fig. 1. The Bay is affected by river runoff and tide from the East China Sea. The Yangtze (Changjiang) River with an average water runoff of $925 \times 10^9 \, \text{m}^3 \, \text{yr}^{-1}$ and sediment load of $480 \times 10^9 \, \text{kg} \, \text{yr}^{-1}$ supplied the dominant amount of sediment to the Hangzhou Bay (Li et al., 2009; Wang et al., 2008), where it is re-deposited by southward coastal currents and tides (Jilan and Kangshan, 1989; Xie et al., 2009). The climate is subtropical monsoonal with annual average temperature and rainfall of 16.3 °C and 1418 mm, respectively. The coastal plain of Cixi is densely covered by rivers, lakes, as well as urban and agriculture areas with main crops being wetland rice, rape, barley, and cotton (Hua and Zhu, 2000).

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Step by step land reclamation on marine tidal mudflat sediments (continuous alluvial plain of Andong Beach) through the building of protective dykes over the past 2000 yr, allows the investigation of a soil chronosequence with different stages of development and well known starting dates of cultivation (Edit Committee of Chorography of Cixi County, 1992; Yu et al., 2003; Cheng et al., 2009; Jahn et al., 2011). Wetland rice cultivation generally started when salt concentration decreases to tolerance levels, commonly after <5 yr. Based on the time of dyke construction and information's of the Edit Committee of Chorography of Cixi County (1992) sites with ongoing wetland rice cultivation for 50, 100, 300, 500, 700, 1000 and 2000 yr were identified.

In this region the generally cropping system constitutes one wetland rice season and one dry inter-crop (vegetables, cotton or cereals) season per year, called paddy-upland rotation. Soils with wetland rice cultivation represent anthraquic anthrosols, or briefly paddy soils. These are exposed to longer phases of irrigation influenced by oxygen deprivation and establishment of reducing conditions. The sampled paddy soils can be differentiated into a stagnic gleyic cambisol (50 year old paddy), a gleyic cambisol (100 to 500 yr old paddy soils) and an endogleyic stagnosol (700 to 2000 yr old paddy soils) as outlined in Jahn et al. (2011).

2.2 Sampling

Sampling was conducted in June 2008 after the harvest of the upland crop from seven paddy sites (P50, P100, P300, P500, P700, P1000, P2000) before flooding. In addition, sediment from a flooded paddy nursery site (P50N), a marine site (TW for tidal wetland), and a lacustrine site (FW for freshwater sediment) were analyzed. From each site the top soil/sediment (roughly 0–20 cm) was sampled. The sample representativeness was ensured by collecting five field replicates at each site. The sampling strategy in Fig. 2 shows that each field replicate constitutes a composite sample of seven subsamples. All soil and sediment samples were freeze dried and homogenized by grinding to fine powder. Samples were stored in glass vials in a freezer prior to further analysis.

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2.3 Laboratory analysis

Total organic carbon (TOC) concentrations of the soils and sediments were determined with a LECO CS-225 analyzer after decarbonatisation with HCl (10 % v/v) and neutralization with distilled water. The total carbon (TC), total nitrogen (TN) and total sulfur (TS) were measured directly with a CNS analyzer (Elementar Vario EL-III). Bulk magnetic susceptibility was analyzed at room temperature with a Kappabridge (KLY-2, noise level 4×10^{-8} SI) to characterize the magnetizability of ferromagnetic particles in the sample. Soil colour was quantified using a Minolta (CM-700d/600d) spectrophotometer by measuring the colour parameters on the surface of air-dried samples as described in Wiesenberg et al. (2006). Determinations of water extractable organic carbon (DOC) and nitrogen (DON) were conducted after extraction of the samples with 0.01 M $CaCl_2$ (solid to liquid ratio of 1:3) with a total organic carbon analyzer DIMA-TOC 100 (Jörgensen and Brookes, 1991). For the detection of microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) content, aliquots of soils/sediments were fumigated with chloroform (24 h) prior to $CaCl_2$ extraction. The nitrate and ammonium concentrations were measured in $CaCl_2$ extracts by a photometric autoanalyzer (CFA-SAN Plus/Skalar Analytic) using the commercial kits NANOCOLOR Nitrat-50 and Ammonium-3.

Bulk elemental analysis-isotope ratio mass spectrometry (EA-IRMS) was conducted with an elementary analyzer (FlashEA1110, ThermoFisherScientific) coupled to a mass spectrometer (DeltaV Advantage, ThermoFisher Scientific). The isotopic compositions were expressed as δ^{13}C and δ^{15}N permil units in relation to internal standards V-PDB and air nitrogen.

The hydrocarbon extractable lipids of soils and sediments were obtained by pressurized solvent extraction (Dionex ASE 200). Briefly, lipids from ca. 8 g dry soil were extracted with a dichloromethane/methanol (3/1; v/v) solvent mixture at 100 °C and 7×10^6 kg m^{-1} s^{-2}. Elemental sulphur was removed from the total lipid extracts by addition of activated copper. For quantification known amounts of perdeuterated $n-$tetracosane were added as internal standard. Total extracts dissolved in $n-$hexane

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were separated into apolar and polar compounds using small scale chromatography (Bastow et al., 2007). The aliphatic hydrocarbons were eluted with $n-$hexane after passing the glass column (4 mm × 8 cm) filled with activated silica gel (2 h at 130 °C). The sample volume was reduced via evaporation prior to transfer to GC-MS vials. Aliphatic hydrocarbon fractions were analyzed on a 30 m, ZB-1ms fused silica capillary column (0.25 mm internal diameter, film thickness 0.25 μm; Phenomenex) in a HP 5890 Series II gas chromatograph equipped with a split/splitless injector coupled to a HP 5971A mass spectrometer operated in EI-mode at 70 EV.

2.4 Statistical analysis

All individual data sets were subjected to a statistical evaluation including calculation of various descriptive statistics such as the average (AV), the standard deviation (SD) and the coefficient of variation (CV), whereby the latter describe the spread and relative proportion of variation in the data set. Non-parametric statistical analyses were applied to compare all measured soil parameters among the different sampling sites. For identification of significant variations between the sites a Kruskal-Wallis Test, suitable for non-Gaussian distributed populations, was operated (null hypothesis was all medians are equal), where the asymptotic significances (p-values) <0.05 indicate one or more medians are different. Additionally, multivariate techniques such as a principal component analysis and a cluster analysis were conducted to identify inter-site variability. The analyses were performed using MS Office Excel and PASW Statistics 18.

3 Results and discussion

3.1 Soil parameters

For the control of sample representativeness five field replicates (each a composite sample of seven individual subsamples covering an area of 2 square meters) were taken from every site and investigated for 23 different biogeochemical parameters. The

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summary of the descriptive statistics with average, standard deviation and the coefficients of variations of all parameters determined for the individual sites are listed in Table 1.

3.2 Macroscale intra-site variability

According to their properties the parameters chosen could be pre-differentiated in two groups. The first group termed as "conservative" parameters contained the soil properties that were assumed to represent time-averaged and well homogenized (puddled) soil properties. The second group termed as "labile" parameters encompassed all fast reacting properties (reflecting daily, weekly or seasonal changes) as shown in Table 1.

3.2.1 Bulk organic and minerogenic parameters

The results show much lower variation of individual parameters in the conservative group with non-uniform distribution pattern over different paddy soil cultivation times (Table 1, Fig. 3a). A coefficient of variation (CV) for soil parameters lower than 20 % generally indicates insignificant variability of these soil properties. The lowest spreading of CV values (< 1.4 %) within all soils and sediments was observed for the bulk soil organic matter (SOM) $\delta^{13}C$ isotope values and the lightness parameter L^* (CIE axis ranging from black to white). A slightly higher but generally low variation of CV values (between 1 to 13 %) was detected for the $\delta^{15}N$ values of bulk SOM, magnetic susceptibility (χ), and soil colour parameters a^* and b^* (Fig. 3a). These parameters and their marginal variation within a sampling site, comparable in soils and sediments provide information about the homogeneity of the parent material on which the respective paddy soils developed. Only the 1000 yr old paddy soil presents an exception (Fig. 3a) with a higher variation in magnetic susceptibility (18.4 %), which can be explained by a mixture of the paddy soil with adjacent upland soils of different mineralogy caused by topsoil removal and mixing in the course of dyke maintenance work (Jahn et al., 2011; Roth et al., 2011).

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Other conservative parameters (TOC, TN, C/N) influenced predominantly by organic matter input at a given site also show minor dispersion with coefficients of variation generally <10 % in soils with more than 100 yr of paddy cultivation. Only in younger paddy soils and in the reference substrates the CV values of these parameters varied in a slightly broader range up to approximately 20 % (Table 1). A range in the CV close to or less than 10 % at older paddy sites is better than expected and indicates a decreasing variability in soil organic matter parameters in longer used cropping sites. In reference substrates the CV values of conservative parameters are generally higher than in paddy soils, exemplified by variation of TN and C/N in marine sediments and of TN and TS in lacustrine sediments (Table 1).

3.2.2 Lipid and alkane concentration and composition

The highest variations of conservative parameters within a sampling site were noted for the concentration of total extractable lipids and the lipid class of n–alkanes. The latter is derived from land plant wax coatings, limnic macrophytes, marine/limnic algae and cyanobacteria. The n–alkane distributions reveal a maximum CV of 22 % in paddy soil sites and of 10 % or 30 % in the TW and FW reference substrates, respectively (Table 1). The reason for the higher variation in the limnic environment could be attributed to sampling in a shallow water environment. This favoured mixed organic matter input from submerged aquatic macrophyte biomass and terrestrial plant matter supplied by the catchment to the near-shore limnic setting.

The range of alkane concentrations in paddy soils is caused by diverse organic matter input from actual crop or weed vegetation, products from incomplete biomass combustion, or fossil fuel contaminants at different "hot-spots" on a site. In general, total extractable lipids in paddy soils represent 5.6 % of the total soil organic carbon and are mainly composed of n–fatty acids, n–alcohols, sterols, long-chain wax esters, sugars and other functionalized lipid classes. On average $2.0\,mg\,kg^{-1}$ of these soil lipids are composed of source-diagnostic n–alkanes (Table 1) but in the 700 yr old paddy soil substantially higher proportions of n–alkanes ($6.5\,mg\,kg^{-1}$) were observed, which

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could be attributed to fossil fuel contamination.

A partial origin of n–alkanes from fossil contamination is evident from the presence of a pronounced "unresolved complex mixture" (UCM) and a high abundance of thermally mature tricyclic and pentacyclic triterpenoids (hopanoids) dominating over the recent microbial triterpene diploptene (Fig. 4). Recent bacteria biosynthesize the unsaturated 17β(H)-, 21β(H)-hope(22,29)ene also termed diploptene, which is only stable under near-surface conditions (Ourisson et al., 1987). This compound is diagenetically transformed into saturated analogues upon sediment burial when reaching thermal maturity (Peters et al., 2005). In petroleum thermally stable hopanoids with 17α(H)-, 21β(H)-isomer configuration and a predominance of the 22S over 22R isomers are found. Such a petroleum derived hopanoid distribution has been encountered in the P700 topsoils (Fig. 4). Fossil fuel contamination in a paddy field could originate from a point source in the field, e.g. caused by breakdown of motorized farming machinery associated with spillage of lubricants or fuels. In such a case, only a small area of a few square meters would be contaminated, due to hydrocarbon hydrophobicity preventing further dispersal. The spatially continuous presence of fossil fuel derived hydrocarbons in the P700 field argues against such a localized point spill, but points towards a diffuse contamination, e.g. by inflow of contaminated irrigation canal waters.

The compositional variation of n–alkanes in the paddy soils can be evaluated using standardized parameters describing the preferential enrichment of individual alkanes. The carbon preference index (CPI) established by Bray and Evans (1961) is used to highlight the predominance of odd-over even numbered n–alkane homologues. High CPI_{long} values for long-chain components ($> nC_{23}$) derive from fresh plant waxes and tend to decline with increasing biodegradation and thermal maturity. The same accounts for short chain n–alkanes ($< nC_{22}$) derived from algae or cyanobacteria. Fossil fuels exhibit CPI values close to unity. Variation in CPI values thus reflects recent diagenetic progress or fossil fuel origin. The short chain alkanes for paddies and TW reveal CPI values <1.7 indicative of minor algal and/or cyanobacterial input with only the FW site giving a higher CPI_{short} of 2.1 pointing to more enhanced algal/cyanobacterial

contributions. In conjunction with a small average CV of <10 % the overall proportion of aquatic microbial biomass has been low. More substantial variation was observed for CPI_{long} values around 6.0 for the young paddy sites (P50–P300) and around 4.0 for the older paddies and reference sites, indicative of progress in diagenetic overprinting. Exceptionally high CPI_{long} values were observed for P500 (Table 1) and indicate an origin of plant waxes from crops other than rice. Based on comparison with recent crop plants, the n–alkane distribution at this site is governed by input of wax lipids from the upland crop rape (*Brassica napus*). Very low CPI_{long} values of 2.5 for P700 are due to admixture of fossil fuel with a CPI near unity and thus support the UCM and hopanoid data. For all CPI_{long} values CV values are between 5 to 20 % showing no preference for source or degree of diagenesis.

The P_{aq}-ratio established by Ficken et al. (2000) has been used to determine the relative contribution of submerged aquatic macrophytes to the sedimentary n–alkane load. Values for all paddies except for P500 are close to that of the TW substrate and only the FW reference sites reveal enhanced values of 0.34 (Table 1). The exceptionally low P_{aq} of P500 is due to an origin of alkanes from non-aquatic plants and emphasizes an origin from upland crops growing under dry conditions. During time of sample acquisition the P500 site was used as a paddy soil and had been under this utilization for more than 3 yr. The molecular composition of lipids, in particular n–alkanes, from this site however clearly reflects its previous long-term use as a non-paddy upland cropping site. The time-integrative manner of conservative molecular biogeochemical indicators unravelling the temporally dominating land-use of soils is well illustrated in the P500 case. Despite the coexistence of older (<3 yr) and fresh lipids, the CV for various molecular parameters is only 12 to 17 %.

For all sites the CV values are below 20 % and confirm that application of molecular proxies for source identification, degree of diagenetic overprinting and fossil fuel contamination are very robust and reliable.

3.2.3 Integrating conservative and labile parameters over cultivation times

Calculation of averages for the most important conservative parameters (TC, TOC, TN, TS, lipid yield, alkane yield, $\delta^{13}C$, $\delta^{15}N$, χ, L^*, a^*, and b^*) resulted in CV values of <20 % for all cultivation times as shown in Fig. 3c. A prominent outlier (exceeding the 95 percentile) was the n-alkane yield at P700 that is controlled by addition of fossil fuel contaminants to this site.

A comparison of the P50 and P50N site reveals differential behaviour that can be explained by management practices. Soil at the P50 site has experienced a long series of redox cycles like all other paddy soils, whereas the P50N site as a rice seedling nursery is kept under flooded conditions for longer times longer and thus has been going through less frequent and less dramatic redox cycles. As a result paddy soil evolution at the P50N site proceeded further compared to other sites of comparable overall cultivation time. A notable exception is the P_{aq} ratio that shows a CV comparable to the P50 or P100 sites (Fig. 3c), indicating that water table fluctuations at the P50N site affect wax lipid composition of rice seedlings, even if the soils do not pass through completely dry cycles.

Except for the P1000 site where interferences due to dyke maintenance have been reported (Jahn et al., 2011; Roth et al., 2011), a decline in the CV values over cultivation time is noted (Fig. 3c). This can be explained by an increasing degree of paddy soil evolution and homogenization of SOM and minerogenic composition, accompanied by stabilization in soil microbial community structure. This establishment of quasi-continuous composition in conservative paddy soil biogeochemical parameters was established after only 300 yr of cultivation time.

The averages for labile parameters according to cultivation age depicted in Fig. 3d show a much higher degree of variability, with the lower and upper boundaries of CV values for the 75 percentile ranging between 20 and 40 % (Fig. 3d). Outliers exceeding the 95 percentile are nitrate for the P50, DON for the P700, and ammonium for the P2000 site. All of these spatially highly variable parameters are associated to reactive

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compounds of the nitrogen cycle and are highly influenced by spatially non-systematic human manipulation such as fertilization. Additionally, the presence of biopores and cracks in the plough pan could contribute to irregular leaching processes coupled with a high variability of these water soluble parameters within a field (Sander and Gerke, 2007). The approximately 20 times higher ammonium content in P2000 could have been induced by uneven manual application of nitrogen fertilizer and an inefficient field management practices just prior to sampling (see also Roth et al., 2011). Other indicators of nitrogen cycling in paddy soil including microbial N ($CV_{avg} = 33$ %), C/N ($CV_{avg} = 5$ %), or $\delta^{15}N$ ($CV_{avg} = 3$ %) behave stable and demonstrate the establishment of a well controlled nitrogen cycle in paddy soils.

3.3 Inter-site variability

Reliable identification of differences in biogeochemistry between individual paddy fields and interpretation of evolutionary trends according to cultivation time, physiogeographical properties, management practises, etc. can only be achieved, if the intra-site heterogeneity is smaller than inter-site differences in biogeochemistry. We thus tested individual parameter relationships and applied statistical approaches to the entire data set employing PCA and non-parametric tests for variance analysis as well as the Kruskal-Wallis test to verify that inter-site exceeds intra-site variation. Finally, a cluster analysis was performed to elucidate, if duration of paddy soil management and associated soil evolution leads to establishment of robust clusters of comparable soil properties for the different paddies.

Examination of pairs of individual parameters revealed that in general values for each site clustered closely and only moderate overlap between site clusters occurred. This is exemplarily shown in Fig. 5a, b, c for $\delta^{13}C$ vs. $\delta^{15}N$, χ vs. a^* and $\delta^{15}N$ vs. P_{aq}, respectively. The binary plots demonstrate that individual single parameters often do not show a clear separation between sites, whereas addition of a second dimension allows full discrimination of the site clusters. The bulk isotope parameters show no

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overlap between site clusters and clearly separate the soils from the marine and limnic substrates (Fig. 5a). Soil magnetic susceptibility and colour parameters depending on minerogenic composition and expression of redoximorph features also show clear separation of site clusters with little to no overlap (Fig. 5b), except for one outlier in soil colour at the FW site. Combinations of molecular compositional and isotope parameters are suitable for site differentiation also exhibiting less intra-site versus inter-site variability (Fig. 5c). Hereby the variance in P_{aq} ratios is substantially higher than for δ^{15}N signatures. In general, overlap due to spread in one parameter is more frequent in young, less well developed or in disturbed soils (P1000).

The Kruskal-Wallis test is employed in ecological, biogeochemical, and environmental quality studies to evaluate, whether variance between sites exceeds variance within sites (Gratton et al., 2000; Katsaunos et al., 2007; Lehndorff and Schwark, 2008). Including all sites and parameters the asymptotic significance gave $p < 0.001$, except for the C/N ratio where a value of 0.004 was reached (Table 2), indicating that sites are less well distinguishable. Nevertheless the critical H values for all sites did not exceed the H-values proposed for the null-hypothesis (Table 2), implying that a full discrimination of all sites using median values of any of the selected parameters was possible. If the data set was reduced to contain only the paddy sites, i.e. P500, TW, and FW excluded (Table 2), asymptotic significance values for the C/N, CPI$_{short}$ and P_{aq}-ratios for $p > 0.01$ could not be met. Furthermore, the median-referred critical H-values exceeded the H-values for the following parameters: TS, C/N, TOC, extract yield and CPI$_{short}$ (Table 2), indicating that the intra-site variance was comparable to or exceeded inter-site variance. As most of these parameters represent concentrations that are primarily related to the absolute amount of soil organic matter rather than its compositional differences, a discrimination of sites using these such indicators is not feasible.

Application of principal component analysis allows evaluating the entire data set and was carried out on all paddy soils using all parameters determined (Fig. 5d), and for all paddy soils employing conservative parameters only (Fig. 5e). A full discrimination of all sites was achieved (Fig. 5d), when using the 1st and 2nd regression factors

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of all parameters, explaining 39.8 and 17.3% of variance in the data set (Table 3). Factor 1 exhibits highest loading by parameters associated with soil organic matter concentration, whereas the 2nd factor is primarily controlled by minerogenic composition parameters (Table 3). No overlap of parametric values between the individual sites was observed, which was taken as direct evidence that the intrinsic heterogeneity of paddy soil does not exclude detailed interpretation of biogeochemical differences between sites.

If the data set was reduced to the conservatively behaving parameters, the discriminative power was reduced (Fig. 5e), whereby preferentially overlap for younger sites was observed. The 1st and 2nd regression factors for the data restricted to conservative parameters explain 50.6 and 14.2% of the variability and are controlled by organic matter concentration and n−alkane compositions, respectively (Table 4). The similarity in biogeochemical properties concerning the conservative parameters, in particular for the P50 and P100 sites, can be attributed to the low evolutionary stage of the paddy soils. All sites under paddy cultivation for 300 yr and more have developed individual soil characteristics as mentioned above when discussing CV for individual age classes. Not only the duration of paddy soil utilization is of critical importance but also the individual management practice. Two sites used for 50 yr of rice cultivation were investigated, whereby one of these sites was used as nursery (P50N) for growing rice seedling prior to transplantation. As the P50N site is consistently kept under flooded conditions, soil evolution proceeds differently from the P50 site. This allows full discrimination of the P50N from the P50 and P100 sites, whereas the latter two do show considerable overlap, when PCA is conducted (Fig. 5e).

Including the reference substrates in PCA for the conservative parameters reveals a more pronounced differentiation of the substrates from the soils that develop on them when 2nd factor and 3rd factor extracted by PCA, explaining 18.3% and 17.0 percent of the variance (Table 5) are used for discrimination (Fig. 5f). The abandonment of factor 1 explaining 37.93 percent of variance in the regression analysis leads to incomplete separation of the individual paddy sites emphasizing the importance of this factor in

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discriminant analysis. The properties exhibiting the highest loading scores on the 1st factor are organic matter concentration-related, those for the 2nd factor are governed by alkane composition, isotope signature and soil brightness, those for the 3rd factor are dominated by properties linked to redox-conditions (Table 5).

Cluster analysis performed to evaluate whether the statistical approach would group individual sites in clusters based on the entirety of all biogeochemical parameters determined is presented in Fig. 6. All older paddy soils not affected by secondary alteration (P500: extended non-paddy use, P1000: removal of surface soil for dyke maintenance) are clustered appropriately, whereas the younger soils exhibit insufficient development of individual biogeochemical paddy soil characteristics. Contamination of the P700 site did not lead to a significant change in time-integrated basic soil biogeochemical parameters but preferentially affected the aliphatic hydrocarbon composition. This indicates that the addition of the petroleum contaminants did not detrimentally affect the soil microbial community or inhibited plant growth by adding toxic substances or providing alternative substrates for microbial utilization. Seen from a temporal perspective, the P700 site acquired its biogeochemical profile over a time span of about 700 yr, whereas the minor petroleum contamination is assumed to have occurred only a few years ago and thus has negligible influence on the overall biogeochemical status. In a similar but opposite fashion, the P500 site reveals the cumulative biogeochemical characteristics of almost 500 yr of use as upland cropping site and only recently (app. 3 yr before sampling) was converted to a paddy field. Consequently, this site still exhibits the time-integrated features of a non-paddy land management and clusters with the very young paddies (P50, P50N, P100) developed on a marine tidal substrate (Fig. 6). Similarly, the P1000 site, though continuously utilized as paddy field, groups with the young soils due to repetitive removal of surface soils and dilution with soil material of non-paddy origin. Both of these sites, the P500 as well as P1000 exhibit four subsamples of close similarity and one subsample of largely deviating character, indicating the large intra-site variation caused by human interference.

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Although several sites of the chronosequence studied have been affected by anthropogenic perturbations, the cluster analysis indicates that paddy soil evolution over time led to the establishment of stable biogeochemical properties and conditions, even if permanent human intervention via soil management and utilization prevailed.

3.4 Organic matter accumulation and sequestration trends

The environmental budget of paddy soils is under debate and considered to show a negative balance due to the emission of greenhouse gases and intensive nitrate loss from paddies (IPPC, 2007; Conrad, 2009; Koegel-Knabner et al., 2010). On the other hand a positive balance could be attributed to paddy soils based on intensive atmospheric CO_2 sequestration via surface soil accumulation and preservation of fresh photosynthate. The chronosequence studied here offers the opportunity to evaluate CO_2 sequestration in paddies, comparison with non-paddy sites (P500) and interferences via intentional management (P1000) or unintentional contamination (P700). The accumulation trends for TOC, lipids and $n-$alkanes over cultivation time are shown in Fig. 7a–c, complemented by the accumulation of lipids and alkanes normalized to TOC (Fig. 7d, e). The TOC concentrations of paddy soil reach app. 1 % after 50 yr of cultivation, i.e. more than double the concentration of the substrate (Table 1). Increase in TOC continues to be rapid until about 300 yr and levels off to reach maximum concentrations after 2000 yr of cultivation. Severely lower TOC concentrations are noted for the P500 site, which might indicate a use as non-paddy for a longer period and thus has accumulated much less TOC compared to a paddy soil. The P1000 site is assumed to have accumulated TOC continuously but has lost about 50 to 70 % of this TOC due to human interference.

The lipid concentration of the paddy soils reveals a similar accumulation pattern though lipids are less stable than recalcitrant TOC (Wiesenberg et al., 2004; Marschner et al., 2006) that includes non-extractable fractions, e.g. black carbon from incomplete combustion of rice straw. Normalization of lipid yield to soil TOC indicates that these constitute app. 5 to 6 % of the organic carbon (Fig. 7d) and that the relative

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concentration increases with cultivation age. This indicates that the labile proportion of organic input into paddy soils and/or the standing microbial biomass is better preserved in long cultivated paddy soils.

The n−alkane accumulation trends exhibit not only negative perturbations as did TOC and lipid yield but also a pronounced increase at the P700 site due to fossil fuel contamination (Fig. 7c). This externally added n−alkane fraction even increases in proportion, if normalized to TOC concentrations (Fig. 7e). Exclusion of the P700 site still results in an increase of n−alkanes over time, which is explained by the lower mineralization of n−alkanes compared to functionalized lipids, e.g. fatty acids or alcohols. These components undergo oxidation and decarboxylation reactions upon diagenesis, finally leading to generation and accumulation of n−alkanes.

Aberrations in the accumulation of organic matter and organic matter fractions can be sufficiently explained by applications of molecular proxies, unravelling deviating sources of organic input or fossil fuel contamination. A different input of plant material, preferentially rape based on comparison of n−alkane distributions with recent reference crops, at the P500 site revealed this site to have been used as upland field for prolonged periods. Exceptional concentrations and compositional differences in aliphatic hydrocarbons, in particular n−alkanes, hopanes and UCM identify human perturbations of the soil ecosystem by petroleum contamination.

4 Conclusions

Biogeochemical proxies determined on five field replicates of paddy soils differing in cultivation age and two substrates on which these paddy soils evolved showed that the intrinsic heterogeneity of paddy soil organic and minerogenic components is smaller than differences in biogeochemical properties between study sites. This conclusion was drawn based on interpretation of individual parameters, descriptive and non-parametric statistical analysis, PCA and cluster analysis. The coefficient of variation for conservative parameters determined in pentuplicate and reflecting time-integrated

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evolution of soil properties in general was 10 % or lower. Based on this study, collection of one composite field sample is considered sufficient for generation of representative biogeochemical data in paddy soils. In field heterogeneity of fast cycling and anthro-pogenically amended nutrients was found very high with coefficients of variation usually between 20 and 40 % and frequent outliers. Sampling strategies covering the heterogeneity of such parameters will require much higher sampling frequency and spatial resolution.

Biogeochemical properties acquired by paddy soils over centennial periods of time behave conservative and do adapt not rapidly, if management conditions or practices are altered. Hence, previous historic land use or management practices can be reconstructed, even after new utilization has been established.

Duration of cultivation as paddy soil leads to establishment of specific soil characteristics that become increasingly stable with cultivation time. For paddies evolving on marine tidal substrates as in this study, the full development of paddy biogeochemical signatures was completed in less than 300 yr.

The environmental/ecological budget of paddy soils in this study revealed a positive balance, when sequestration of atmospheric CO_2 was considered. Perturbation of paddy soils leading to severely reduced sequestration potential can be identified by application of molecular source proxies. Thus the integrity of the carbon accumulation history of paddy soils in unknown areas can be critically evaluated.

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Table 1. Descriptive statistics of all biogeochemical parameters determined for the 10 study sites. P50N designates the seedling nursery paddy, TW designates the marine tidal flat substrate and FW designates the freshwater limnic substrate. AV = average value, SD = standard deviation, CV = coefficient of variation. Conservative parameters were grouped TC to b^*(D65), labile parameters were grouped N_{mic} to DOC.

	P50			P50N			P100			P300			P500		
	AV	SD	CV	AV	SD	CV	AV	SD	CV	AV	SD	CV	AV	SD	CV
TC [%]	1.49	0.17	11.3	1.33	0.11	8.1	1.40	0.27	19.2	2.41	0.10	4.1	1.39	0.07	4.8
TOC [%]	0.99	0.14	14.2	1.04	0.07	6.3	1.39	0.28	20.0	2.25	0.12	5.3	1.33	0.11	8.4
TIC [%]	0.50	0.06	12.8	0.29	0.08	26.6	0.02	0.02	98.2	0.17	0.10	63.0	0.06	0.08	140.1
TN [%]	0.118	0.017	14.5	0.122	0.014	11.1	0.157	0.026	16.7	0.261	0.010	3.9	0.158	0.012	7.5
TS [%]	0.032	0.006	19.6	0.031	0.001	4.3	0.030	0.005	16.9	0.056	0.010	17.7	0.030	0.004	14.1
C/N	8.37	0.19	2.3	8.54	0.78	9.1	8.85	0.59	6.6	8.63	0.40	4.6	8.44	0.37	4.4
Lipids [mg kg^{-1} dw]	553	115	20.8	544	42	7.7	735	154	21.0	1210	51	4.3	651	50	7.6
Lipids [g kg^{-1} TOC]	56	5	9.0	52	3	6.1	53	4	8.4	54	4	6.7	49	3	5.5
n-Alkanes [µg kg^{-1} dw]	1531	310	20.3	1271	96	7.6	2141	446	20.8	3440	221	6.4	2346	186	7.9
n-Alkanes [mg kg^{-1} TOC]	155	30	19.0	123	15	12.0	154	16	10.5	153	13	8.6	176	10	5.7
CPI$^b_{short}$	1.8	0.2	11.9	1.6	0.2	10.1	1.4	0.1	3.6	1.3	0.1	9.2	1.4	0.2	12.9
CPI$^c_{long}$	5.7	0.8	14.6	4.2	0.9	21.4	5.8	1.0	18.1	6.1	0.5	8.4	10.4	1.8	17.0
P^d_{aq}	0.24	0.05	19.2	0.22	0.05	22.2	0.19	0.03	16.1	0.19	0.01	3.4	0.07	0.01	17.6
δ^{13}C [‰]	−28.0	0.4	−1.4	−27.1	0.3	−1.2	−28.2	0.2	−0.6	−28.5	0.1	−0.4	−28.0	0.1	−0.4
δ^{15}N [‰]	3.1	0.1	2.9	5.7	0.3	4.4	4.6	0.1	2.9	2.7	0.3	11.4	5.6	0.1	2.6
χ [10^{-8} m^3 kg^{-1} dw]	26.1	1.1	4.1	24.7	0.3	1.4	14.8	1.1	7.2	11.8	0.9	7.7	21.3	0.8	3.9
L^* (D65)	55.6	0.4	0.7	56.3	0.6	1.0	55.3	0.4	0.7	54.1	0.6	1.1	55.0	0.6	1.0
a^* (D65)	3.7	0.1	2.8	3.6	0.1	4.1	3.7	0.1	3.5	3.3	0.1	2.0	3.2	0.0	1.3
b^* (D65)	15.3	0.2	1.3	15.1	0.5	3.0	14.8	0.2	1.1	14.0	0.3	2.1	13.6	0.4	2.6
N_{mic} [µg g^{-1} dw]	33.4	8.5	25.5	45.1	5.8	12.9	37.6	3.3	8.8	4.1	1.1	27.9	15.6	5.3	33.7
C_{mic} [µg g^{-1} dw]	293	103	35.0	558	61	11.0	522	108	20.6	167	33	19.5	490	93	18.9
Nitrate [µg N g^{-1} dw]	2.7	1.8	67.1	3.6	1.1	30.8	7.5	3.9	52.1	27.5	12.0	43.5	12.0	3.2	27.0
Ammonium [µg N g^{-1} dw]	0.4	0.1	33.3	0.5	0.4	83.8	0.2	0.1	30.8	0.6	0.3	59.0	0.1	0.0	13.1
DON [µg g^{-1} dw]	2.2	0.9	42.1	3.0	0.6	19.9	5.2	1.3	24.3	5.8	1.4	23.3	12.0	2.6	21.8
DOC [µg g^{-1} dw]	21.5	7.7	36.0	9.3	2.8	29.9	14.7	4.6	31.2	16.4	5.2	31.5	23.6	4.2	17.9

[a] $\sum$ n-alkanes = n-C$_{13-40}$

[b] CPI$_{short}$: 0.5* ([(n-C$_{15}$ + n-C$_{17}$ + n-C$_{19}$ + n-C$_{21}$)/(n-C$_{14}$ + n-C$_{16}$ + n-C$_{18}$ + n-C$_{20}$)]+ [(n-C$_{15}$ + n-C$_{17}$ + n-C$_{19}$ + n-C$_{21}$)/(n-C$_{16}$ + n-C$_{18}$ + n-C$_{20}$ + n-C$_{22}$)])

[c] CPI$_{long}$: 0.5* ([(n-C$_{25}$ + n-C$_{27}$ + n-C$_{29}$ + n-C$_{31}$)/(n-C$_{24}$ + n-C$_{26}$ + n-C$_{28}$ + n-C$_{30}$)]+ [(n-C$_{25}$ + n-C$_{27}$ + n-C$_{29}$ + n-C$_{31}$)/(n-C$_{26}$ + n-C$_{28}$ + n-C$_{30}$ + n-C$_{32}$)])

[d] P_{aq}: (n-C$_{23}$ + n-C$_{25}$)/(n-C$_{23}$ + n-C$_{25}$ + n-C$_{29}$ + n-C$_{31}$)

Table 1. Continued.

	P700			P1000			P2000			TW			FW			all soils		
	AV	SD	CV	AV	SD	CV	AV	SD	CV	AV	SD	CV	AV	SD	CV	AV	SD	CV
TC [%]	2.10	0.19	8.9	1.16	0.11	9.4	3.05	0.13	4.3	1.28	0.02	1.6	1.62	0.17	10.3	1.80	0.65	36.2
TOC [%]	2.00	0.09	4.7	1.10	0.10	8.9	2.85	0.10	3.5	0.49	0.06	12.4	1.51	0.21	14.0	1.71	0.70	41.0
TIC [%]	0.12	0.12	98.8	0.06	0.03	60.7	0.20	0.13	67.7	0.79	0.05	6.3	0.10	0.11	101.2	0.09	0.12	126.2
TN [%]	0.208	0.022	10.5	0.128	0.017	13.2	0.361	0.018	4.9	0.052	0.011	21.4	0.148	0.028	19.1	0.191	0.082	42.9
TS [%]	0.050	0.006	12.2	0.026	0.004	17.0	0.057	0.005	9.6	0.047	0.004	8.0	0.035	0.007	20.1	0.039	0.013	34.1
C/N	9.68	0.77	8.0	8.67	0.57	6.5	7.93	0.61	7.7	9.63	1.99	20.6	10.38	1.34	12.9	8.99	0.74	8.2
Lipids [mg kg^{-1} dw]	1358	143	10.5	639	54	8.4	1764	73	4.2	862	248	28.7	1438	177	12.3	941	440	46.8
Lipids [g kg^{-1} TOC]	68	8	11.7	59	10	16.7	62	2	3.4	176	49	28.1	95	6	5.9	54	8	13.9
n-Alkanes [µg kg^{-1} dw]	6557	1456	22.2	1690	146	8.7	5806	961	16.6	975	102	10.5	4551	1341	29.5	3.138	2.017	64.3
n-Alkanes [mg kg^{-1} TOC]	330	80	24.4	153	8	5.1	204	34	16.9	200	11	5.6	299	74	24.8	175	65	37.1
CPI$_{short}^{b}$	1.6	0.1	5.7	1.5	0.2	16.3	1.7	0.2	10.4	1.3	0.1	6.6	2.1	0.2	10.5	1.5	0.2	13.5
CPI$_{long}^{c}$	2.5	0.1	5.0	4.2	0.9	20.3	4.1	0.2	4.1	3.4	0.3	9.5	4.0	0.4	9.6	5.4	2.4	44.9
P_{aq}^{d}	0.24	0.01	5.8	0.24	0.04	17.5	0.25	0.01	5.2	0.26	0.02	6.0	0.34	0.03	7.6	0.21	0.06	30.2
δ^{13}C [‰]	-28.0	0.0	-0.2	-25.9	0.2	-0.6	-29.4	0.1	-0.3	-24.4	0.1	-0.2	-22.6	0.4	-2.0	-27.9	1.0	-3.6
δ^{15}N [‰]	4.9	0.2	4.0	5.3	0.2	3.7	2.1	0.3	13.4	4.6	0.2	3.7	1.6	0.2	11.6	4.3	1.3	31.3
χ [10^{-8} m^3 kg^{-1} dw]	15.5	0.8	4.9	15.5	2.9	18.4	12.0	1.0	8.7	62.8	0.7	1.1	16.0	2.1	13.1	17.5	5.3	30.3
L^* (D65)	55.1	0.7	1.2	57.1	0.8	1.4	57.2	0.2	0.4	56.4	0.4	0.7	58.7	0.2	0.3	55.7	1.1	2.1
a^* (D65)	2.8	0.1	2.9	2.9	0.1	4.8	1.8	0.2	8.5	3.7	0.2	4.1	2.7	0.3	12.4	3.1	0.6	19.9
b^* (D65)	14.5	0.2	1.2	15.2	0.5	3.4	12.4	0.7	5.7	14.5	0.3	2.2	14.3	0.7	5.2	14.3	1.0	6.9
N_{mic} [µg g^{-1} dw]	30.0	6.2	20.8	37.7	3.8	10.1	32.6	12.4	38.0	27.4	5.6	20.3	57.2	26.6	46.4	29.4	14.1	47.8
C_{mic} [µg g^{-1} dw]	535	43	8.1	331	71	21.4	4043	1591	39.4	255	41	16.2	1006	616	61.3	882	1340	151.9
Nitrate [µg N g^{-1} dw]	7.0	2.8	40.0	6.5	0.8	12.3	15.1	5.0	33.5	3.9	2.3	60.9	21.9	20.9	95.5	10.4	9.0	85.9
Ammonium [µg N g^{-1} dw]	0.2	0.0	19.3				6.6	5.8	87.9	0.6	0.2	32.6	12.4	8.4	68.2	1.1	2.9	262.7
DON [µg g^{-1} dw]	9.7	6.4	65.4	4.4	0.9	20.6	10.7	3.0	27.7	0.7	0.5	72.8	21.0	9.5	45.5	6.8	4.3	63.5
DOC [µg g^{-1} dw]	21.1	4.0	18.9	8.1	2.1	26.0	34.7	7.7	22.1	13.6	2.8	20.3	30.7	20.4	66.3	18.6	9.4	50.5

Table 2. Non-parametric variance analysis by Kruskal-Wallis test, suitable for non-normal distributed data sets, performed for all sites ($n = 49$) and for paddy sites only ($n = 34$). Significant variation between sites is indicated, H values from Chi-squared test exceed the critical H-values of the null-hypothesis. Parameters indistinguishable between sites because intra-site variance exceeds inter-site variance are plotted in italic.

	all sites				all paddy sites			
	H	df	p	Hcrit	H	df	p	Hcrit
TC [%]	40.824	9	0.000	28.992	29.524	6	0.000	27.800
TOC [%]	43.880	9	0.000	39.396	27.995	6	0.000	29.200
TIC [%]	33.971	9	0.000	28.191	22.712	6	0.001	14.800
TN [%]	43.005	9	0.000	36.195	29.282	6	0.000	29.200
TS [%]	38.561	9	0.000	37.995	26.502	6	0.000	27.800
C/N	24.233	9	0.004	17.187	16.390	6	0.012	16.400
TOC/S	30.601	9	0.000	23.389	19.565	6	0.003	16.400
Lipids [mg kg^{-1} dw]	41.197	9	0.000	40.997	28.920	6	0.000	29.200
Lipids [g kg^{-1} TOC]	37.719	9	0.000	29.992	17.955	6	0.006	18.200
n-Alkanes [µg kg^{-1} dw]	44.868	9	0.000	39.396	30.076	6	0.000	29.200
n-Alkanes [mg kg^{-1} TOC]	41.218	9	0.000	36.195	26.051	6	0.000	18.200
CPI$_{short}$	30.716	9	0.000	28.191	16.890	6	0.010	18.000
CPI$_{long}$	39.956	9	0.000	36.195	25.216	6	0.000	22.800
P_{aq}	35.325	9	0.000	25.190	14.853	6	0.021	14.000
δ^{13}C [‰]	45.219	9	0.000	37.995	30.028	6	0.000	23.600
δ^{15}N [‰]	46.165	9	0.000	40.997	31.663	6	0.000	29.200
χ [10^{-8} m^3 kg^{-1} dw]	44.184	9	0.000	36.195	29.069	6	0.000	21.200
L^* (D65)	42.415	9	0.000	40.997	27.689	6	0.000	23.600
a^* (D65)	44.108	9	0.000	39.396	30.311	6	0.000	29.200
b^* (D65)	37.053	9	0.000	28.191	26.286	6	0.000	21.200
N_{mic} [µg g^{-1} dw]	33.908	9	0.000	24.190	21.355	6	0.002	16.600
C_{mic} [µg g^{-1} dw]	41.157	9	0.000	36.195	28.730	6	0.000	26.000
Nitrate [µg N g^{-1} dw]	35.111	9	0.000	28.191	25.720	6	0.000	19.600
Ammonium [µg N g^{-1} dw]	41.891	9	0.000	37.995	26.021	6	0.000	15.000
DON [µg g^{-1} w]	43.210	9	0.000	39.396	27.202	6	0.000	21.200
DOC [µg g^{-1} dw]	31.265	9	0.000	28.992	25.586	6	0.000	21.400

Table 3. Factor loadings table obtained from PCA performed with all paddy soils and all parameters illustrated in Fig. 5d.

analysis 1	factor loadings			
	1	2	3	4
TC	0.932	0.306	−0.054	−0.061
TN	0.882	0.427	−0.062	0.127
TS	0.836	0.267	−0.102	−0.336
C/N	−0.028	−0.122	0.108	−0.790
TOC/S	0.373	0.611	−0.041	0.396
TOC	0.885	0.437	−0.063	−0.056
CPI_{short}	0.274	−0.488	0.509	0.005
CPI_{long}	−0.028	−0.264	−0.842	0.265
P_{aq}	0.100	−0.155	0.687	0.017
$\delta^{15}N$	−0.860	0.085	0.317	−0.127
$\delta^{13}C$	−0.874	0.021	0.222	−0.035
$L^*(D65)$	−0.075	0.044	0.651	0.556
$a^*(D65)$	−0.646	−0.522	−0.439	−0.157
$b^*(D65)$	−0.817	−0.369	−0.019	−0.149
χ	−0.370	−0.839	0.263	−0.096
Lipids	0.835	0.502	0.122	−0.117
n-Alkanes	0.677	0.460	0.320	−0.389
TIC	−0.170	−0.926	0.078	0.002
N_{mic}	−0.410	−0.183	0.571	0.415
C_{mic}	0.711	0.143	0.318	0.541
Nitrate	0.464	0.370	−0.634	−0.033
Ammonium	0.648	0.065	0.186	0.533
DON	0.529	0.507	0.205	0.038
DOC	0.845	−0.026	0.218	0.155

Table 4. Factor loadings table obtained from PCA performed with all paddy soils and all conservative parameters illustrated in Fig. 5e.

analysis 2	factor loadings			
	1	2	3	4
TC	0.987	0.009	0.006	−0.018
TN	0.964	0.028	−0.191	−0.111
TS	0.897	0.001	0.304	−0.051
C/N	−0.019	0.137	0.815	0.035
TOC/S	0.533	0.093	−0.512	−0.383
TOC	0.983	0.052	−0.018	−0.133
CPI_{short}	0.094	0.195	−0.025	0.812
CPI_{long}	−0.064	−0.909	−0.173	−0.101
P_{aq}	−0.008	0.608	−0.133	0.532
$\delta^{15}N$	−0.799	0.383	0.170	−0.249
$\delta^{13}C$	−0.840	0.322	0.006	−0.158
$L^*(D65)$	−0.106	0.579	−0.617	0.233
$a^*(D65)$	−0.748	−0.543	0.283	0.047
$b^*(D65)$	−0.899	−0.081	0.233	0.036
χ	−0.614	−0.054	0.230	0.654
Lipids	0.950	0.247	0.031	−0.141
n-Alkanes	0.796	0.445	0.306	−0.106

Table 5. Factor loadings table obtained from PCA performed with all sites and all conservative parameters illustrated in Fig. 5f.

analysis 3	factor loadings			
	1	2	3	4
TC	0.942	−0.161	0.207	0.061
TN	0.829	−0.245	0.477	−0.022
TS	0.871	−0.211	−0.345	0.174
C/N	0.055	0.629	−0.391	0.075
TOC/S	0.271	0.050	0.894	−0.200
TOC	0.871	−0.139	0.451	0.001
CPI_{short}	0.131	0.758	0.302	0.061
CPI_{long}	−0.154	−0.207	0.152	−0.910
P_{aq}	0.093	0.568	−0.085	0.773
$\delta^{15}N$	−0.625	−0.465	−0.114	−0.131
$\delta^{13}C$	−0.307	0.771	−0.365	0.162
$L^{*}(D65)$	0.003	0.798	0.089	0.279
$a^{*}(D65)$	−0.750	−0.293	−0.406	−0.047
$b^{*}(D65)$	−0.813	−0.038	−0.039	0.416
χ	−0.261	0.067	−0.904	0.033
Lipids	0.905	0.260	0.100	0.187
n-Alkanes	0.797	0.124	0.286	0.170
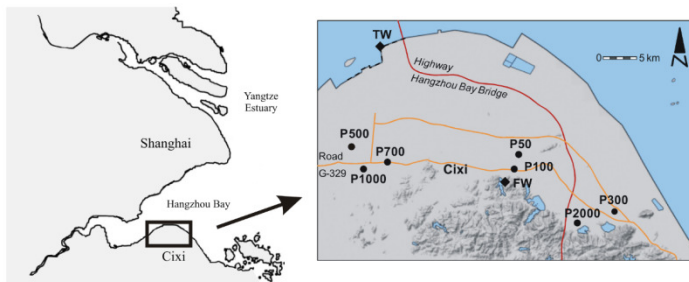
Fig. 1. Map of study area, depicting generations of dykes constructed for land reclamation purposes (adapted from Jahn et al., 2011) and sampling locations.
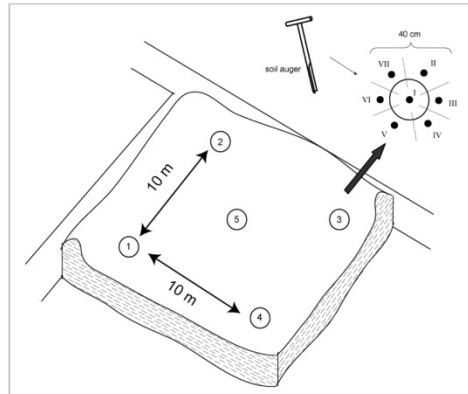
Fig. 2. Design for recovery of field replicates at 10 m regular spacing, each of which is a composite of 7 subsamples taken at 40 cm regular spacing.
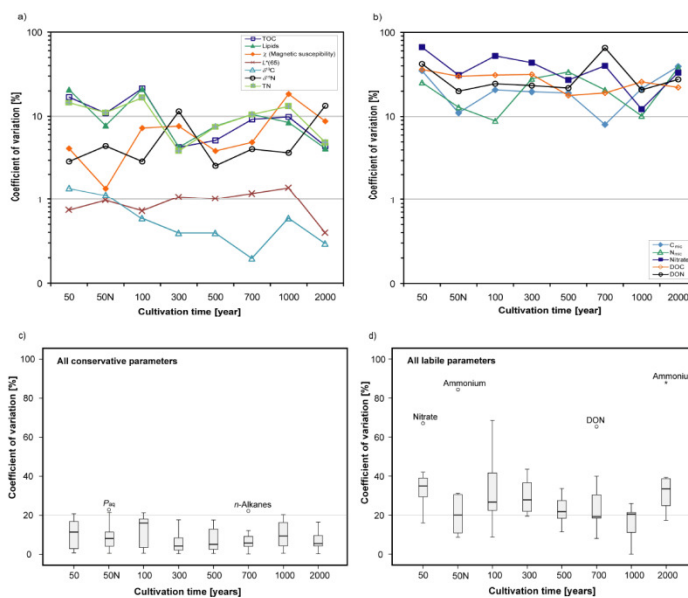
Fig. 3. Coefficients of variation for paddy soil sites sampled in pentuplicate with **(a)** conservative parameters, **(b)** labile parameters, **(c)** box and whisker-plots showing median value, 75 percentile, 90 percentile and outliers for conservative parameters, **(d)** same as **(c)** but for labile parameters.
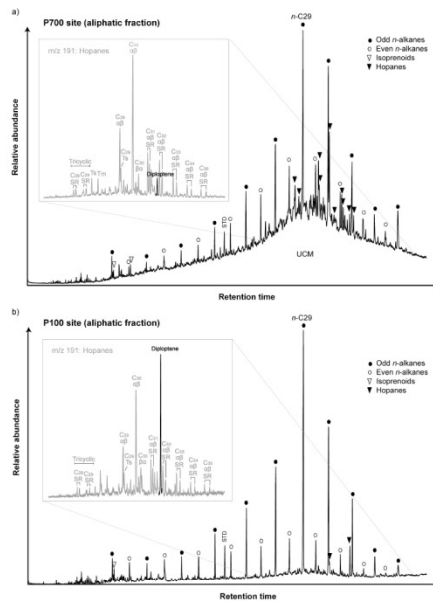
Fig. 4. GC/MS total ion traces obtained from aliphatic hydrocarbon fractions, with major peaks labelled for identification. The inset shows the extracted mass fragmentogram of m/z = 191, indicative for tri- and pentacyclic triterpenoids. Diploptene marked black is indicative of recent bacteria, hopanes and tricyclics in grey derive from fossil fuel contamination. Peaks are labelled according to number of carbon atoms per molecule and isomerisation at position C17, C21 and C22. Ts = trisnorhopane, Tm = Trisnorneohopane. Note high abundance of fossil fuel hopanes vs. recent diploptene in P700.

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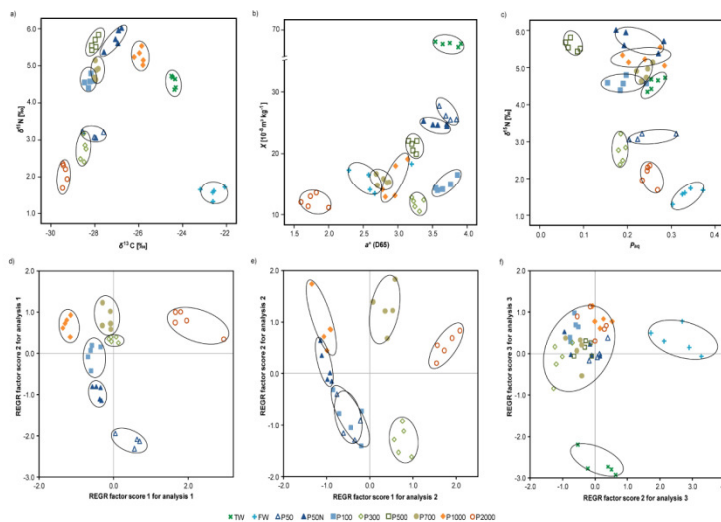
Fig. 5. Discrimination of variance between sites versus in-site using descriptive statistics **(a)** to **(c)** and non-parametric and multivariate methods **(d)** to **(f)**. The factor plots obtained from PCA are shown for **(d)** all paddy soils using all parameters, **(e)** all paddy soils using exclusively conservative parameters, and **(f)** using all paddy soils, non-paddy soil P500 and substrates FW and TW. Note that discrimination of substrates was achieved best, when using the 2nd and 3rd factor rather than 1st and 2nd factor as in **(d)** and **(e)**.

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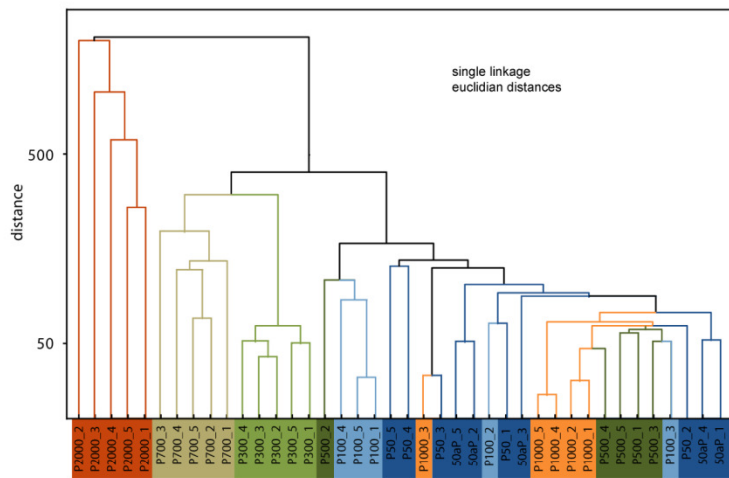
Fig. 6. Cluster diagram for paddy soils and non-paddy soil P500, constructed using all parameters.
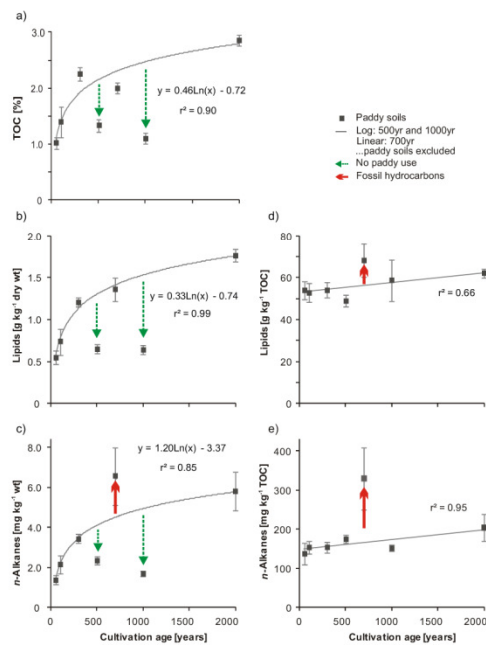
Fig. 7. Accumulation trends of **(a)** TOC, **(b)** lipid yield and **(c)** *n*-alkane yield normalized to dry sample weight and **(d)** lipid yield and **(e)** *n*-alkane yield normalized to TOC over cultivation time. Arrows denote deviations from natural accumulation trends due to human disturbance of the paddy soil system. The P500 was used as upland field and only recently converted to paddy soil use, the P1000 site experienced topsoil removal and admixture of other soil material in the course of dyke maintenance work, the P700 site suffers from petroleum contamination.

Publication V

Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils

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ABSTRACT

We developed an improved protocol, allowing the simultaneous extraction of DNA and RNA from soil using phenol-chloroform with subsequent column-based separation of DNA and RNA (PCS). We compared this new approach with the well established protocol published by Griffiths et al. (2000), where DNA and RNA are separated by selective enzymatic digestions and two commercial kits used for DNA or RNA extraction, respectively, using four different agricultural soils. We compared yield and purity of the nucleic acids as well as abundance and diversity profiles of the soil bacterial communities targeting the *nosZ* gene via quantitative real-time PCR and terminal restriction fragment length polymorphism on DNA and RNA level. The newly developed protocol provided purer nucleic acid extracts compared to the used kit-based protocols. All protocols were suitable for DNA- and RNA-based gene quantification, however high variations between replicates were obtained for RNA samples using the original Griffiths protocol. Diversity patterns of *nosZ* were highly influenced by the extraction protocol used both on the DNA and RNA level. Finally, our data showed that the new protocol allows a simultaneous and reproducible extraction and separation of DNA and RNA, which were suitable for reliable analyses of gene and transcript copy numbers and diversity pattern.

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1. Introduction

Soils represent one of the most complex environments on earth providing living space for a huge number of different microbes. Enormous progress has been made in recent years by means of molecular methods like quantitative real-time PCR (qPCR), fingerprinting techniques and sequencing to gain information about abundance, composition and diversity of microbial communities in these habitats (Rajendhran and Gunasekaran, 2008; Roose-Amsaleg et al., 2001). Nucleic acid extraction from environmental samples forms the basis for further molecular analyses (McIlroy et al., 2009). However DNA and RNA extraction from complex matrices like soil is highly challenging due to low yields of nucleic acids, difficulties in reproducibility (McIlroy et al., 2009) and contamination of nucleic acid extracts, mainly due to humic substances, which can inhibit PCR amplification (Rajendhran and Gunasekaran, 2008).

In principal, two different approaches for extracting nucleic acids from environmental samples exist (Herrera and Cockell, 2007). The indirect approach, is based on an initially separation of cells from the soil matrix by elution, followed by cell lysis and nucleic acid extraction

(Delmotte et al., 2009; Holben et al., 1988), whereas the direct approach is based on a lysis of cells directly in the soil and subsequent extraction of nucleic acids from the environmental sample (Roose-Amsaleg et al., 2001). While numerous articles have described DNA *or* RNA extraction procedures, only very few publications deal with DNA/RNA co-extraction (Costa et al., 2004; Griffiths et al., 2000; Hurt et al., 2001; McIlroy et al., 2009; Ogram et al., 1995; Orsini and Romano-Spica 2001; Yu and Mohn, 1999). However if gene abundance pattern should be linked to transcript rates there is a need for co-extraction of DNA and RNA due to the different types of bias, that is linked to each individual nucleic acid extraction protocol. One of the most cited protocols for DNA/RNA co-extraction has been published by Griffiths et al. (2000). As the DNA/RNA separation is based on DNA or RNA digestion by DNase or RNase treatment, respectively, subsequent purification and separation steps are needed, which in consequence may induce selective ranges of error, despite the same extraction procedure biases. Therefore we developed a protocol for DNA/RNA co-extraction based on nucleic acid precipitation with polyethylene glycol (PEG) (according to Arbeli and Fuentes (2007)) and subsequent DNA and RNA separation via silica-based columns. In this study we compared the new protocol based on a phenol-chloroform extraction with subsequent column-based separation of DNA and RNA (PCS) with the original protocol by Griffiths et al. (2000) and a commercially available DNA and RNA extraction kit, respectively. We used four

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different agricultural soils and compared the amount and purity of the extracted nucleic acids as well as abundance and diversity of the gene *nosZ* which encodes the denitrifying enzyme nitrous oxide reductase (Philippot et al., 2007).

2. Material and methods

2.1. Soil description

The four different soils derived from Germany, the Czech Republic and Burkina Faso and were managed as arable land and pasture, respectively. All soil characteristics are given in Table 1. Soil samples were taken in four replicates and were immediately sieved trough a 2 mm mesh, homogenized and frozen at −80 °C for molecular investigations.

2.2. Nucleic acid extraction and purification

2.2.1. Extraction with kits from MP Biomedicals

DNA and RNA were extracted from 0.5 g soil using the FastDNA® SPIN Kit for Soil and the FastRNA® SPIN Kit for Soil from MP Biomedicals (France), respectively, according to the manufacturers' protocols. Bead beating was performed on the Precellys®24 Homogenizer (Bertin Technologies, France). Extracts were stored at −80 °C until use.

2.2.2. Extraction according to Griffiths et al. (2000)

DNA and RNA from soil samples were co-extracted by phenol–chloroform extraction and subsequently enzymatically digested according to Griffiths et al. (2000). Briefly, 0.5 g of moist soil were extracted in Lysing Matrix E tubes (MP Biomedicals, France) with 0.5 ml of hexadecyltrimethylammoniabromide (CTAB) extraction buffer and 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1) (pH 8). The samples were lysed for 30 s using the Precellys®24 Homogenizer and centrifuged at 16100 ×*g* for 5 min at 4 °C. The aqueous layer was removed and mixed with an equal amount of chloroform–isoamyl alcohol (24:1). After centrifugation (5 min, 4 °C), 2 volumes of the precipitation solution containing 30% polyethylene glycol (PEG) 6000 and 1.6 M NaCl were added to the aqueous phase, incubated for 2 h on ice for nucleic acid precipitation, and then centrifuged for 10 min at 4 °C. The nucleic acid pellet was washed in ice-cold 70% ethanol, again centrifuged for 10 min at 4 °C, air dried and re-suspended in 50 ml of pure and nuclease-free water. Afterwards, the extract was divided in two aliquots to obtain pure DNA and RNA, respectively. For pure DNA, 700 ng of the extracted nucleic acids were incubated at 37 °C for 10 min with RNase A (Sigma, Germany) at a final concentration of 100 μg ml⁻¹. Pure RNA was obtained by treating 250 ng of the extracted nucleic acids with DNase (Applied Biosystems, Germany) as recommended by the manufacturer. Afterwards, the digestions were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and stored at −80 °C until further use.

2.2.3. Extraction according to the improved PCS protocol

The PCS protocol basically rested upon the protocol by Griffiths et al. (2000) but was significantly modified in different steps as specified in the following: (i) The CTAB extraction buffer additionally contained 10 μl ß-mercaptoethanol ml⁻¹ to inactivate RNases and DNases, which could be released after cell disruption. (ii) The chloroform extraction step was performed twice to completely remove phenol residues. (iii) Only one volume of the precipitation solution containing however 10% PEG 8000 and 1.2 M NaCl was added to the aqueous phase and incubated on ice for 2 h (according to Arbeli and Fuentes, 2007). (iv) The total DNA/RNA extract was used to separate DNA from RNA by the AllPrep DNA/RNA Mini Kit (Qiagen, Germany) without any further digestions, purification steps or sample divisions. To obtain pure DNA and RNA a maximum of 20 μg of the DNA/RNA mixture could be added to the silica columns.

2.3. Purity and yield of nucleic acids

2.3.1. RNA purity

Contamination of RNA samples with co-extracted DNA was excluded by PCR assays targeting the 16S rRNA genes using the universal primers 986f and 1401r (Nübel et al., 1996). The PCRs resulted in no amplicons for all samples except the Cambisol_pasture soil being extracted with the RNA extraction kit. After a reduction of the soil to 0.2 g for extraction weak bands of PCR products were still observed in a few samples. For further analyses these samples were examined critically.

2.3.2. Amount of DNA and RNA

DNA and RNA quantities were determined by ultrasensitive fluorescent nucleic acid staining using the Quant-iT PicoGreen Kit and the Quant-iT RiboGreen Kit (Invitrogen, Germany), respectively, with detection ranges of 0.025–1 ng μl⁻¹ for DNA and 0.001–0.05 ng μl⁻¹ for RNA. The determined nucleic acid concentrations were used to calculate the DNA/RNA content g⁻¹ dry soil. The purity of the DNA and RNA extracts was measured spectrometrically via absorbance at 320 nm (NanoDrop Technologies, USA) (Miller, 2001).

2.4. cDNA synthesis

The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany) in a total volume of 40 μl and was afterwards purified with the QIAquick PCR Purification Kit (Qiagen, Germany).

2.5. Quantitative real-time PCR (qPCR) assay

For the real-time PCR quantification of the single copy gene *nosZ* primers from Henry et al. (2006) were used. The chemical composition and the amplification were conducted as recently published (Töwe et al., 2010). To confirm the specificity of the amplicons after each PCR run a melting curve and a 2% agarose gel were conducted. The amplification efficiencies were calculated by $Eff = [10^{(-1/slope)} - 1] * 100\%$ and resulted in 86.2% (±5.1). The r² of the regression amounted to more than 0.99 and the y-intercept value accounted for 35.8 (±2.7).

2.6. T-RFLP assay

Diversity analysis by T-RFLP was also performed targeting the *nosZ* gene. For amplification primer pairs and PCR profiles were performed

Table 1
Soil properties.

Soil type	Land use	Location	Coordinates	% Clay	% Silt	% Sand	% C	% N	pH
Luvisol	Arable	Germany, Munich	48°29′ N, 11°26′ E	15.3	44.6	40.1	1.4	0.13	6.1
Cambisol_arable	Arable	Germany, Munich	48°29′ N, 11°25′ E	10.2	22.7	67.1	1.1	0.1	5.9
Lixisol	Arable	Burkina Faso, Saria	12°16′ N, 2°9′ W	9.3	–	59.7	0.22	0.015	5.6
Cambisol_pasture	Pasture	Czech Republic, Budweis	48°52′ N, 14°13′ E	6–14	14–32	60–80	10.6	0.9	8.96

as described for q-PCR, but the forward primer was labeled with 5′-FAM (6-carboxyfluorescein). The PCR products were first purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and afterwards digested using the restriction enzyme *HpyCH4V* (New England Biolabs, Germany) according to the manufacturer's protocol. The restriction enzyme was selected based on *in silico* T-RFLPs using the program REPK (Restriction Endonuclease Picker) (Collins and Rocap, 2007). Digested amplicons (50 ng) were desalted and purified by the MinElute Reaction Cleanup Kit (Qiagen, Germany). One μl was then mixed with 13 μl of Hi-Di formamide (Applied Biosystems, Germany) containing a 400-fold dilution of a 6-carboxy-X-rhodamine-labeled MapMarker 1000 ladder (Bio-Ventures, USA.), denatured (3 min at 95 °C), cooled on ice, and size-separated on a 3730 DNA analyzer (Applied Biosystems, Germany). Electrophoresis was performed with POP-7 polymer in a 50 cm capillary array under the following conditions: 10 s injection time, 2 kV injection voltage, 7 kV run voltage, 66 °C run temperature, and 63 min analysis time. Electropherograms were analyzed using the GeneMapper 3.5 software package (Applied Biosystems, Germany).

2.7. Statistics

Gene abundance data were analyzed by one way ANOVA (SPSS 11.5). Prior to analysis data were tested for normal distribution by Q–Q plots and the Kolmogorov–Smirnoff test. Homogeneity of variances was checked by the Levene test. If the requirements were met pairwise comparisons were conducted by Tukey's test. If homogeneity of the variances was not fulfilled a Games–Howell test was applied. The T-RFLP data set was normalized to percent of the total peak height of a sample (Anderson, 2003) excluding peaks smaller than 0.5%. A permutation-based multivariate analysis of variance was conducted with the PerMANOVA software (Anderson, 2001; McArdle and Anderson, 2001) using an experimental design consisting of the three different treatments (three levels) for each soil with the null hypothesis "no difference between soils" and a significance level of 0.05 ($n = 4$ replicates per soil). The different treatments were defined as orthogonal and fixed. For each analysis term, 4999 random permutations of the raw data were conducted to obtain *p* values (Manly, 1997). Euclidean distance was used as distance measure (Anderson, 2005).

3. Results

3.1. Nucleic acid content and purity

DNA and RNA concentrations measured in the purified extracts were converted to amounts related to g^{-1} dry soil and ranged from 0.02 to 159 μg DNA g^{-1} soil for DNA (Fig. 1A). While the two co-extraction based protocols (Griffiths and PCS) revealed no differences in yield, significantly higher values were obtained with the DNA extraction kit for the Luvisol and Cambisol$_{arable}$. RNA contents ranged from 0.2 to 83 μg RNA g^{-1} soil (Fig. 1B). Significant differences between the extraction methods were only observed for the Cambisol$_{pasture}$ with the highest value for the PCS protocol and lowest for the RNA extraction kit.

Considering the purity of DNA and RNA, significantly (up to 100 times) higher absorption values were obtained for all soil samples extracted with the kits indicating major contaminations with humic substances (Fig. S1; supplemental material).

3.2. Abundance of nosZ genes and transcripts

DNA and RNA extraction efficiencies of the different protocols were evaluated and compared by quantification of *nosZ* genes and transcripts using qPCR. The *nosZ* gene and transcript abundance levels were related to g^{-1} soil (Fig. 2A) as well as to ng^{-1} DNA (Fig. S2). Overall, the gene copy numbers ranged from 3×10^5 (Lixisol, PCS) to 3×10^9 copies g^{-1}
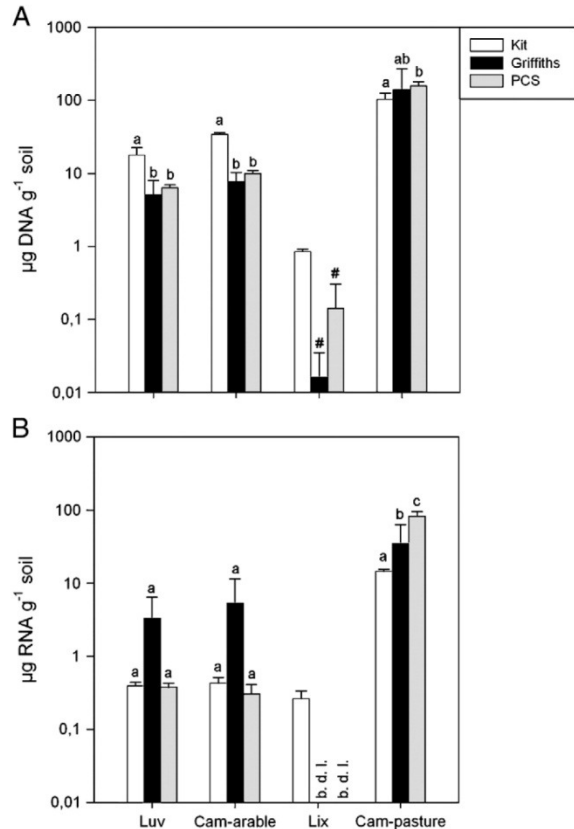

Fig. 1. DNA (A) and RNA (B) contents g^{-1} soil are displayed ($n = 4$, error bars represent standard deviations). Significant differences between the three extraction protocols are shown by different letters and were checked by oneway ANOVA. "b.d.l." indicates that all replicates were below the detection limit of 0.001 ng μl^{-1}. #The mean is only composed of two replicates as the DNA concentration was below the detection limit for the remaining samples. Thus, no statistic could be performed for this soil.

soil (Cambsiol$_{pasture}$, Griffiths) and reflected approximately the patterns of extracted DNA g^{-1} soil (Fig. 1A). The abundance of *nosZ* genes within one soil differed in part significantly between the protocols, but without favoring a certain protocol. When gene copy numbers were related to ng^{-1} DNA significantly higher gene copies were achieved with the PCS method compared to the DNA extraction kit for the Luvisol and Cambisol$_{arable}$. Highest abundance levels were measured in all soils being extracted according to the Griffiths protocol, but due to high standard deviations, the differences were not significant.

For the copy numbers of *nosZ* transcripts, larger differences between the methods were observed compared to *nosZ* genes (Fig. 2B, S2; supplemental material). The use of the Griffiths protocol resulted in higher copy numbers related to g^{-1} soil and ng^{-1} DNA, respectively, but again the differences were not significant due to huge standard deviations.

3.3. Diversity analysis of the extracted DNA and RNA

In order to test whether the different nucleic acid extraction protocols select for different microbial phylotypes, we performed T-RFLP analyses of *nosZ* genes and transcripts. The results are presented in Figs. 3 and 4 with only T-RFs contributing to more than 5% to the total community. Results of the respective Principal Component Analysis (PCA) are shown in Figs. S3 and S4 (supplemental material).
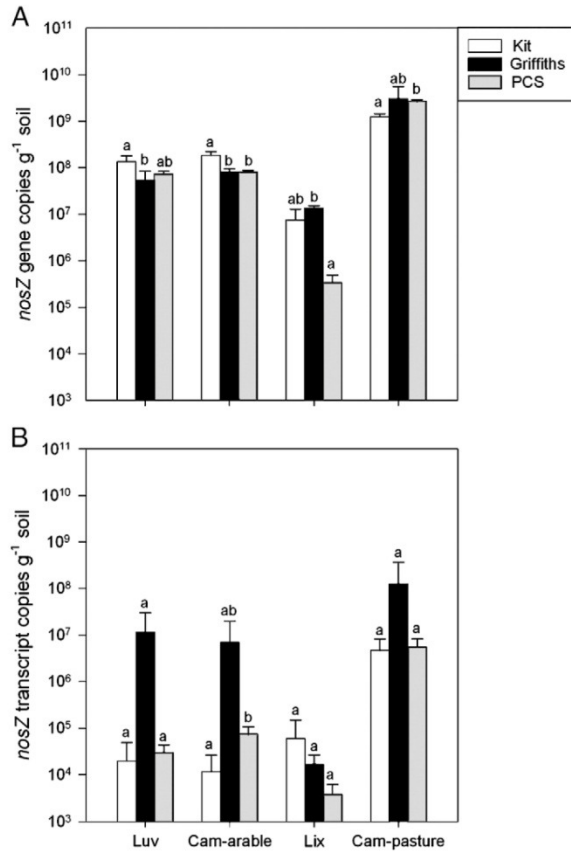
Fig. 2. Copy numbers of the *nosZ* gene per g dry soil are displayed for DNA (A) and cDNA (B) samples ($n = 4$, error bars represent standard deviations). Significant differences between the three extraction protocols are shown by different letters and were checked by oneway ANOVA.

Data revealed significantly different communities when comparing the DNA extraction kit and the PCS-method for all investigated soils except the Luvisol (Fig. 3). The number of dominant (>5%) T-RFs obtained by the PCS protocol always exceeded those by the kit, while the Griffiths protocol ranged in between. Only for the Lixisol highest numbers of dominant T-RFs were found in those samples being extracted according to the Griffiths protocol. The Principal Component Analysis revealed a strong clustering of the communities according to the soil type, with the Lixisol being again the only exception.

In all soils the active bacterial communities markedly differed from the overall community exhibiting different and fewer dominant T-RFs (Fig. 4). Moreover, almost for all combinations of soils and extraction protocols the number of total T-RFs was higher for the *nosZ* transcripts (15–37) than genes (10–22). Both, the lowest (Lixisol) and highest (Cambisol~pasture~) number of total T-RFs of *nosZ* transcripts were obtained using the Griffiths protocol. With exception of the Lixisol, the different protocols led to in part significantly different communities within a soil, however without any clear pattern. The PCA analysis revealed a clear separation of the communities received with the PCS protocol for the arable soils from Munich.

4. Discussion

In the last decades many studies focused on the improvement of single steps in nucleic acid extraction like cell lysis, homogenization,

precipitation and purification from soils and sediments (Arbeli and Fuentes, 2007; Chandler et al., 1999; de Boer et al., 2010; Inceoglu et al., 2010; Miller et al., 1999). However, only few studies targeted the simultaneous extraction of DNA and RNA from one and the same sample (Duarte et al., 1998; Griffiths et al., 2000; Hurt et al., 2001; Yu and Mohn, 1999). Beside the extraction steps, a crucial point in these protocols is the accurate performance of DNA and RNA separation. The presented PCS protocol allows the complete separation of DNA and RNA in one step. In contrast, many protocols are based on a subdivision of the extract and a subsequent digestion (Griffiths et al., 2000), meaning that a part of the extracted DNA and RNA, respectively, is enzymatically degraded. Alternatively, previous protocols included a separation with resin columns (Hurt et al., 2001), but an additional digestion step was still necessary.

To proof the reliability of newly derived protocols many studies refer to 16S rRNA-based community analysis or gene quantification methods (Krsek and Wellington, 1999; Sagova-Mareckova et al., 2008; Schneegurt et al., 2003; Wang et al., 2009). However, 16S rRNA operon numbers can differ considerably depending on the bacterial strain (Klappenbach et al., 2000), which might be unfavorable for quantitative comparisons. In this study we used instead the widely spread, functional single copy gene *nosZ* (Kandeler et al., 2006) that is known to be congruent with 16S rRNA-based taxonomic classification (Jones et al., 2008).

Extraction and quantification of DNA and RNA in our study were generally possible for all soils with all protocols except the Lixisol, where the reproducibility was only given for the DNA and RNA extraction kit, respectively. Zhou et al. (1996) found out that the carbon content is often positively related to the DNA yield. In our survey, the Lixisol revealed ten times lower carbon and nitrogen contents compared to the other soils, which might have been a reason that the amount of the extractable nucleic acids was remarkably low (Fig. 1A). Nevertheless, it was possible to analyze the gene abundance and diversity of the nitrous oxide reductase gene *nosZ* even in the Lixisol. In contrast, the Cambisol~pasture~ was characterized by high microbial biomass and carbon content, which led to RNA being contaminated with DNA when using the RNA kit-based extraction protocol. Furthermore, the obtained RNA yields and *nosZ* transcript copy numbers were similar or lower compared to the other methods. In contrast the PCS protocol enabled a complete separation of DNA and RNA even in soils with high microbial biomass, provided that the loading capacity of the silica columns was not exceeded. Interestingly, replicates extracted with the PCS protocol always revealed comparable RNA yields and *nosZ* transcript copy numbers, whereas samples extracted with the Griffiths protocol resulted in high variations between replicates. This might reflect the drawback of the sample processing with the Griffiths protocol, where repeated purification and sample division result in high standard deviations.

In most cases maximum DNA yields and *nosZ* gene abundance values could be detected within samples that were extracted with the kit-based protocol; however the amount of co-extracted humic substances (revealed by spectrophotometry) was high. This contamination problem frequently arises due to similar physicochemical properties of nucleic acids and humic acids, which bind competitively to the same absorption sites when using the minicolumns for purification (Harry et al., 1999; Lear et al., 2010). However, it was possible to overcome the inhibition by serial dilutions of the DNA extracts.

Regarding the diversity pattern, we could obtain T-RFLP profiles for all soils with all protocols, even if several repetitions of the PCR were necessary for the Lixisol to get sufficient amounts of PCR product due to the overall low nucleic acid yield. Between 10 and 22 *nosZ* T-RFs were obtained for DNA samples, which is in line with previous studies (Enwall and Hallin, 2009; Perryman et al., 2008); for cDNA the numbers ranged between 15 and 37 *nosZ* T-RFs. Interestingly,
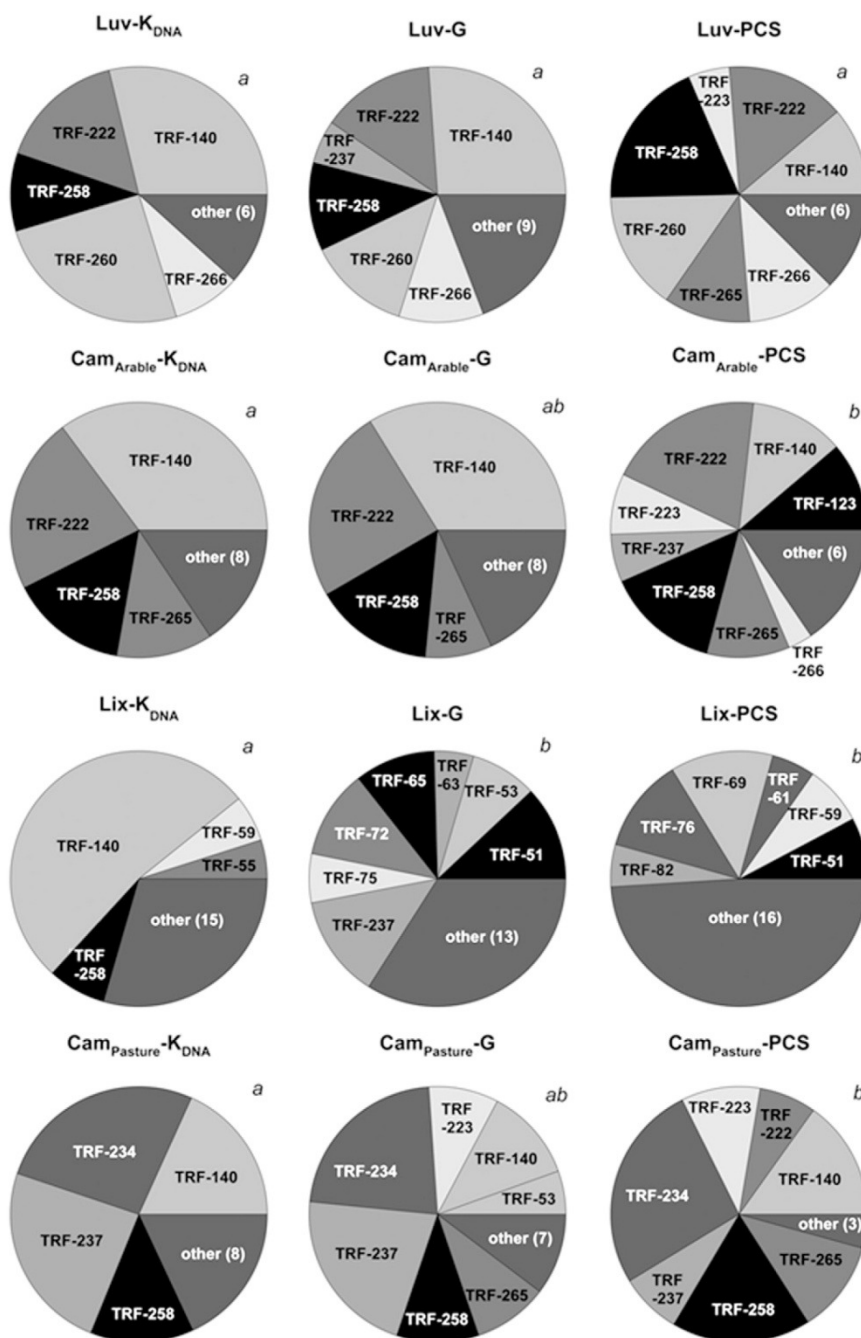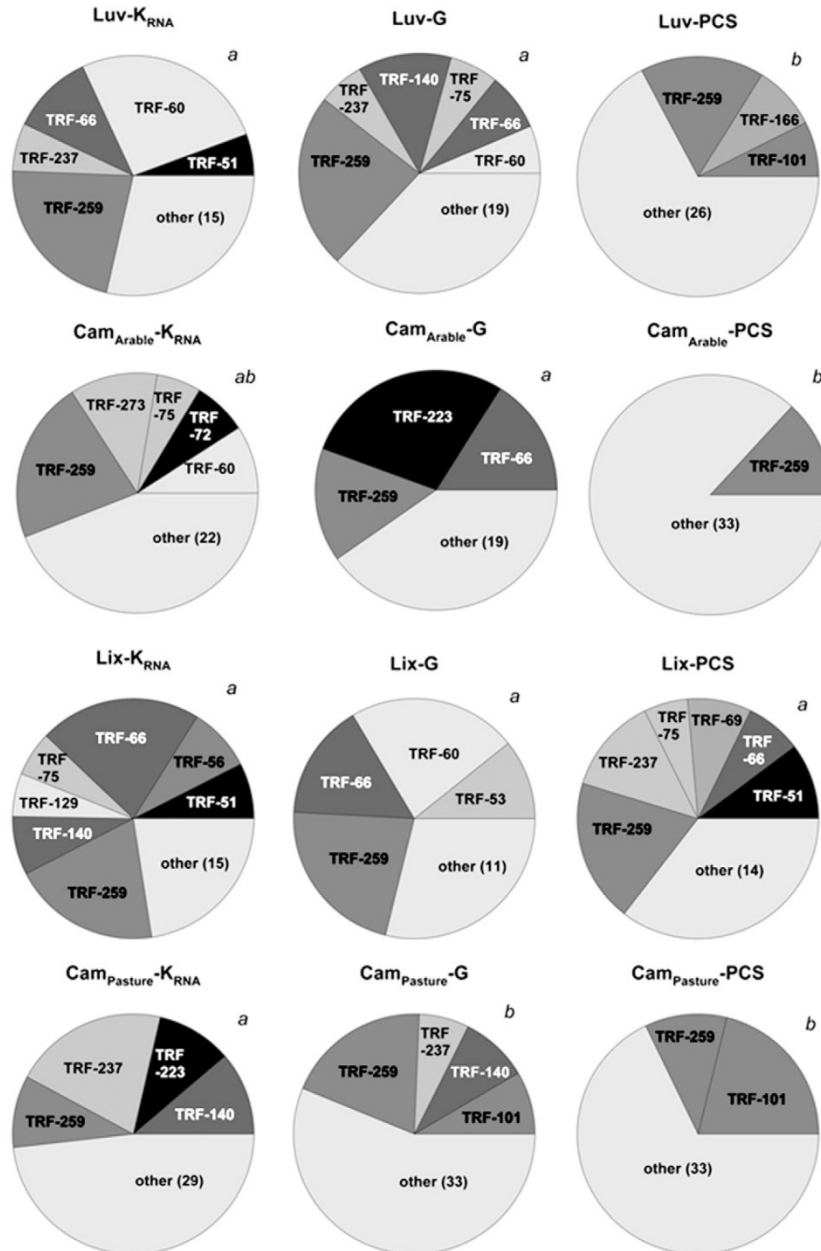
Fig. 3. Contribution of dominant T-RFs to total *nosZ* gene fragment diversity in the DNA samples. T-RFs which contributed to less than 5% were summarized as "other". Significant differences are indicated by different letters ($n = 4$).

different dominant T-RFs appeared in the active community compared to the potential community involved in N_2O reduction. The phenomenon that the most dominant microbes are not necessarily the most active ones is corroborated by a study from Bürgmann et al. (2005), who compared the potential and active diazotrophic community. Anyway, no clear preference for one protocol could be revealed by diversity analysis neither for DNA nor cDNA samples. Depending on soil type and extraction protocol, in part significant differences in diversity pattern were revealed. These observations reflect the contradictory opinions about the influence of the extraction method on the community composition (Carrigg et al., 2007; Inceoglu et al., 2010). Moreover, the quite scattered DNA-based

Fig. 4. Contribution of dominant T-RFs to total transcribed *nosZ* gene fragment diversity (cDNA samples). T-RFs which contributed to less than 5% were summarized as "other". Significant differences are indicated by different letters.

diversity pattern of the Lixisol (Fig. S3), underlines the challenge to reproducibly extract DNA or RNA from low biomass environments (Barton et al., 2006).

The results clearly demonstrated that on DNA level quantification and diversity analyses of a functional gene were possible with all protocols. While the RNA extraction kit and the PCS method revealed comparable results for cDNA, samples obtained from the Griffiths protocol were characterized by high standard deviations. However, extraction using the kits had two disadvantages: (i) Two independent extractions for DNA and RNA have to be performed and (ii) the extracts seem to be more contaminated with humic substances. In contrast, it was possible to extract DNA and RNA simultaneously from one and the same sample and to completely separate the DNA and RNA in one additional step with the improved PCS protocol. However, further investigations and adaptations are needed for the reliable nucleic acid extraction of extreme soils, e. g., with low microbial biomass or high clay content. Generally, it is important to stick to one protocol during sample processing and to introduce as less working steps as possible to reduce the method-specific biases especially for quantitative analysis.

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mimet.2010.12.028.

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Figure S1. Detection of humic substances at A_{320nm} ($n = 4$, error bars represent standard deviations). Significant differences between the three extraction protocols are displayed by different letters and were checked by oneway ANOVA.
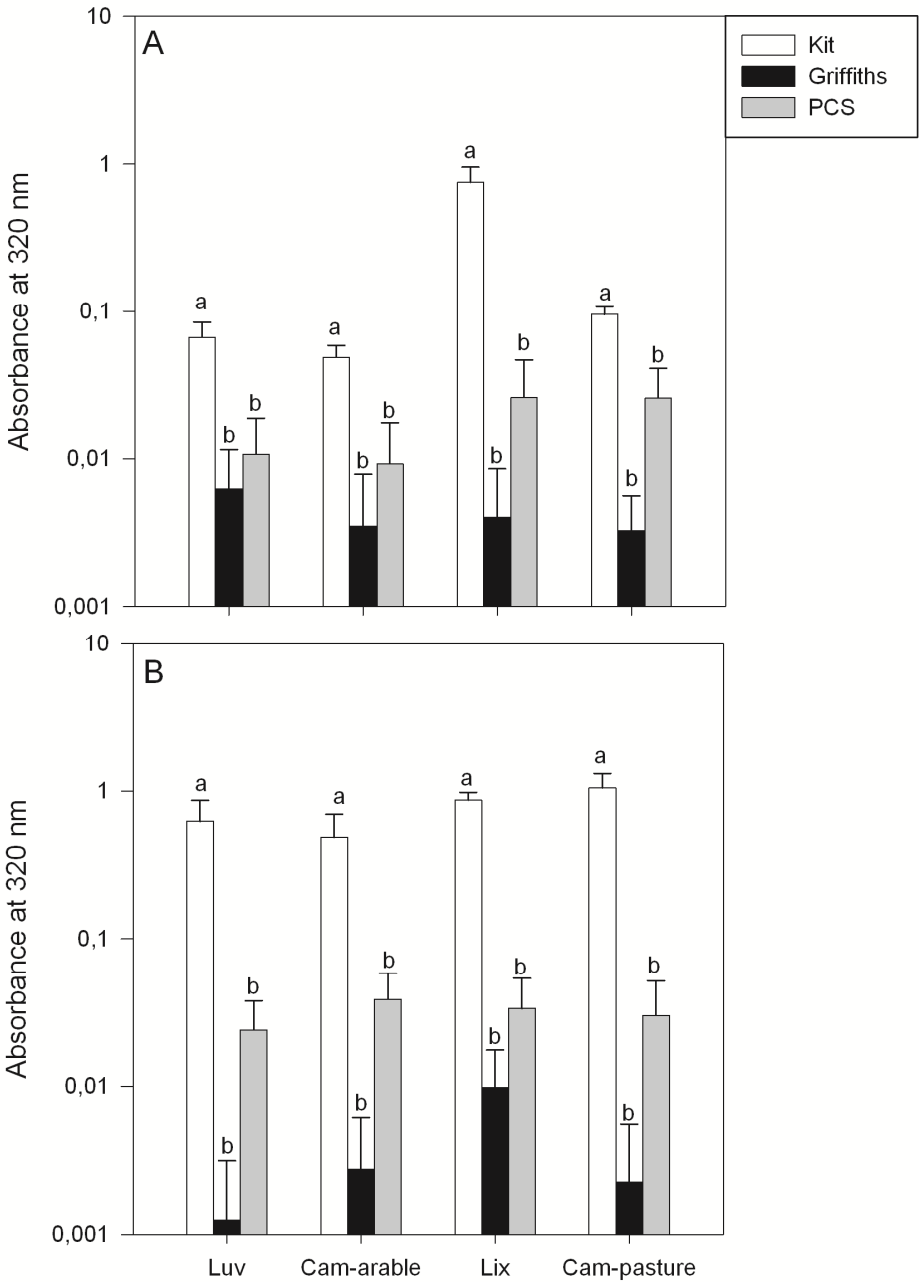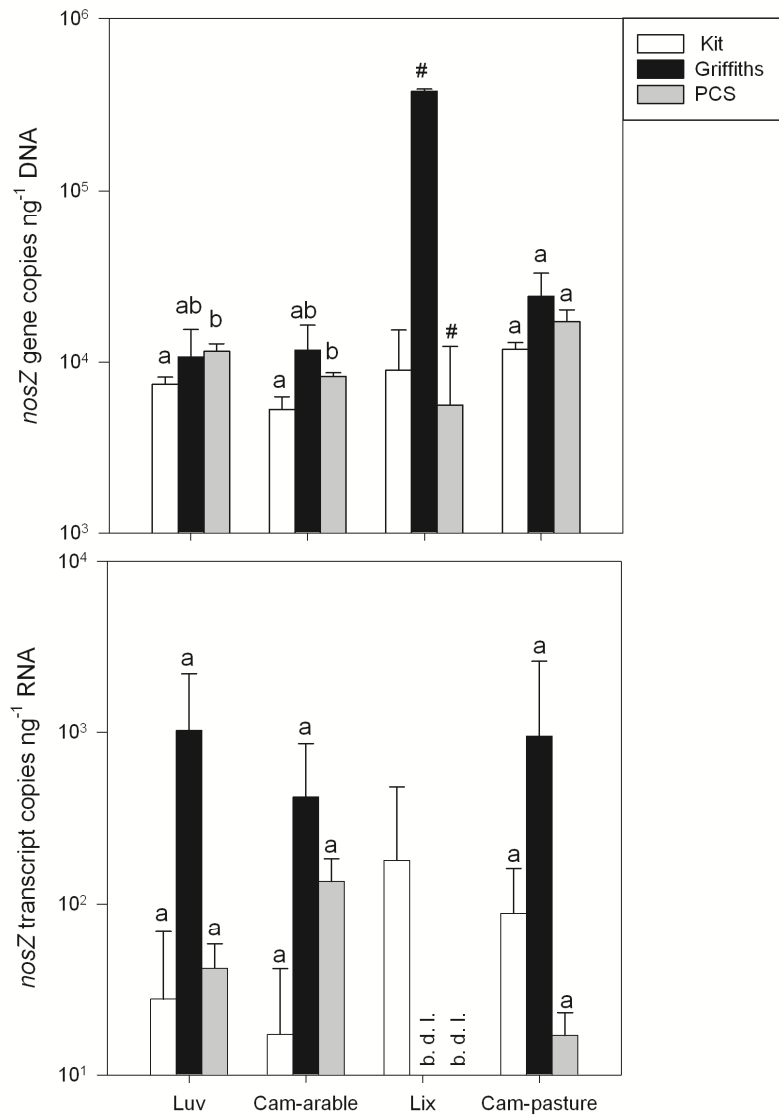
Figure S2. Copy numbers of the *nosZ* gene per ng DNA are displayed ($n = 4$, error bars represent standard deviations). Significant differences between the three extraction protocols are shown by different letters and were checked by oneway ANOVA. "b.d.l." indicates samples where the RNA concentration was below the detection limit of 0.001 ng μl^{-1} (see Figure 1).

The mean is only composed of two replicates as the DNA concentration is below the detection limit for the remaining samples. Thus no statistic was performed for this soil.

Figure S3. Principal Component Analysis of the T-RFLP dataset for *nosZ* gene fragments in the DNA samples. The ordination plots of the first two principal components (PC) show mean values of the replicates per extraction method. The four soils are displayed by four different symbols and each of the three similar symbols per soil stands for an extraction method
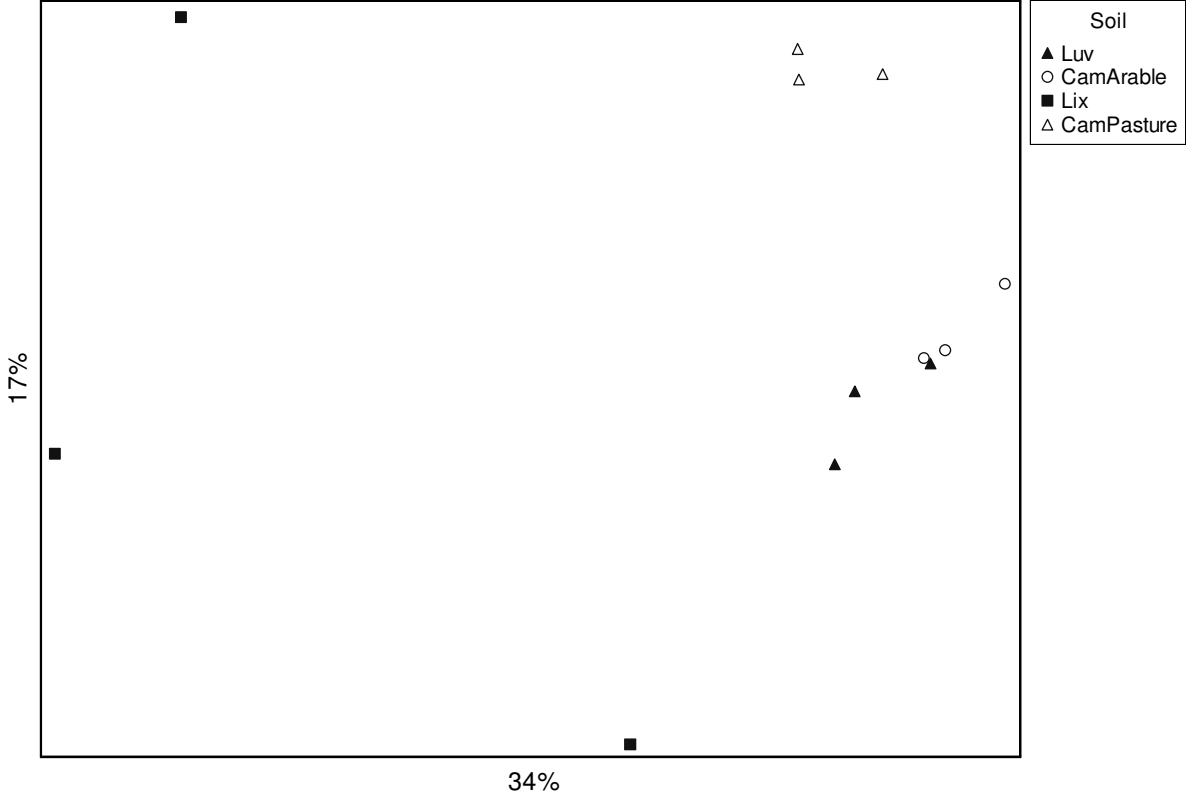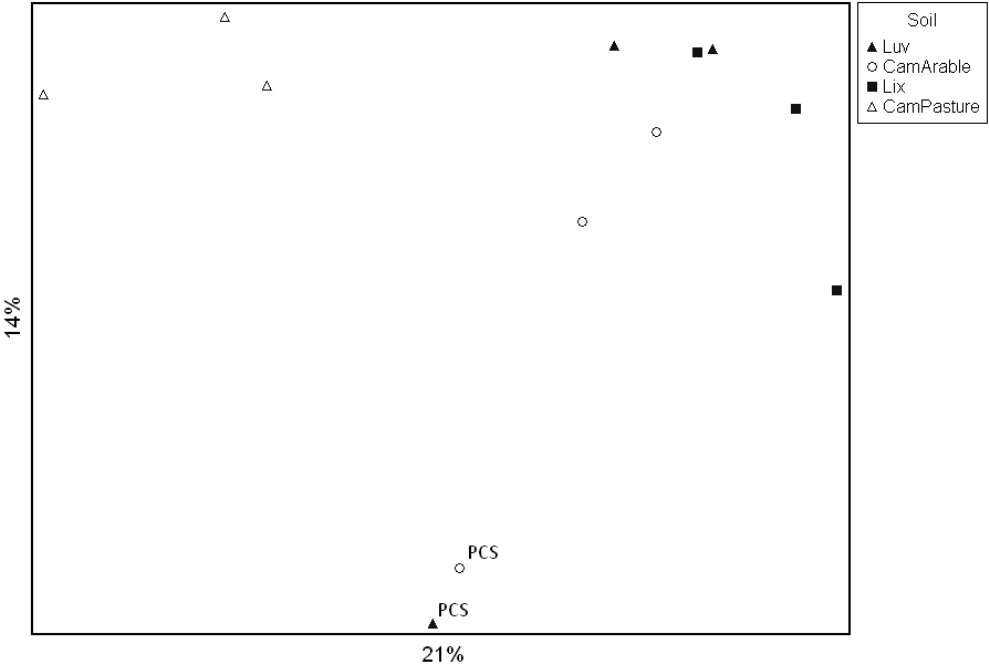
Figure S4. Principal Component Analysis of the T-RFLP dataset for *nosZ* gene fragments in the cDNA samples. The ordination plots of the first two principal components (PC) show mean values of the replicates per extraction method. The four soils are displayed by four different symbols and each of the three similar symbols per soil stands for an extraction method

Publication VI

Nitrogen turnover in soil and global change

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Abstract

Nitrogen management in soils has been considered as key to the sustainable use of terrestrial ecosystems and a protection of major ecosystem services. However, the microorganisms driving processes like nitrification, denitrification, N-fixation and mineralization are highly influenced by changing climatic conditions, intensification of agriculture and the application of new chemicals to a so far unknown extent. In this review, the current knowledge concerning the influence of selected scenarios of global change on the abundance, diversity and activity of microorganisms involved in nitrogen turnover, notably in agricultural and grassland soils, is summarized and linked to the corresponding processes. In this context, data are presented on nitrogen-cycling processes and the corresponding microbial key players during ecosystem development and changes in functional diversity patterns during shifts in land use. Furthermore, the impact of increased temperature, carbon dioxide and changes in precipitation regimes on microbial nitrogen turnover is discussed. Finally, some examples of the effects of pesticides and antibiotics after application to soil for selected processes of nitrogen transformation are also shown.

Introduction

Nitrogen is one of the crucial nutrients for all organisms (LaBauer & Treseder, 2008), as it is an essential component of important biopolymers. However, most of the N in nature occurs as dinitrogen gas or is fixed in organic compounds, like proteins or chitin, both of which cannot be directly used by plants and animals. Only specialized microorganisms are able to transform the gaseous dinitrogen into ammonia or to make organically bound N bioavailable by mineralization. Not surprisingly, N input by fertilization has always been a key factor for high crop yields and plant quality. Therefore, crop production is by far the single largest cause of human alteration of the global N cycle (Smil, 1999). Whereas in preindustrial times exclusively organic fertilizers had been used, the invention of the Haber Bosch procedure in the 20th century made huge amounts of mineral fertilizer available. The doubling of world food production in the past four decades could only be achieved with a strong land-use intensification including an almost sevenfold increase of N fertilization (Tilman, 1999) as well as wide-ranging land reclamations. These developments have contributed to the doubling of N loads to soil since the beginning of the 20th century (Green et al., 2004). The total global N input in the year 2000 was about 150 TgN (Schlesinger, 2009), whereas supply in croplands via mineral fertilizer was the single largest source accounting for almost half of it. Surprisingly, N entry from N-fixation was the second largest factor and contributed to 16%, while manure and recycled crop residues provided similar amounts and each accounted for only 8–13% of the total global supply. Remarkably, the entry of N via atmospheric deposition was in the same range. In regions with high mineral fertilizer application, the highest N accumulation potential in ecosystems could be observed, whereas the accumulation of N leads to high impacts on environmental quality like loss of diversity (Cragg & Bardgett, 2001), dominance of weed species (Csizinszky & Gilreath, 1987) and soil acidification (Noble et al., 2008). Additionally, land-use intensification also results in an increased use of bioactive chemicals, like pesticides and herbicides as well as antibiotics, which enter the environment via manure (Lamshöft et al., 2007).

According to Liu et al. (2010), 55% of the global applied N was taken up by crops. The remainder was lost in leaching

(16%), soil erosion (15%) and gaseous emission (14%). Such N depletion of soils leads to eutrophication (Stoate *et al.*, 2009), surface- and groundwater pollution (Spalding & Exner, 1993) and emission of the greenhouse and ozone-depleting gas nitrous oxide (N_2O) (Davidson *et al.*, 2000), impacting on human health and climate change (Fig. 1).

To reduce these threats, Schlesinger (2009) suggested that policy makers and scientists should focus on increasing N-use efficiency in fertilization, reducing transport of reactive N fractions to rivers and groundwater and maximizing denitrification to N_2.

Because of the use of advanced molecular tools (Gabriel, 2010) and stable isotopes (Baggs, 2008) in recent years, scientists have been able to identify new key players of N turnover for selected processes like nitrification (Leininger *et al.*, 2006) or N-fixation (Chowdhury *et al.*, 2009) as well as completely new processes like anammox (Op den Camp *et al.*, 2006). All these findings have revolutionized our view of N transformation processes in soils, although the relevance for the overall understanding of N transformation is not entirely clear yet and discussed controversially in the literature. However, despite numerous studies and a large amount of collected data, we have to admit that N turnover and factors driving the corresponding populations are not yet completely understood.

Furthermore, according to the *UN Millenium Ecosystem Assessment* (http://www.maweb.org/), global change will highly affect N turnover in soils to a so far unknown extent. According to the definition given in Wikipedia, the term 'global change' encompasses interlinked activities related to population, climate, the economy, resource use, energy development, transport, communication, land use and land cover, urbanization, globalization, atmospheric circulation, ocean circulation, the C cycle, the N cycle, the water cycle and other cycles, sea ice loss, sea-level rise, food webs, biological diversity, pollution, health, overfishing and alteration of environmental conditions including climate change as well as land-use changes and effects of xenobiotic substances. Therefore, there is a need for experimental approaches to study the consequences of altering environmental conditions including climate change as well as land-use changes and the effects of xenobiotic substances on N turnover in soil. In the following review, state-of-the-art knowledge is summarized concerning the impact of selected global change scenarios on microbial N turnover as well as the abundance and diversity of key players. Additionally, implications for future research strategies and priorities are given.

Ecosystem development

Natural and anthropogenic activities lead to new terrain for soil development. In this context, different chronosequences of ecosystem development like glacier forefields, sand dunes,

volcanoes or restoration sites have emerged. These are interesting aspects to study the development of N-cycling processes as well as the contributing functional microbial groups. Overall, three phases can be postulated: initial, intermediate and mature phases. Depending on the investigated ecosystem, these phases can range from a few days or weeks (Jackson, 2003) to hundreds of years (Kandeler *et al.*, 2006; Brankatschk *et al.*, 2011), respectively.

Most of the initial ecosystems are characterized by nutrient shortage, barren substrate and scarce vegetation (Crews *et al.*, 2001; Nemergut *et al.*, 2007; Smith & Ogram, 2008; Lazzaro *et al.*, 2009; Brankatschk *et al.*, 2011). The total N concentrations are often far below 0.1% and only traces of ammonia and nitrate can be measured (Brankatschk *et al.*, 2011). Additional N input by the weathering of bedrock material is unlikely as it only contains traces of N. Thus, the colonization with N-fixing microorganisms seems to be the only way for N input, despite the high energy demands for the transformation of N_2 into ammonium. Crews *et al.* (2001) demonstrated that the total N input in young lava flows was mainly driven by N fixation, although fixation rates were low. This has been confirmed in several other studies, which demonstrated a high abundance of nonheterocystous N-fixing cyanobacteria like *Microcoleus vaginatus* (Yeager *et al.*, 2004; Nemergut *et al.*, 2007; Abed *et al.*, 2010). It is obvious that in initial ecosystems, cyanobacteria play a prominent role in ecosystem engineering. They not only improve the N status of soils by N-fixation, but also secrete a polysaccharide sheath, resulting in the formation of soil crusts. This leads to a stabilization of substrates, capture of nutrients and an increase of the water-holding capacity, which paves the way for other organisms and processes (Garcia-Pichel *et al.*, 2001; Schmidt *et al.*, 2008). Therefore, at early stages of soil development heterotrophic microorganisms, which are able to mineralize the N derived from air-driven deposition (e.g. chitin) or ancient and recalcitrant materials are able to find their niches and stimulate N turnover (Bardgett *et al.*, 2007; Brankatschk *et al.*, 2011). However, this process is highly energy demanding and thus the turnover rates typically low. Obviously, as only limited competition for N resources exists at this stage (due to a lack of plants), the amount of ammonia is sufficient for the development of microbial communities involved in nitrification. This process results in the formation of nitrate, which leaves the ecosystem mainly by leaching. Therefore, N accumulation rates at initial sites are low (Tscherko *et al.*, 2004).

If the total N concentrations in soil exceed 0.2%, plant development starts and cyanobacterial soil crusts are displaced by shadowing by plant growth (Brankatschk *et al.*, 2011). Therefore, the intermediate stage of ecosystem development is characterized by increasing plant coverage and surface stabilization resulting in an increased C input
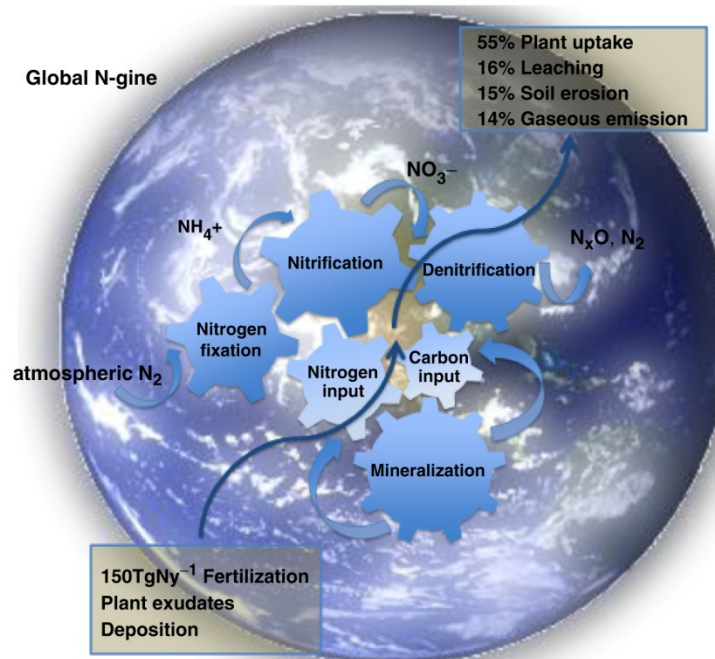
Fig. 1. Nitrogen turnover at the global scale.

via exudation and litter material. However, ammonium and nitrate contents are still much lower (Kandeler *et al.*, 2006; Brankatschk *et al.*, 2011) than in well-developed grassland sites (Chronáková *et al.*, 2009). Although it has been argued that this stage of ecosystem development is characterized by a competition between microorganisms and plants for N (Schimel & Bennett, 2004; Hämmerli *et al.*, 2007), associative or symbiotic networks between N-fixing microorganisms (mainly bacteria) and plants become a central element at this stage (Duc *et al.*, 2009). This results in an increased N-fixation activity in the rhizosphere and a highly efficient share of nutrients between plants and microorganisms. Because of the patchy distribution of C and N concentrations at those sites, many studies have revealed the highest microbial diversity at intermediate stages of ecosystem development by targeting functional genes like *nifH* (Duc *et al.*, 2009) or general microbial diversity by 16S rRNA gene (Gomez-Alvarez *et al.*, 2007). This fits with the intermediate-disturbance hypothesis, postulating that medium disturbance events cause the highest diversification (Molino & Sabatier, 2001). However, besides the development of plant–microorganism interactions, the intermediate phase of ecosystem development is also characterized by highly efficient degradation of litter and subsequent N mineralization (Esperschütz *et al.*, 2011) as well as an increase in fungal biomass (Bardgett & Walker, 2004), probably also of arbuscular mycorrhiza, which may contribute to a better distribution of the N in soil with ongoing succession. At this stage, the abundance and activity of nitrifiers (Nicol *et al.*, 2005)

and denitrifiers (Smith & Ogram, 2008) is still low due to the high N demand of the plants. Whether typical plants at those sites are able to produce nitrification inhibitors to better compete for ammonium might be a highly interesting question for future research (Verhagen *et al.*, 1995).

In contrast, when total N concentrations above 0.7% are reached in soils at well-developed sites and vegetation is no longer dominated by legumes, nitrification becomes a highly significant process. Interestingly, in ecosystems of glacier forefields, nitrification activity seems to be driven by ammonia-oxidizing archaea (AOA), although being lower in abundance than their bacterial counterpart [ammonia-oxidizing bacteria (AOB)]. This might be due to the better adaptation to relative ammonium-poor environments (Di *et al.*, 2009) and low pH (Nicol *et al.*, 2008). In combination with pronounced root penetration resulting in increased exudation, enhanced water retention potential and less oxygen diffusion (Deiglmayr *et al.*, 2006), denitrification becomes a key process for the overall N budget at those sites in soil. Interestingly, Brankatschk *et al.* (2011) only found a good correlation of a part of the functional genes of the denitrification cascade, for example, *nosZ* (nitrous oxide reductase) gene abundance and potential denitrification activity, whereas *nirK* and *nirS* (nitrite reductases) gene abundance did not correlate with the rates of potential activity. Moreover, the highest relative gene abundance of *narG* was observed in early development stages of soils (Kandeler *et al.*, 2006), while the nitrate reductase activity peaked at late stages of soil development (Deiglmayr *et al.*,
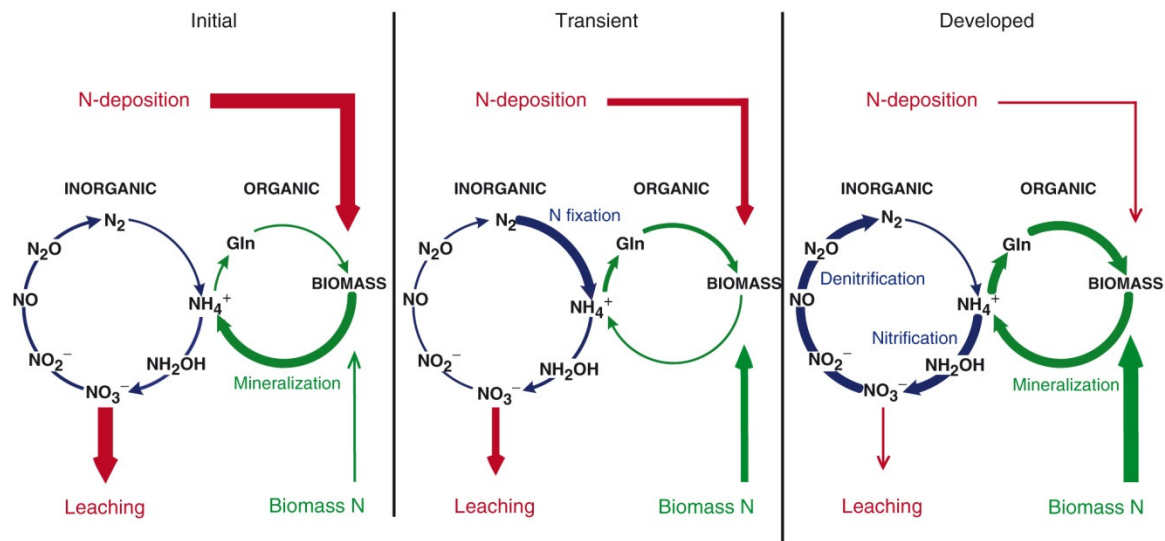
Fig. 2. Scheme of the development of the nitrogen cycle during ecosystem development (initial, transient and developed). The size of the arrows represents the impact of the corresponding process for nitrogen turnover.

2006). Similar observations were made by Smith & Ogram (2008) along a restoration chronosequence in the Everglades National Park. The mechanistic bases for these observations are still not clear. In addition to high activities of nitrifiers and denitrifiers at well-developed sites, the highest values of mineralization activity have been observed there in several studies (Tscherko *et al.*, 2004; Brankatschk *et al.*, 2011). These data are congruent with the observations of Frank *et al.* (2000), who found a positive correlation between nitrification, denitrification and N mineralization processes in Yellowstone Park grasslands.

Overall, the studies performed so far using the chronosequence approach to describe ecosystem development have revealed surprisingly similar patterns of the participation of different functional groups of microorganisms involved in N cycling at the three different phases (Fig. 2). In summary, all systems described were characterized by very low C and N concentrations in soil as well as less pronounced organismic networks of interaction at the initial stages of soil development.

Changing land-use patterns

A generalization of the results described above to other scenarios of global change related to ecosystem development, for example, in response to natural disasters (earthquakes), after manmade destructions (clear cuts of forest sites) or due to land-use changes is not possible. This is due to the different quality and amount of C and N present in soil as well as the biodiversity, mainly related to soil animals and plants at the initial stages in these disturbed systems. Whereas the consequences of natural disasters for N turn-

over have been rarely addressed, the impact of land-use changes on N turnover and the corresponding functional communities has been studied extensively. However, in this context, it is difficult to identify *one* main driver for shifts in the microbial population structure, as land-use changes often encompass a combination of different forms of management. For example, the use of extensively used grassland for crop production will not only change aboveground biodiversity, but will also result in changes in pesticide application, tillage and fertilizer management.

Overall, the conversion of forests or grasslands to agricultural land has an impact on almost all soil organisms (Postma-Blaauw *et al.*, 2010). Therefore, the functional diversity of microorganisms involved in N cycling is also highly influenced by land-use changes. This has been well documented for nitrifiers and denitrifiers, whereas surprisingly for N-fixing bacteria, clear response patterns have been described in only a small number of cases. In some cases, even no response of *nifH* towards land-use changes was detected (Colloff *et al.*, 2008; Hayden *et al.*, 2010), which might be related to the high concentrations of ammonium and nitrate before land-use change. In terms of nitrification, good correlations between gene abundance and land use have been described for AOB in several studies. Colloff *et al.* (2008) found higher gene abundance of the bacterial *amoA* gene in agricultural soils compared with soils from rainforests. By contrast, Berthrong *et al.* (2009) observed consistently reduced nitrification rates in soils that were converted from grassland into forest. These trends were also confirmed by Bru *et al.* (2010) comparing land-use changes between forests, grassland and agricultural soils in different

parts of the world. The authors found a strong correlation between AOB and the form of land use. Interestingly, in the same study, no differences were observed for archaeal ammonia oxidizers (AOA) in relation to the investigated land-use types. Hayden *et al.* (2010) almost consistently observed a greater abundance of AOB *amoA* genes in managed compared with remnant sites. The good correlation between AOB and land use might be related to the different ammonium concentrations in soil in response to different land-use types. AOB often colonizes habitats with high ammonium concentrations, whereas for AOA abundance, so far, no general dependency on ambient ammonium concentration has been documented. Furthermore, the results might be related to the high sensitivity of AOB towards low pH, which is often present in forest soils and leads to low availability of ammonia.

It was reported that land-use changes from forest to grassland soils are often accompanied by high N losses from soil (reviewed by Murty *et al.*, 2002). However, no clear trends are visible so far, if these losses occur in general due to increased denitrification rates or leaching of the nitrate formed during nitrification, as both observations have been described in the literature. This might be explained by the different soil types under investigation in the various studies. Whereas in loamy soils, which tend to have more anoxic microsites, denitrification might be stimulated (Rich *et al.*, 2003; Boyle *et al.*, 2006), in sandy soils, the nitrate formed may leach fast to the groundwater (Murty *et al.*, 2002). For denitrifiers, land-use changes overall influence the abundance and diversity patterns of selected functional groups. Attard *et al.* (2010) described, for example, higher potential denitrification rates in grassland soils compared with soils under cropping management. This was in accordance with a 1.5–5-fold higher abundance of denitrifiers (based on the abundance of *nirK* genes) in grassland soils than arable soils found in various studies (Baudoin *et al.*, 2009; Attard *et al.*, 2010), including shifts in the diversity patterns of *nirK*-harboring bacteria. Whereas a strong correlation between gene copy numbers of *nirK* and potential denitrification rates has been described, no correlation was found between the diversity patterns of *nirK* and turnover of nitrate. This indicates highly similar ecophysiological patterns of nitrite reducers of the *nirK* type.

Agricultural management

Not only changes in land-use patterns, but also shifts in agricultural management practice can result in alterations of functional microbial communities involved in N cycling. In general, there is consensus that an intensification of agriculture and subsequent increased fertilization regimes result in higher nitrification and denitrification rates as well as an increase of both functional groups (Le Roux *et al.*, 2003, 2008; Patra *et al.*, 2006). In the case of ammonia oxidizers, mainly AOB benefit from the increased availability of ammonium in soil (Schauss *et al.*, 2009a). For N-fixing prokaryotes, several studies have indicated a reduction based on the abundance of *nifH* and consequently also lower N-fixation activity in highly fertilized soils (Coelho *et al.*, 2009). Interestingly, the inoculation of seeds from legumes with rhizobia, which is a common practice in low-input farming to enhance N-fixation, does not only increase *nifH* abundance in the rhizosphere, but also leads to higher abundance of nitrifiers and denitrifiers (Babic *et al.*, 2008). This indicates that at least a part of the fixed N is released into soil, despite the symbiotic interaction (Babic *et al.*, 2008). As the use of monocultures and the intensification of agriculture *per se* (including the transformation of sites, which are less suited for agriculture, for the production of renewable resources) is often accompanied by a loss in nutrients (Malézieux *et al.*, 2009), which is primarily compensated by the application of inorganic fertilizers, changes in N turnover and the corresponding microbial communities might be primarily a result of changed fertilization regimes, as described by Drury *et al.* (2008). It has been confirmed in several studies that the type of fertilizer (mineral vs. organic fertilizer) has a clear influence on the N budget of soils and the corresponding functional microbial groups (Hai *et al.*, 2009; Ramirez *et al.*, 2010). As expected, the application of a mineral fertilizer based on ammonia-nitrate increases the nitrification and denitrification patterns in soil shortly after application, when the fertilizer is not taken up by the plant due to increased availability of the corresponding substrates. In contrast, the application of an organic fertilizer leads to higher abundance of microorganisms involved in mineralization and only relatively slight increases of nitrifiers and denitrifiers and their activity in the long run. Because of the overall more balanced N budget in soil when organic fertilizers are applied, N-fixing microorganisms are favored by this practice (Pariona-Llanos *et al.*, 2010). Not surprisingly, the effects observed in soils that have been used for grazing can be compared with those where manure has been applied, including clear shifts mainly in the diversity patterns of ammonia- and nitrite-oxidizing microorganisms as well as denitrifiers (Chroňáková *et al.*, 2009) Furthermore, grazing also induces shifts in root exudation patterns (Hamilton & Frank, 2001), which may further influence the abundance and activity of microorganisms involved in N turnover.

In the last decades, the influence of tillage management on N turnover has been studied in several projects, as nontillage systems have been described to be of advantage in terms of nutrient supply and are very popular in organic farming (Hansen *et al.*, 2011). Overall, changes in nitrification activity after modifying the tillage practice were well

explained by the accumulation of ammonium in the top soil due to nontillage and the corresponding changes in the abundance of nitrifiers (Attard *et al.*, 2010). In most studies, performed so far, a higher nitrification activity and subsequent higher nitrate concentrations in soil were linked to increased denitrification rates in the top soil layer in nontillage compared with tillage treatments (Petersen *et al.*, 2008; Baudoin *et al.*, 2009; Attard *et al.*, 2010). This is due to tillage-induced higher C concentrations in top soils and a stronger formation of aggregates with anoxic microsites due to a lack of tillage-induced mixing. In addition, tillage results in a merging of the surface soil layers with the lower layers, the latter being characterized by lower denitrification potential (Attard *et al.*, 2010), which causes overall lower denitrification rates and abundance of the corresponding functional genes (especially *nirK*). However, as stated above, in most cases, changes in tillage management are accompanied by changes in pest management and cropping sequences. The changes observed in long-term studies therefore cannot be linked conclusively to tillage management alone. Thus, most studies performed so far in this area were linked to short-term perturbations. They may not reflect the typical response patterns of the soil microorganisms to the new conditions after the change of the tillage management, as they do not account for microbial adaptation, in the context of the intermediate-disturbance hypothesis (Molino & Sabatier, 2001) as well as the increasing C contents in the top soils over time where nontillage practice has been performed.

Changing climatic conditions

Because of ongoing climate change, various modifications in land use and agricultural management have been implemented. Thereby, climate and land management are highly interlinked and cannot be separated. In addition, it is well accepted that climatic conditions notably influence microbial performance in soil. Thus, several studies have been performed to estimate the consequences of increased atmospheric temperature or carbon dioxide (CO_2) concentrations as well as shifts in precipitation on N turnover and the corresponding functional communities.

In general, it is difficult to simulate increased temperature scenarios in experiments, as an increase of the average temperature of $3\,°C$ over the next 50 years would at most result in an annual increase of $< 0.2\,°C$. Therefore, experiments comparing soils with ambient temperature with soils increased in temperature by $2–5\,°C$ do not simulate climate change, but are more appropriate to understand the overall stress response of the soil microbial community. An air temperature increase of $3\,°C$ for example, induced shifts in the AOB community structure, decreased AOB richness and concurrently increased potential nitrification rates in the

rhizosphere of legumes. It remains open whether AOA adopted the ability to transform ammonia, while their bacterial counterparts were sensitive to the elevated temperature (Malchair *et al.*, 2010a). Besides questioning the relevance for studying climate change effects, it is unclear whether the observed shifts were a direct effect of the temperature or were rather related to changes of the plant performance, for example, increased exudation, in response to the increased temperature.

More relevant in the context of temperature-related effects are questions addressing changes in soils of permafrost regions, as here, only a slight increase of air temperature results in a prolonged period in which soils are unfrozen during the summer time. In these studies, the focus has mostly been on C turnover and methane emission, although clear effects on N transformation have been described. There is broad agreement that thawing of permafrost soils leads to a rapid increase of denitrification and hence high N_2O emissions, due to the high water saturation and the availability of easily degradable C and nitrate in those soils (Repo *et al.*, 2009; Elberling *et al.*, 2010). Measured emissions were comparable to values from peat soils ($0.9–1.4\,\mathrm{g}\,N_2O\,\mathrm{m}^{-2}$ and year). In contrast, nitrifying communities did not benefit from the changed environmental conditions in the short run. Metagenomic analysis and clone library studies revealed a low diversity and a relatively low abundance for ammonia oxidizers (AOA and AOB) (Liebner *et al.*, 2008; Yergeau *et al.*, 2010). Obviously, the high concentrations of available C as well as the anoxic conditions do not favor the growth of AOA and AOB. Therefore, not surprisingly, in permafrost soils, clear evidence for anaerobic ammonia oxidation has been obtained (Humbert *et al.*, 2010), in contrast to many other soil ecosystems. N-fixing microorganisms did not play a major role in the investigated sites and did not change in abundance and diversity after thawing (Yergeau *et al.*, 2010).

However, also in moderate climatic zones, small shifts in the temperature affect freezing and thawing regimes in soil during winter time and increased numbers of freezing–thawing cycles are expected. Therefore, this topic is of interest for agricultural management practice, notably when intercropping systems are used over winter. Like in permafrost regions, soil thawing is mainly accompanied by an accelerated release of nutrients, but also by the emission of greenhouse gases, such as N_2O and nitric oxide (NO), as well as CO_2 and methane. Considerable research was focused on gaseous N losses and the N_2O/N_2 ratio in the last two decades (Philippot *et al.*, 2007). A modeling study by De Bruijn *et al.* (2009) indicated that N_2O emissions resulting from freezing–thawing are not monocausal and mainly depend on the amount and quality of available C and N, the microbial biomass and the redox conditions in soil after thawing. Although N_2O emissions were reported from

soils that are generally characterized by a low temperature ($< 15\,°C$), these values are far lower than the N_2O concentrations emitted from thawing soils (Koponen & Martikainen, 2004). Wolf *et al.* (2010) could show that up to 70% of the annual N_2O emissions from agricultural fields might occur in the winter period. Peak emissions of N_2O were reported from arable soils during or shortly after thawing (Dörsch *et al.*, 2004) and could only be attributed in part to N_2O physically trapped in soil aggregates during freezing (Teepe *et al.*, 2001). A large part of N_2O arises from the microbial denitrification process, which fits with decreased oxygen and increased C and N availabilities in soils that were subject to freezing–thawing cycles (Öquist *et al.*, 2004). Sharma *et al.* (2006) observed an increase in transcripts of the nitrate and nitrite reductase genes *napA* and *nirK*, respectively, straight after thawing began. Other studies have shown a significant increase in N mineralization compared with nonfrozen soils (De Luca *et al.*, 1992). In contrast to permafrost soils, where aerobic ammonium oxidation did not play an important role, increased nitrification rates were measured after thawing in soils from moderate climatic zones. Su *et al.* (2010) demonstrated that bacterial ammonia oxidizers were impaired by freezing and thawing, whereas their archaeal counterparts even increased in abundance. This is in accordance with the hypotheses by Schleper *et al.* (2005) and Valentine (2007), who presumed that archaea are more tolerant to stress conditions than bacteria. Therefore, archaea could be the main contributors to ammonia oxidation after freezing and thawing.

Studies on the effects of changes in precipitation on microbial N turnover are rare, notably when questions about the effects of extreme weather events are addressed, although it is well accepted that the increased variability in precipitation and the resulting soil water dynamics directly alter N cycling in terrestrial ecosystems (Corre *et al.*, 2002; Aranibar *et al.*, 2004). Not surprisingly, irrigation increased, on the one hand, nitrate leaching rates mainly in sandy soils (Olson *et al.*, 2009). On the other, increased denitrification activities were measured. For example, scenarios simulating high rainfall events resulted in 2.4–13-fold increases in ammonia, nitrate, NO and N_2O fluxes in clay loam, whereas NO and N_2O fluxes decreased in sandy soils in response to water drainage (Gu & Riley, 2010). Ruser *et al.* (2006) found maximum N_2O emission rates in differently compacted soils after rewetting of dry soil that increased with the amount of water added. Muhr *et al.* (2008) postulated that rather than the intensity of rewetting, the length of the drought period might be more important for the process patterns and the microbial communities involved in N_2O and NO emissions. Again, the effects of precipitation depend on other factors like agricultural management. For example, it could be shown that the effects of irrigation depend on the type of cover crop in soil (Kallenbach *et al.*, 2010).

Overall, studies mainly focused on the effects of precipitation on denitrification rates. Other processes of the N cycle as well the corresponding communities have been rarely studied so far. It must also be assumed that these processes are also highly affected directly or indirectly by dryness and precipitation, respectively. Interestingly, Zavaleta *et al.* (2003) demonstrated changes in plant diversity patterns in different grasslands in response to different precipitation regimes, which may indicate indirect effects of different precipitation regimes on nitrifiers as well as on N-fixing microorganisms.

The same authors could show that enhanced CO_2 concentrations in the atmosphere decrease plant diversity at grassland sites. However, C input into the soil via exudation was enhanced, which resulted in an overall stimulation of most microorganisms. Mainly N-fixing bacteria benefited from the additional C input, as their abundance was increased at grassland sites with increased CO_2 (He *et al.*, 2010). As expected, enhanced CO_2 concentrations also stimulated denitrifiers in soil due to a general reduction of the redox potential in soil as a result of the increased microbial activity (Pinay *et al.*, 2007). Furthermore, a stimulation of N mineralization has been proven (Muller *et al.*, 2009). Consequently, elevated CO_2 values in the atmosphere resulted in reduced abundance of autotrophic microorganisms like ammonia oxidizers (Horz *et al.*, 2004) in combination with reduced activity patterns (Barnard *et al.*, 2006) due to competition from heterotrophs as well as lower and lower activity in grassland soils. In addition, several studies have described a positive correlation between plant species richness and AOB richness in grassland soils. Malchair *et al.* (2010b) hypothesized that this link could be due to the spatial heterogeneity of ammonia, promoted by the plant species richness. In contrast, AOB were unaffected by increased atmospheric CO_2 (Nelson *et al.*, 2010) in soils under intensive agricultural use (e.g. soybean or maize cultivation), probably as the present ecotypes in these soils are already adapted to higher C input into the soil, for example, by manuring, litter application and intensive exudation by the cultivated crop. However, when relating those results to ongoing climate change, it must be considered, as described above for temperature effects, that we are challenged with an continuous increase in CO_2 concentrations in the atmosphere and not with a doubling from 1 day to another as simulated in most experiments.

Xenobiotics

New climatic conditions and changed agricultural practice have led to an emerging pressure from weeds and phytopathogens, which complicates farming practice and has resulted in the increased use of (new) chemical substances

worldwide. Pesticides, i.e. herbicides, fungicides and insecticides, can exert collateral effects on soil microorganism and important functions such as N cycling. Some of these compounds also represent a source of N to microbial communities through mineralization. For example, the ability of microorganism to use atrazine as a sole N source has been demonstrated (Mandelbaum *et al.*, 1995; Struthers *et al.*, 1998). As bioavailability of pesticides depends on the formulation as well as on diverse crop and soil factors (e.g. percentage crop cover of the soil surface, soil type, structure, pH, N and C contents, pore volume, water-holding capacity) determining sorption, leaching and degradation of the compound, the response of the microbial biomass is expected to be linked to both the soil type and the pesticide used. Moreover, herbicides are typically applied onto bare soil while fungicides and insecticides are used on dense crops and the exposure of the soil is consequently lower (Johnsen *et al.*, 2001).

The effects of pesticides on bacterial groups involved in N transformation have been thoroughly studied using cultivation-dependent methods in the past, for example, *Rhizobium* fixing N in symbiosis with leguminous plants (Aggarwal *et al.*, 1986; Kishinevsky *et al.*, 1988; Mårtensson, 1992; Revellin *et al.*, 1992; Ramos & Ribeiro, 1993; Singh & Wright, 2002), free-living diazotrophs *Azotobacter* and *Azospirillum* (Banerjee & Banerjee, 1987; Jena *et al.*, 1987; Martinez-Toledo *et al.*, 1988) and nitrifying bacteria (Doneche *et al.*, 1983; Banerjee & Banerjee, 1987; Martinez-Toledo *et al.*, 1992a, b). On the contrary, only a few recent studies have used culture-independent approaches to better gain insight into the effects on the structure and function of soil microbial communities (Engelen *et al.*, 1998; Rousseaux *et al.*, 2003; Seghers *et al.*, 2003; Devare *et al.*, 2004; Saeki & Toyota, 2004; Bending *et al.*, 2007). In many cases, pesticides applied at the recommended field rate concentration did not have a significant impact on the structure and function of the soil microbial communities (Saeki & Toyota, 2004; Ratcliff *et al.*, 2006). Seghers *et al.* (2003) demonstrated that the community structure of AOB in bulk soil of a maize monoculture was unaltered by 20 years of atrazine and metolachlor application. Some other studies have indicated more pronounced effects. Thus, Chang *et al.* (2001) observed a severe impact of atrazine on both the abundance and the community structure of AOB. However, in this study, short-term microcosm experiments were performed with high herbicide concentrations (*c.* three orders of magnitude higher than the field rates). There is also increasing evidence that chloropicrin and methyl isothiocyanate can stimulate N_2O production (Spokas & Wang, 2003; Spokas *et al.*, 2005, 2006). For other herbicides like prosulfuron, glyphosate and propanil as well as the fungicides mancozeb and chlorothalonil, decreased N_2O emissions were observed, possibly because the compounds inhibited

nitrification and denitrification (Kinney *et al.*, 2005). Cernohlávková *et al.* (2009) confirmed this hypothesis and demonstrated that mancozeb and dinocap can impair nitrification at a field rate in an arable and a grassland soil.

Besides pesticides, antibiotics are also extensively used in agricultural production systems, predominantly in livestock husbandry. As slurry and manure are usually applied as organic fertilizers in agricultural farming, a substantial fraction of the administrated compounds enters the environment (Lamshöft *et al.*, 2007). Unlike pesticides, antibiotics are explicitly designed to affect microorganisms. The impact of, for example, sulfadiazine, a broad-spectrum bacteriostatic agent, has been intensively evaluated due to its frequent use, high excretion rate and persistence in soil (Thiele-Bruhn, 2003; Lamshöft *et al.*, 2007; Schauss *et al.*, 2009a). Similar to pesticides, soil and crop characteristics are major factors influencing the response patterns of the microbial communities toward antibiotics in soil (Heuer & Smalla, 2007; Hammesfahr *et al.*, 2008; Kotzerke *et al.*, 2008; Schauss *et al.*, 2009a; Ollivier *et al.*, 2010). Potential nitrification activity remained unchanged under low sulfadiazine concentration conditions in bulk soil when applied in combination with manure (Kotzerke *et al.*, 2008). This might have been due to a substitution of the highly affected AOB by their archaeal counterparts (Schauss *et al.*, 2009b). Similar observations concerning sulfadiazine effects on the abundance patterns of AOB and AOA were made in the rhizosphere of maize and clover (Ollivier *et al.*, 2010). Also, both functionally redundant groups of nitrite reducers were negatively influenced by antibiotic addition to manure. Hence, not surprisingly, potential denitrification rates decreased in treatments where sulfadiazine was applied (Kotzerke *et al.*, 2008). While nitrite reducers harboring the *nirS* gene increased in abundance after bioavailable sulfadiazine had declined, the abundance of *nirK*-harboring nitrite reducers remained on the level of the nonmanured control treatment (Kleineidam *et al.*, 2010). Clearly, pronounced effects of sulfadiazine on the denitrifying bacteria were also observed in the rhizosphere of maize and clover, where the dominating *nirK*, but also the *nirS* nitrite reducers as well as the *nosZ*-harboring N_2O reducers were significantly impaired (Ollivier *et al.*, 2010). Furthermore, the abundance of *nifH* genes, coding for key enzyme of N fixation, was significantly impacted by sulfadiazine in the rhizosphere of both plant types, but to a greater extent in the rhizosphere of the legume.

Conclusions and outlook

The research over the last two decades linking N transformation processes in soil to the corresponding functional microbial communities has improved our knowledge

significantly about the factors driving the abundance, diversity and activity mainly of microorganisms involved in the inorganic N cycle as well as the dynamics of the corresponding turnover processes and nutrient fluxes. Overall, most studies that addressed questions linked to the consequences of land-use changes or agricultural management included data for nitrifiers, denitrifiers and N-fixing microorganisms, whereas studies in the area of climate change in most cases focused only on consequences for denitrification and N_2O emissions. This reflects well the areas of interest of the various scientific communities involved in the different research areas. However, it must be taken into account that the processes of the N-cycle are closely interlinked and thus influence each other. Thus, even if the focus is on trace gas emissions from soil, knowledge of processes like nitrification and N-fixation is of key importance too. In general, data on the diversity and abundance of N-mineralizing microorganisms are rare in microbial ecology, due to the huge variety of different biochemical pathways, which are so far mostly unknown. Therefore, not surprisingly, in most studies that are of relevance for consequences of global change on N-transformation, this functional group of microorganisms has been excluded from analyses. Nevertheless, it is generally accepted that the amount of mineralized nitrogen is one major driver for the inorganic nitrogen cycle mainly in nonfertilized natural soils.

From the recently published data, the following conclusions can be drawn generally: (1) global change-related modifications of environmental factors affect nitrifiers, denitrifiers and N-fixing microorganisms and alter the corresponding processes. (2) The abundance of the autotrophic ammonia oxidizers and nitrite oxidizers in soil is negatively correlated with additional C input by plants as a result of land-use changes towards agricultural land or a more intensive agriculture as well as enhanced CO_2 concentrations in the atmosphere. This results in soils, where no inorganic fertilizer has been applied, in reduced nitrate concentrations and consequently, despite the presence of easily degradable carbon sources, in reduced denitrification activity under anoxic conditions. Although N-fixing microorganisms benefit from the additional carbon input, their activity is only increased under low ammonia concentrations in soil, for example, conditions where most of the ammonia is taken up by the plant or by soil microorganisms for biomass production. Overall, plants might benefit from this scenario due to reduced competition for ammonium with ammonia-oxidizing microorganisms in soil. Furthermore, such conditions may reduce the amount of leached nitrate as well as emissions of N_2O. (3) By contrast, ammonia oxidizers might benefit from the application of xenobiotics as AOA in particular seems to tolerate a number of compounds that, like antibiotics, are toxic for other prokaryotes (Schauss *et al.*, 2009a, b).

This may result in increased nitrification rates if enough ammonia is available and consequently in the formation of nitrate. As denitrifiers might be reduced in their activity under the given scenario, nitrate could leach to the ground water, if it is not taken up by the plants. (4) Water conditions and the oxygen content in soil highly influence nitrifiers and denitrifiers. Under anoxic conditions, however, the activity of denitrifiers again depends on the amount of available nitrate and, therefore, either on fertilization regimes or the activity of nitrifiers in non-water-logged habitats in soil.

As stated in the introduction, 'global change' encompasses *interlinked* activities of the different scenarios described above. Because each scenario results in a different response pattern of the investigated microbial communities, a prediction of what happens if two or more scenarios are mixed is almost impossible. For example, whether the addition of xenobiotics and increased carbon inputs by increased atmospheric CO_2 concentrations will lead to higher or lower concentrations of nitrate in soil cannot be predicted from currently available data. However, these types of predictions are needed to transform scientific results into concrete recommendations for practice. Another important aspect of research linked to global change is to understand the long-term consequences of changes in the environment for microbial life in soil. As yet, most studies in the past have concentrated on short-term effects using sometimes highly unrealistic predictions of future conditions. Therefore, in many cases, results represent data more relevant for disturbance ecology than for global change research. As described above, this is true for many experimental setups in the frame of climate change. Finally, the different scales of relevance must be taken into account. Microorganisms act on the μm^2 scale; however, the scales that need to be addressed in terms of political recommendations are at regional or even at a global scale. And conceptual approaches to overcome the scale problem are far from being 'on the market'. This holds true for 'upscaling' from 1 g of soil to the ha or km^2 scale, but also for 'downscaling' 1 g of soil to microsites of μm^2, where microbial life occurs. In this respect, research addressing questions about the relevant scale that must be considered for different scenarios of global change is currently absent.

Authors' contribution

J.O. and S.T. contributed equally to this work.

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Curriculum Vitae

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Publications

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