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SHORT COMMUNICATION

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

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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 monooxygenase 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

abundance, caldarchaeol was assessed as an overall lipid marker being present in all major archaeal phyla (De Rosa and Gambacorta 1988).

In this study, we analyzed the ratio of archaeal *amoA* genes to crenarchaeol as well as the amount of caldarchaeol in a natural tidal wetland and four agriculturally used paddy soils in the southeast of China. The abundance of *amoA* genes was determined by real-time PCR while crenarchaeol and caldarchaeol, respectively, were measured via pressurized liquid extraction.

Materials and methods

Site description and soil sampling

The study sites are located in Cixi, Zhejiang Province, China, in a subtropical monsoon area, with a mean annual temperature of 16.3°C and precipitation of 1,325 mm (Zhang et al. 2004). We sampled five flooded soils, one of them being a natural tidal wetland (TW) and four cultivated paddy soils (P50, P100, P300, and P2000). The coordinates of the sampled sites are: TW: 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. All five sites under investigation are located within 40 km. The paddy soils which had been obtained by land reclamation from the tidal wetland are used for rice cultivation for 50, 100, 300, and 2,000 years, respectively, and differ in pH value, total organic C and total N content (Table 1). The duration of rice cultivation at the respective sites was estimated according to Cheng et al. (2009).

All soils were sampled in July 2009 at the beginning of the vegetation period. As all paddy fields are located in the same region and the agricultural management is centrally controlled in China since 1949 by instructions of the technical service bureau, a comparable management has been performed for all sites. Five independent field replicates were taken at each site with a soil auger from 0–20 cm depth. Soil aliquots for DNA extraction were shock-frozen in liquid N directly after sampling and stored at −80°C.

DNA was extracted with the FastDNA Spin Kit for soil (MP Biomedicals, USA), according to the protocol of the manufacturer. Quality and quantity of the DNA extracts were checked with a spectrophotometer (Nanodrop, PeqLab, Germany).

Quantitative real-time PCR of archaeal as well as bacterial *amoA* genes was carried out according to Töwe et al. (2010). Dilution series of the different DNA extracts were tested in a pre-experiment with all soils to avoid

Table 1 Characterization of the five examined soils (tidal wetland 50, 100, 300, and 2,000 years cultivated paddy soils) by different parameters: soil texture, pH value ($CaCl_2$), total organic C, and total N, nitrate and ammonium concentrations, microbial biomass C, microbial biomass N, and DNA content

Soil parameters	TW	P50	P100	P300	P2000
Soil texture (% sand, silt, clay)	7.4	0.4	2.0	3.4	4.0
	80.4	83.6	81.2	81.2	85.1
	12.2	16.0	16.8	15.4	10.9
pH	8.1 a	7.6 b	7.6 b	7.5 b	7.3 c
	(0.13)	(0.08)	(0.13)	(0.08)	(0.10)
TOC (%)	0.58 a	1.7 b	1.7 b	2.5 c	3.1 d
	(0.17)	(0.14)	(0.16)	(0.16)	(0.11)
TN (%)	0.060 a	0.17 b	0.19 c	0.27 d	0.36 e
	(0.012)	(0.014)	(0.015)	(0.020)	(0.019)
Nitrate (μg Ng^{-1} dw)	2.1 a	12 ab	8.3 ab	16 b	2.2 a
	(0.70)	(1.2)	(2.9)	(6.5)	(1.7)
Ammonium (μg Ng^{-1} dw)	0.42 a	6.0 a	25 a	27 a	22 a
	(0.12)	(3.5)	(19)	(30)	(11)
Cmic (μg g^{-1} dw)	150 a	720 b	1,000 b	1,800 b	5,100 c
	(58)	(120)	(330)	(780)	(1,300)
Nmic (μg g^{-1} dw)	39 a	28 a	110 ab	92 ab	150 b
	(6.8)	(18)	(55)	(36)	(30)
DNA content (μg g^{-1} dw)	140 a	760 bc	630 b	810 bc	1,100 c
	(35)	(24)	(120)	(220)	(140)

Standard deviations are given in parentheses (n=5). Significant differences are indicated by different letters

TW tidal wetland, *TOC* total organic C, *TN* total N, *Cmic* microbial biomass C, *Nmic* microbial biomass N

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=[$10^{(-1/\text{slope})}-1$]×100%, 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

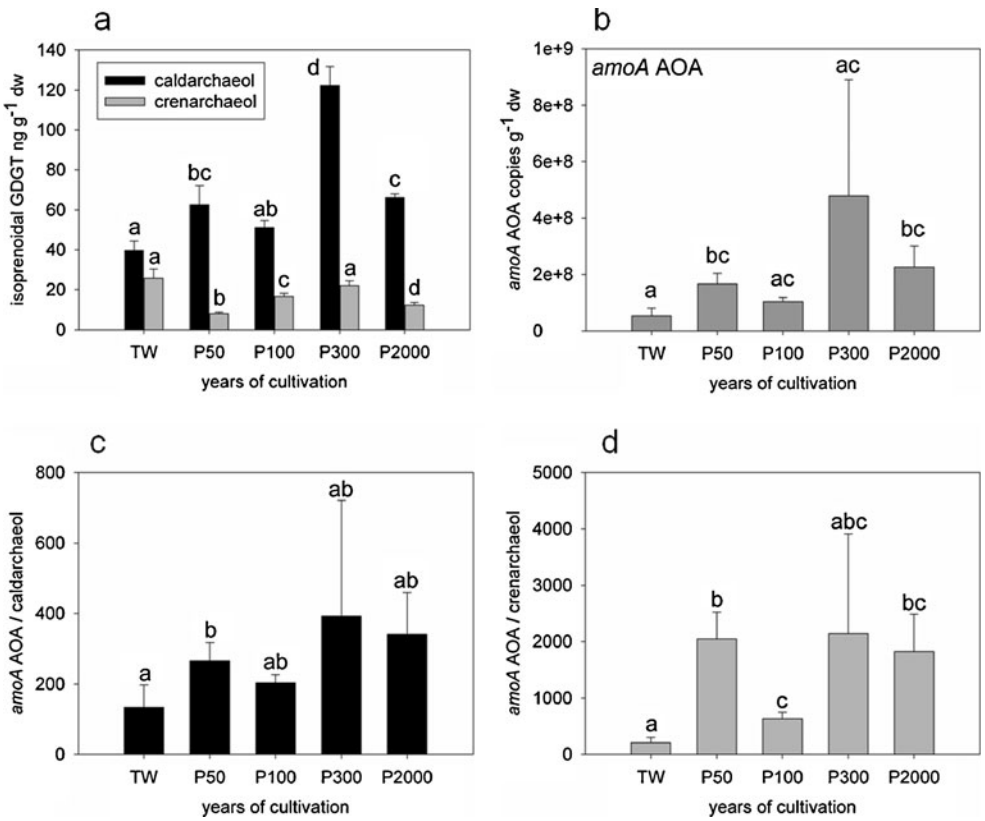

Fig. 1 Total copy numbers of *amoA* AOA genes (**a**) and values of isoprenoidal glycerol dialkyl glycerol tetraether lipids (caldarchaeol and crenarachaeol) (**b**) as well as ratios of total *amoA* AOA copy numbers to amounts of caldarchaeol (**c**) and crenarchaeol (**d**), respectively (×10^7 copies 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 ($\times10^7$ 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 ($\times10^7$ 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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