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SHORT COMMUNICATION

Influence of different litter quality on the abundance of genes involved in nitrification and denitrification after freezing and thawing of an arable soil

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Abstract Due to disruption of soil aggregates and cell lysis and the subsequent release of organic C and N, increased microbial N transformation processes can be observed after freeze–thaw cycles. In a microcosm study, we investigated the influence of plant residues with different C/N ratios (lucerne-clover-grass-mix and wheat straw) on N transformations and the abundance pattern of the corresponding functional genes in an arable soil after freezing and thawing. Unfrozen soil samples, continuously incubated at 10°C, served as control. Concentration of soil NH_4^+, NO_3^-, and water-extractable organic C (WEOC) as well as genes involved in nitrification and denitrification, quantified by real-time PCR, were determined before freezing and 1, 3, and 7 days after thawing. The amounts of inorganic N and WEOC as well as the investigated gene abundance pattern did hardly differ between control samples and samples subjected to freezing and thawing that have been amended with straw. In contrast, clear alterations of the measured parameters and abundances were observed after freezing and thawing in samples being amended with the lucerne-clover-grass-mix compared to the control samples.

Keywords Freeze–thaw · Organic amendment · Nitrification (*amoA* AOA; *amoA* AOB) · Denitrification (*nirK*; *nirS*)

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Introduction

Factors driving microbial N cycle in soils during the vegetation period have been studied in detail in the last decades. It has been clearly shown that many factors such as mineral N and available C contents, oxygen concentration, soil water content, pH value, and temperature (Davidson and Kingerlee 1997) influence the N transformation rates. Although high denitrification rates during the winter months have been observed accounting for up to 30% of the annual N_2O emissions from agricultural soils (Ruser et al. 1998), there is still a lack of understanding how N turnover is regulated during the cold time of the year (Phillips 2008).

Legumes and legume–grass mixtures, frequently used as green manure in organic farming, might be one factor influencing N turnover in winter. These plants are cropped in autumn, remain on the fields over winter, and supply plants in the following vegetation period with N. Due to the low C/N ratio of green manures compared to other plant residues (e.g., straw of cereals, which is left on the fields after harvest in autumn), more N is also available for microbial activities. However, it is poorly understood how and under which conditions the N of the plant residues is transformed during the winter months. We expected that due to freeze–thaw processes and the increased amounts of available C, e.g., microbes involved in denitrification will become more abundant depending on the quality and amount of applied N.

Therefore, we investigated the influence of two common agricultural plant residues (wheat straw and lucerne-clover-grass-mix) considerably differing in their C quality and C/N ratio on nitrifiers and denitrifiers based on abundance of the functional genes for the ammonia monooxygenase gene *amoA* and the nitrite reductase genes *nirK* and *nirS*,

Table 1 Primers used for real-time PCR quantification of functional genes [archaeal *amoA* (AOA) and bacterial *amoA* (AOB), *nirK*, and *nirS*]

Gene	Primer	Literature
amoA (AOB)	amoA 1F	(Rotthauwe et al. 1997)
	amoA 2R	(Rotthauwe et al. 1997)
amoA (AOA)	19F	(Leininger et al. 2006)
	CrenamoA616r48x	(Schauss et al. 2009)
nirS	cd3af	(Michotey et al. 2000)
	R3cd	(Throbäck et al. 2004)
nirK	nirK 876	(Henry et al. 2004)
	nirK 5R	(Braker et al. 1998)

respectively, and on mineral N fractions (NO_3^- and NH_4^+) after a freeze–thaw cycle.

Materials and methods

The experimental site is located on the research farm "Klostergut Scheyern" in Southern Germany (48°30.0′ N; 11°20.7′ N). Soil samples were taken from an arable field (Cambisol) in September 2008 several weeks after harvest of summer wheat. The upper 20 cm of the plowed soil horizon were sampled and characterized as follows: 22% clay, 36% silt, and 42% sand; 1.5% organic C, C/N ratio of 10.0; pH value 5.8 (0.01 M $CaCl_2$). The soil was sieved at 2 mm and air-dried at room temperature.

Wheat straw and growth of a lucerne-clover-grass-mix were chosen as organic amendment reflecting common agricultural situations at field sites in autumn and winter. The different plant residues exhibited similar C contents (43.6% and 42.1%, respectively) but differed considerably in their C/N ratios (126.6 and 10.1, respectively), recalcitrance, and biodegradability (Mueller et al. 1998; Nicolardot et al. 2007; Wang et al. 2004). The residues were air-dried and ball-milled (Retsch MM2, Germany) before application.

The experiment was set up in the following way: 50 g soil was amended with 2 g of the respective plant residues and incubated in cylinders of 4-cm height and a volume of 100 cm^3. Thus, similar amounts of C were added to the soil (corresponding to 222 dt C ha^{-1}), however of different quality and C/N ratios. Per treatment and sampling time point, four independent replicate cylinders were prepared. All samples were equilibrated during a pre-incubation period of 2 weeks at 10°C and 60% water holding capacity. Half of the samples were then frozen for 3 days at −20°C and afterwards returned to 10°C; the other half of the samples remained constant at 10°C. Sampling was carried out at day 0 (day before freezing) and days 4, 6, and 10 (1, 3, and 7 days after thawing), respectively.

Aliquots of soil samples were immediately extracted with 0.01 M $CaCl_2$ [soil/$CaCl_2$ ratio 1:2 (wt/wt)] according to Zsolnay (2003). Both NH_4^+–N and NO_3^-–N concentrations were measured using the Nanocolor Ammonium 3

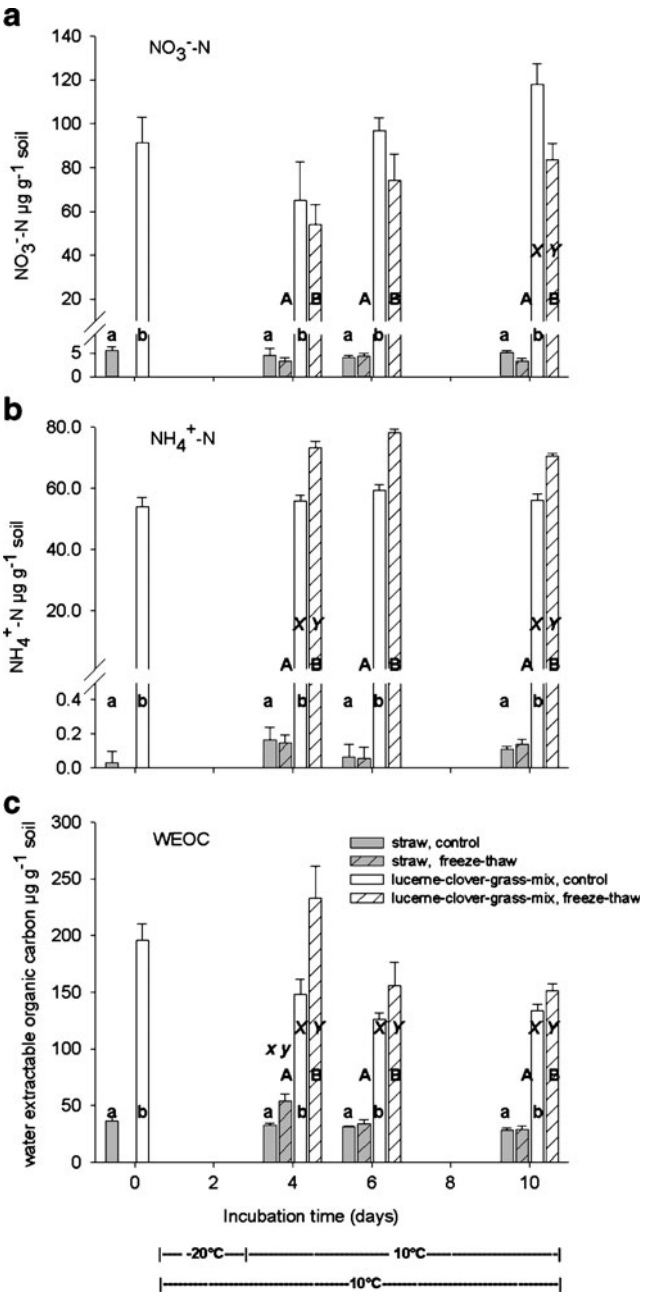

Fig. 1 Amounts of ammonium, nitrate, and water-extractable organic carbon in the differently treated soils during incubation. Samples were taken at day 0 (day before freezing), day 4 (first day after thawing), day 6 (third day after thawing), and day 10 (1 week after thawing). *Error bars* indicate standard deviation of mean (n=4). Different *letters* display at a given time point a significant difference between the freeze–thaw treatment and the unfrozen control treatment in the straw-amended (*x*/*y*) and the lucerne-clover-grass-mix-amended soil (*X*/*Y*) or a significant difference between the straw amendment and the lucerne-clover-grass-mix amendment in the unfrozen control treatment (*a*/*b*) and the freeze–thaw treatment (*A*/*B*)

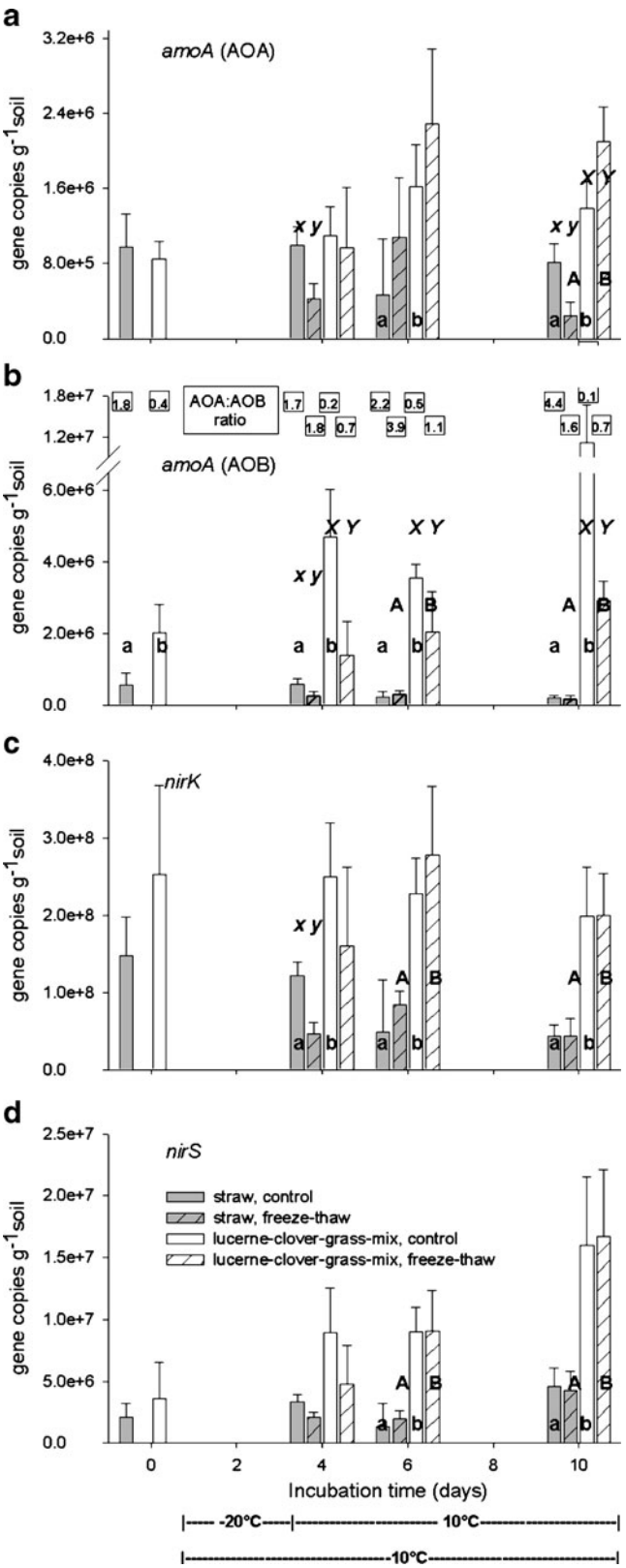

Fig. 2 Abundances of the functional genes *amoA* (AOA and AOB), *nirK*, and *nirS* expressed as copy numbers per gram of dry soil in the differently treated soils during incubation. Samples were taken at day 0 (day before freezing), day 4 (first day after thawing), day 6 (third day after thawing), and day 10 (1 week after thawing). *Error bars* indicate standard deviation of mean (n=4). For explanation of the different *letters*, please refer to Fig. 1. Ratios of AOA to AOB *amoA* copies are shown in *boxes* in Fig. 1b

and Nanocolor Nitrat 50 kit (Merck, Germany), respectively. The concentrations of water-extractable organic C (WEOC) were determined by DIMATOC 100 (DIMATEC, Germany) in the extracts.

The remaining soil was stored at −20°C until soil DNA was extracted according to Griffiths et al. (2000). Gene abundances of functional genes related to nitrification [archaeal *amoA* (AOA) and bacterial *amoA* (AOB)] and denitrification (*nirK* and *nirS*) were measured by SybrGreen-based quantitative real-time PCR, using the 7300 Real-Time PCR System (Applied Biosystems, Germany) as described by Hai et al. (2009). Briefly, the reaction volumes consisted of 25 µl including 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µl 3% BSA (Sigma-Aldrich, Germany), 0.625 µl DMSO (Sigma, Germany), 2 µl DNA template, 0.5 µl of each primer (10 µM, Table 1), and 8.375 µl 0.1% DEPC water. All samples and standard curves (serial plasmid dilutions from 10^1 to 10^6 gene copies μl^{-1}) were performed in triplicates, and at least four negative controls without DNA template were run per plate. Amplification efficiencies of 81–86% (*amoA* AOA), 79–82% (*amoA* AOB), 85–90% (*nirK*), and 86–91% (*nirS*) were achieved. Copy numbers were related to 1 g of dry soil.

Statistical analyses were carried out using SPSS 11.5. Prior to analysis, data were checked for normal distribution and homogeneity of variances by the Kolmogorov–Smirnov test and the Levene test, respectively. The effect of the freeze–thawing, respectively, of the organic amendments at a given time point were tested on significance with the t test (P<0.05).

Results and discussion

Due to the low N input in the straw treatment, soil NH_4^+ and NO_3^- concentrations did not differ between the samples treated by freezing and thawing and the control samples constantly kept at 10°C. The measured concentrations remained low (3.3–5.5 µg NO_3^-–N and up to 0.3 µg NH_4^+–N g^{-1}, respectively) and did not change during the investigation period (Fig. 1a, b). Similar observations were made for the soil WEOC contents in this treatment (28.3–54.2 µg C g^{-1}; Fig. 1c). In contrast, addition of the lucerne-clover-grass-mix significantly increased the soil NO_3^-–N (56.5–113.6 µg g^{-1}), NH_4^+–N (54.0–70.6 µg g^{-1}), and

WEOC (126.3–195.8 μg g^{-1}) concentrations compared to the straw amendment (Fig. 1a–c). In this treatment, freezing and thawing significantly increased the amount of NH_4^+–N and WEOC and led to lower NO_3^-–N concentrations compared to the non-frozen control samples.

It is generally accepted that the amount of available inorganic N is one of the factors regulating denitrification rates in soil (Phillips 2008). Our results support our hypothesis that this is not only true for the vegetation period but can be extended to the winter period. Clark et al. (2009) could show that nitrification was the cause of NO_3^- accumulation in frozen soils, and this process was consequently limited by NH_4^+ availability. Therefore, higher NH_4^+ concentrations are often associated with increased NO_3^- concentrations (Elliott and Henry 2009).

High amounts of NH_4^+ during mid-winter thawing periods might be a result of increased soil protease activity, which is also related to the availability of C (Mrkonjic Fuka et al. 2009). In our study, we found higher concentrations of WEOC in the samples amended with the lucerne-clover-grass-mix (Fig. 1c) and correlating higher NH_4^+ concentrations. Moreover, WEOC determines denitrification activities (Phillips 2008). Thus, WEOC affects N turnover in two ways: on the one hand it stimulates the protease activity and the formation of inorganic N, and on the other hand it is a prerequisite for denitrification.

According to the different amounts of soil inorganic N and readily available organic C in the two treatments, higher abundances of genes involved in nitrification (AOA $0.8–1.6\times10^6$ and AOB $0.2–1.1\times10^7$ *amoA* copies g^{-1}; Fig. 2a, b) and denitrification (*nirK* $2.0–2.5\times10^8$ and *nirS* $0.4–1.6\times10^7$ copies g^{-1}; Fig. 2c, d) were determined in the soil amended with lucerne-clover-grass-mix. Interestingly, somewhat higher archaeal *amoA* (AOA; $4.7–9.7\times10^5$ copies g^{-1}) than bacterial *amoA* (AOB; $1.8–5.8\times10^5$ copies g^{-1}) gene copies were found in the straw-amended samples, whereas lucerne-clover-grass-mix-amended samples showed the opposite behavior (Fig. 2a, b). However, the influence of freezing and thawing resulted in both treatments in increased AOA/AOB ratios compared to the non-frozen control soil (Fig. 2b).

The result that AOB are more affected by the freeze–thaw event compared to AOA also supports the hypotheses by Schleper et al. (2005) and Valentine (2007) who presumed that archaea are more tolerant to stress conditions than bacteria. Several studies also showed that AOA/AOB ratios increase with decreasing availability of C and N (Leininger et al. 2006). It was also speculated that AOA might be more closely attached to soil particles which might result in a certain protection of AOA compared to AOB.

Concerning the genes involved in denitrification, the straw-amended samples also revealed lower copy numbers of both, *nirK* ($0.4–1.5\times10^8$ copies g^{-1}) and *nirS* ($1.4–4.6\times10^6$ copies g^{-1}) genes (Fig. 2c, d). However, both treatments exhibited considerably more *nirK* than *nirS* gene copies and showed decreased copy numbers after 1 day of thawing compared to the non-frozen control.

The higher *nirK* copy numbers could reflect a possible niche differentiation for microbes harboring one of the two functionally redundant nitrite reductases, where *nirK*-possessing bacteria could preferentially colonize habitats with more available C and N. This trend has also been observed when hotspots in soil exhibiting high amounts of available C and N have been compared with low activity areas (e.g., rhizosphere vs. bulk soil) (Sharma et al. 2005). In the legume mix-amended samples, a fast recovery of *nirK* copy numbers was detected, which was also found by Sharma et al. (2006), whereas the straw-amended soil showed a decrease in gene copies also in the control samples during the incubation period.

Our results indicate that the amount and quality of applied N have distinct effects on the abundance of genes involved in N cycling and the corresponding N transformations during freezing and thawing events. This is in line with previous studies showing the close link between the different transformation processes of the nitrogen cycle (Nannipieri and Paul 2009). This study indicates that the nitrogen stored in legumes, which are often used as green manure in agriculture, can fast be mineralized, nitrified, and denitrified during winter time as a result of freezing and thawing cycles. It could be considered if nitrification inhibitors applied after thawing might help to reduce transformations of the formed NH_4^+ into NO_3^- and thus reduce N losses via leaching of NO_3^- and emission of N_2O and N_2 by denitrification.

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