Soil Biology & Biochemistry 80 (2015) 283–292

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Mycorrhizal effects on nutrient cycling, nutrient leaching and N_2O production in experimental grassland

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ARTICLE INFO

Article history:
Received 4 August 2014
Received in revised form
7 October 2014
Accepted 17 October 2014
Available online 28 October 2014

Keywords:
Arbuscular mycorrhizal fungi
Nitrous oxide
Nitrogen
Phosphorous
Resource efficiency
Agriculture
Organic N
Unreactive P

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) can enhance plant nutrition and growth. However, their contribution to nutrient cycling in ecosystems is still poorly understood. Using experimental grassland microcosms filled with two different soil types (pasture and heath soil) and fertilized with different N forms (NO_3^- or NH_4^+), we tested the AMF contribution to N and P cycling including measurements of organic and inorganic leaching losses and N_2O fluxes. We hypothesized that AMF enhance the sustainability of plant-soil systems by reducing nutrient losses and enhancing plant nutrient uptake. AMF reduced reactive and unreactive P leaching by 31%, enhanced plant P contents by 15% and increased P mobilization from soil by 18%. AMF reduced N_2O fluxes and NH_4^+ leaching in both soils. Leaching of dissolved organic N was reduced by 24% in the heath soil only. Plant N contents were increased by 13% in the pasture soil but not affected in the heath soil. The microbial biomass N content was higher with AMF. This is the first comprehensive assessment of the influence of AMF on N and P cycling, including effects on inorganic and organic nutrient leaching losses and N_2O emissions in a single study. We conclude that AMF can promote sustainable nutrient cycling but the effects on N cycling are context dependent.

1. Introduction

In agriculture, huge amounts of chemical fertilizers are applied to fields, of which around 50% remain unused by crops and are prone to getting lost from the system (Smil, 1999; Liu et al., 2010). Nutrient losses are among the top environmental threats to ecosystems worldwide, as they can result in the pollution of waterways, harm the integrity of downstream ecosystems and add greenhouse gases to the atmosphere (Galloway et al., 2003). Moreover, the global phosphorus reserves suitable for the production of fertilizers are limited and might experience depletion within the next century (Cordell et al., 2009; Van Vuuren et al., 2010), while the production of mineral N fertilizers is highly depended on declining fossil energy resources (Vance, 2001). Thus, there is an urgent need to increase nutrient use efficiency and reduce fertilizer application and nutrient losses in agro-ecosystems (Schlesinger, 2009). Increased nutrient use efficiency will help to maintain agricultural yields sufficient to feed a growing global population, and reduce environmental impacts.

Soil biota form an indispensable component of nutrient cycling. It is well established (Robertson and Groffman, 2007; van der Heijden et al., 2008) that soil biota regulate nutrient transformations in soil and consequently determine plant nutrient availability. In addition to this, there is increasing evidence that soil biota influence the amount of nutrients being lost from soil via leaching or as gaseous forms (Plante, 2007; Philippot et al., 2009; Wagg et al., 2014; Bender and van der Heijden, 2014). However, the role of many specific groups of soil biota in regulating nutrient cycling is still poorly understood and the contribution of soil biota to several specific processes within the nutrient cycle (e.g. leaching of organic nutrients, denitrification and N_2O production) is unclear and has large uncertainties.

Arbuscular mycorrhizal fungi (AMF) are a very widespread group of soil fungi, whose contribution to nutrient cycling processes is increasingly becoming acknowledged (Smith and Smith, 2011a; Veresoglou et al., 2012; Hodge and Storer, 2014). They form symbiotic relationships with the majority of land plants and it has been recognized that these fungi can enhance plant growth by

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http://dx.doi.org/10.1016/j.soilbio.2014.10.016
0038-0717/

improving plant P nutrition (Sanders and Tinker, 1971; Clark and Zeto, 2000). AMF have also been shown to transfer N from soil to plants and in some (George et al., 1995; Atul-Nayyar et al., 2009; Cavagnaro et al., 2012), but not all (Ames et al., 1983; Hawkins et al., 2000; Reynolds et al., 2005) cases improve plant N nutrition. The relevance of AMF for plant N nutrition under ecological relevant conditions is still unclear (Fitter et al., 2011).

While a substantial body of research focused on AMF effects on plant nutrition and performance, their involvement in other ecosystem processes, in particular in nutrient cycling has received relatively little attention (Rillig, 2004) and is not well understood. A limited number of studies has, so far, experimentally investigated the influence of AMF on nutrient leaching losses (Asghari et al., 2005; van der Heijden, 2010; Asghari and Cavagnaro, 2011; Corkidi et al., 2011; Asghari and Cavagnaro, 2012), mostly focusing on mineral leaching. It was shown that AMF can reduce leaching losses of mineral compounds of P and N, leading to the suggestion that AMF increase the nutrient use efficiency and sustainability of plant-soil systems (van der Heijden, 2010). The results obtained in these studies are, however, based on a limited set of soil conditions, and none investigated all different forms of P and N potentially being prone to leaching. For example all but one study used substrates with sand contents ≥80%, while one study used an artificial growth substrate (Corkidi et al., 2011). Moreover, all but one study determined leaching losses of inorganic N and P compounds only, while one study (Asghari et al., 2005) also determined total P losses but did not differentiate between different P fractions.

Apart from dissolved PO_4^{3-}–P, directly available to plants and therefore also defined as reactive P, P can also be leached in unreactive forms comprising all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates and particulate inorganic material, e.g. clays (Pote et al., 2009). These fractions can make up a substantial part of total leaching losses. For example, Ulén (1999) found up to 88%, and Neumann et al. (2012) up to 60% of P leaching in unreactive forms.

Non-mineral N leaching losses in dissolved organic form were found to make up to 64% by Dijkstra et al. (2007), while Ghani et al. (2010) found losses up to 118 kg ha^{-1} yr^{-1} in the form of dissolved organic N (DON), making up 97% of total N leaching loss. Because of the potentially important quantitative contribution of leaching losses in non-mineral form to total leaching, it remains unclear whether AMF can reduce overall N and P leaching losses, or only losses of certain nutrient compounds. To understand whether AMF contribute to improved nutrient recycling and enhanced sustainability, it is necessary to investigate the effects of AMF on nutrient leaching under a wider range of soil conditions and to assess all forms of N and P being leached.

Nitrogen can also be lost in gaseous forms. Estimates of N losses via denitrification, when nitrate is transformed to N_2O and N_2, are very variable and can range from 0 to more than 300 kg N ha^{-1} yr^{-1} lost from agricultural soils (Jambert et al., 1997; Hofstra and Bouwman, 2005; Seitzinger et al., 2006; van der Salm et al., 2007). In earlier work we observed that AMF can reduce gaseous losses of N as N_2O (Bender et al., 2014) and results of a recent study point in a similar direction (Lazcano et al., 2014). However, another study found no effects of AMF on N_2O emissions (Cavagnaro et al., 2012). To our knowledge, only very few studies addressed the effects of AMF on N_2O emissions so far and their universality is still unclear. It is also not known, to which extent AMF influence N_2O emissions under different soil conditions.

The relevance of AMF for N uptake and plant N nutrition is believed to be higher when N is provided in the form of NH_4^+ (Hamel, 2004; Govindarajulu et al., 2005; Tanaka and Yano, 2005). If AMF N uptake is higher under NH_4^+ dominated conditions, one would also expect stronger effects of AMF on N leaching under NH_4^+ dominated conditions, as AMF would reduce the availability of mineral soil N prone to leaching.

The aim of this study is to examine the influence of AMF on N and P cycling by testing effects on plant nutrient uptake, nutrient mobilization, nutrient leaching and gaseous losses of N_2O.

We set up experimental grassland microcosms with two different soils (pasture soil and heath soil) and fertilized with different N forms (NO_3^- or NH_4^+). Because NH_4^+ is in most soils quickly transformed into NO_3^- through the process of nitrification, we chose a *Calluna vulgaris* dominated acid heath soil, which often have a low nitrification activity and in which NH_4^+ is the dominant N form (Troelstra et al., 1990). We assessed the plant and soil nutrient pools, nutrient losses via leaching and fluxes of the greenhouse gas N_2O.

We hypothesized that

1) AMF reduce P losses through leaching. AMF reduce N losses, this effect is stronger under NH_4 fertilization, especially in the heath soil.
2) AMF affect N_2O emissions from denitrification. N_2O emissions are higher under NO_3^- fertilization and are negligible in the heath soil with NH_4 addition.
3) AMF improve plant P and N nutrition.

2. Material and methods

2.1. Experimental system

Grassland microcosms were established in PVC tubes with a diameter of 15 cm, a height of 40 cm, and a volume of approx. 7 L (see Fig. S1). A drain tap was inserted in the bottom of the tubes to allow leachate collection. A sleeve with a rubber seal and a removable cap was fit on the tubes to close the headspace airtight in order to collect gas samples to asses N_2O production. For N_2O measurements, the cap contained two valves in which tubes for gas sampling could be inserted. The sleeve could be moved vertically along the tube surface to form the headspace chamber. The microcosms were filled with 5.0 L of sterilized soil-sand-mixture (see below for details) containing 5.4% AMF inoculum and were planted with *Lolium multiflorum* var. Oryx, a common grass species in Swiss agricultural and natural grasslands often dominating temporary pastures in Switzerland (Nyfeler et al., 2009). For better drainage and filtering purposes 1.3 kg of an autoclaved sand–gravel mixture was added to the bottom of the pots.

The experimental setup comprised 2 soil types, 2 AMF treatments and 2 N fertilizer treatments (see below), each treatment combination being replicated 7 times, resulting in a total of 56 microcosms.

2.2. Soil, inoculum and planting

The pasture soil was a calcaric cambisol collected from a long-term pasture site on an ecological farm near Research Station Agroscope ART in Zürich, Switzerland (47°43′11.83″ N, 8°53′65.25″ E). The pasture had been regularly manured.

The heath soil was a dystric cambisol collected from a dwarf shrub heath land dominated by *C. vulgaris* (L.) *and Vaccinium myrtillus* (L.) in the Black Forest, Germany (47°83′38.83″ N, 8°07′08.74″ E). Collected soils were 4 mm sieved, air dried and mixed with quartz sand to a soil:sand-ratio of 7:3 (v/v). These mixtures were then gamma irradiated with a maximum dose of 32 kGy to eliminate indigenous AMF. Four weeks after irradiation, the soil-sand mixtures were filled into the microcosms, moistened and incubated at room temperature for 2 weeks to allow

stabilization of soil chemical properties before the experiment was initiated.

AMF inocula of *Funneliformis mosseae* (T.H. Nicolson & Gerd., previously named *Glomus mossae*, isolate HG 505/SAF 10), *Rhizophagus irregulare* (Blaszk., Wubet, Renker & Buscot, previously named *Glomus intraradices*, *isolate* SAF 22), and *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm., previously named *G lomus claroideum*, isolate HG 181/SAF 4) (Schüßler and Walker, 2010), common AMF species in Swiss grassland and arable soils (Jansa et al., 2002; Oehl et al., 2010) were used in this experiment. All fungal isolates used can be found in the Swiss collection of AMF (SAF; http://www.agroscope.admin.ch/bodenoekologie/08050/08067/index.html?lang=en). The fungal isolates had been propagated separately on *Plantago lanceolata* L. plants in 3 L pots containing a 3:17 (v/v) soil:sand-mixture in the greenhouse. A volume of 270 ml of a mixture of the three AMF inocula was mixed into the microcosms to create the mycorrhizal ('M') treatment. A control inoculum not containing AMF propagules was produced under exactly the same conditions and 270 ml of this inoculum were mixed into the microcosms to create the non-mycorrhizal ('NM') treatment.

Soil irradiation not only eliminated indigenous AMF but also removed a significant proportion of other soil biota. Therefore, to include microbes from natural grassland and to allow a similar microbial background among the AMF and control inoculums, 100 ml of a microbial wash was mixed into the substrate for each microcosm (Koide and Li, 1989; van der Heijden et al., 2006). The microbial wash was produced by suspending fresh field soil (either the pasture or the heath soil) and all used inocula in deionized water and subsequent filtering through a Schleicher and Schüll, No. 598 ½ filter paper (Schleicher & Schüll, Dassel, Germany).

The characteristics of the final soil mixtures in the microcosms are summarized in Table S1. A volume of 270 ml of sterilized soil-sand mixture was added on top of the microcosms to reduce the risk of contamination between pots.

Before planting, seeds of *L. multiflorum* (Lam.) var. Oryx were surface sterilized by stirring in 1.25% bleach for 10 min and rinsing with deionized water. They were allowed to germinate on 1.5% water agar for one week before planting 30 evenly spaced seedlings into the microcosms.

2.3. Growth conditions

The microcosms were placed in a greenhouse with a 16 h, 20 °C day, and an 8 h, 15 °C night. Plants received natural light and supplemental illumination was provided by 400 W high-pressure sodium lamps to maintain a light level above 300 W m^{-2}. Pots were watered regularly by weight with deionized water to keep soil water content between 10 and 20%. Shoots were cut 5 cm above soil surface at 9 and 14 weeks after planting and were allowed to regrow.

2.4. Fertilization and water pulse

Weekly, each microcosm received 10 ml of a nutrient solution containing 1.5 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM Na_2MoO_4, 20 μM Fe-(Na)EDTA and either 9.98 mM KNO_3 for the NO_3^- fertilization or 4.99 mM $(NH_4)_2SO_4$ for the NH_4^+ fertilization treatment, starting 8 weeks after planting. This corresponds to 0.77 kg N ha^{-1} and 0.26 kg P ha^{-1} or 1.4 mg N and 0.47 mg P per microcosm and fertilization event. After 18 weeks, the microcosms were watered to 90% water filled pore space (WFPS) with deionized water mixed with 10 ml of a nutrient solution (29.3 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM Na_2MoO_4, 20 μM Fe-(Na)EDTA and either 778 mM KNO_3 for the NO_3^- fertilization treatment or 389 mM $(NH_4)_2SO_4$ for the NH_4^+-fertilization treatment. This corresponded to a fertilizer pulse of 60 kg N ha^{-1} and 5 kg P ha^{-1} or 109 mg N and 9.1 mg P per microcosm for both fertilization treatments. The higher water and nutrient loadings were introduced to provide conditions conducive for nutrient leaching and denitrification and related N_2O emissions. Both soils are situated in regions with high annual rainfall (>1000 mm yr^{-1}) and, hence, commonly experience wet conditions as applied here.

2.5. N_2O flux measurements

N_2O fluxes were measured 24 h after fertilization and watering for 6 of the 7 replicates. For the N_2O measurements, the headspace was adjusted to a height of 20 cm above soil surface (4 L volume) and closed for a period of 10 min with the headspace gas pumped through a TEI 46c automated N_2O analyser (Thermo Fisher Scientific, Waltham, MA, USA).

Watering, fertilization, and gas measurements proceeded in 15 min intervals, so that the time between fertilization and N_2O measurement was the same for all microcosms. The cap used to close the headspace was non-transparent.

2.6. Artificial rain and harvest

After the N_2O measurements, the microcosms were exposed to a simulated rainfall of 1.5 L with a rain simulator as described in Knacker et al. 2004. The drain tap in the bottom of the pots was opened and leachate was collected. After approximately 2 h, when no more leachate dripped out of the microcosms, leachate was weighed and a subsample was taken for nutrient analysis. Shoots where cut at soil surface, dried at 60 °C and weighed. The substrate was removed from the microcosms and all visible roots where collected, rinsed with water and a weighed subsample was taken and stored in 50% Ethanol. Remaining roots were dried and weighed. The soil was mixed thoroughly and samples were taken for soil and microbial analyses.

2.7. Nutrient analyses

2.7.1. Leachates

The leachates, which passed through the sand-gravel mixture at the bottom of the microcosms, were very clear and were not additionally filtered before analyses. Leachates were chemically analyzed for nutrient concentrations. NO_3^-–N, NO_2^-–N and dissolved PO_4^{3-}–P were determined using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA, USA). Total P in leachate was determined using Oxisolv® (Merck, Darmstadt, Germany) oxidation prior to the photometric analysis with a spectrophotometer (Helios Gamma, Thermo Scientific, Digitana AG, Switzerland) using the molybdenum blue ascorbic acid method (Watanabe and Olsen, 1965). NH_4^+–N was analyzed using a Skalar segmented flow analyser (Skalar, Breda, the Netherlands) according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL, RAC, FAW, 1996). Total dissolved N (TDN) was measured by chemoluminescence (DIMA-TOC® 2000 coupled with a DIMA-N analyser, Dimatec, Essen, Germany). For PO_4^{3-}–P, NO_3^-–N, NO_2^-–N and NH_4^+–N, 46%, 25%, 37% and 27% of the samples yielded concentrations below the detection limit, respectively.

The measured nutrient concentrations were multiplied with the leachate volume to get the total amount lost per microcosm. Amounts of NO_2^-–N were low and were added to the NO_3^-–N values. Dissolved organic N (DON) was calculated by subtracting

the amounts of mineral N (NO_3^-–N and NH_4^+–N) from TDN. In cases where no mineral N leaching was detected (7% of the samples), the complete amount of TDN leached was assumed to be in dissolved organic form.

The amount of PO_4^{3-}–P in the samples was termed reactive P. The difference between total P and reactive P was termed unreactive P. This fraction comprises all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates and particulate inorganic material, e.g. clays (Pote et al., 2009). In cases where no reactive P leaching was detected, the complete amount of total P leached was assumed to be in unreactive form.

2.7.2. Plant and soil

Dried shoot and root samples were ground with a centrifuge mill (0.12 mm) and a dried soil subsample was milled in a ball mill. All shoot harvests were pooled and N concentrations of the shoot samples, roots and soils were determined with a FLASH Elemental Analyser 1112 (Thermo Finnigan, Waltham, MA, USA). Shoot and root P concentrations were determined photometrically using the molybdenum blue ascorbic acid method (Watanabe and Olsen, 1965) after dry ashing.

Soil texture, particle density, organic C, $CaCO_3$ and soil pH, available soil P extracted with CO_2-saturated water and mineral soil N (NO_3^-–N and NH_4^+–N), extracted with 0.0125 M $CaCl_2$ were all analyzed using standard methods according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL, RAC, FAW, 1996). For soil mineral NO_3^-–N and soil mineral NH_4^+–N, 39% and 29% of the data points were below the detection limit, respectively. The particle density of the soil was determined to be able to calculate the WFPS in the microcosms as described in Elliott et al. 1999 but using the actual particle density determined from our substrates.

2.7.3. Soil microbial biomass C and N

Soil Microbial Biomass C and N was determined by Chloroform Fumigation Extraction (Vance et al., 1987) in duplicate on 20 g (on a soil dry weight basis) subsamples extracted with 80 ml of a 0.5 M K_2SO_4 solution. Organic C (TOC) was determined by infrared spectrometry after combustion at 850 °C (DIMATOC® 2000, Dimatec, Essen, Germany). Total N was subsequently measured in the same sample by chemoluminescence (TNb, Dimatec, Essen, Germany). Microbial biomass C and N was calculated according to Jörgensen 1996, Jörgensen and Mueller 1996. These measurements also comprise the C and N contents of AMF hyphae as these structures are also decomposed by the chloroform treatment (Olsson et al., 1995).

2.8. AMF root colonization

The percentage of root length colonized by AMF was determined from root samples stored in 50% ethanol after staining with pen ink (Vierheilig et al., 1998) and using a modified line-intersection method for 100 intersections (McGonigle et al., 1990).

2.9. Statistical analyses

All leachate, plant and soil data were analyzedusing ANOVA with Soil-type, N fertilizer and AMF treatment as factors and all interactions. To account for the blocking design of the experiment, the Block effect was added first in the model. In case of significant interactions, means were compared using Tukey's HSD test. ANOVA assumptions were controlled by plotting residuals against fitted values. Some nutrient concentration variables contained several values below the detection limit resulting in not available data points. For variables with more than 30% of the values being not available, no ANOVA was performed.

Pearson correlations were performed to test for linear relationships between plant N and P contents and N and P mobilized from soil during the experiment. All statistical analyses were performed using the R statistical software, Version 2.14.1 (R Core Team, 2011).

3. Results

3.1. Leaching losses

Leaching of total P was reduced by 31% in presence of AMF (Fig. 1a, Table 2). Leaching of unreactive P made up the biggest fraction of total P leaching (approx. 64% and 90% for the pasture and heath soil, respectively) and was significantly reduced by AMF (24% reduction) (Fig. 1b, Table 2). Leaching losses of reactive P were low. In the heath soil, only 6 of 28 samples yielded detectable reactive P concentrations, exclusively being derived from the NM treatments (Fig. 1c).

There was on average a 69% reduction of NH_4^+ leaching by AMF, irrespective of soil type and N fertilizer (Fig. 2a, Table 3). Leaching losses of NO_3^- were not affected by AMF and higher in the heath soil and with NO_3^- fertilization (Fig. 2b, Table 3). In the heath soil fertilized with NH_4^+, no NO_3^- leaching could be detected (Fig. 2b).

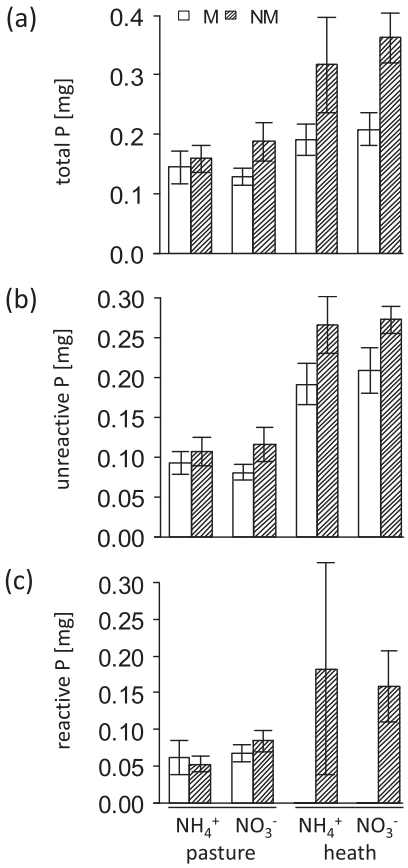

Fig. 1. Leaching losses of total (a), unreactive (b) and reactive (PO_4^{3-}) (c) P from grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars). Error bars indicate ±1 SE ($n = 7$, for reactive P, n is partially lower because several data points were below the detection limit, see Table 1).

Table 1
Mean values of the measured response variables of grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M) or receiving a non-mycorrhizal control inoculum (NM). Numbers in brackets indicate ±1 SE; for some nutrient analyses, several values were below the detection limit resulting in a reduced number of replicates (*n*) as indicated; ND: not detectable.

	Pasture soil											Heath soil										
	NH_4^+-Fertilization						NO_3^--Fertilization					NH_4^+-Fertilization						NO_3^--Fertilization				
	M		*n*	NM		*n*	M		*n*	NM		*n*	M		*n*	NM		*n*	M		*n*	NM		*n*
Leaching losses																						
Reactive P [mg]	0.06	(0.02)	6	0.1	(0.01)	7	0.1	(0.01)	5	0.1	(0.01)	6	ND		–	0.2	(0.08)	2	ND		–	0.2	(0.04)	4
Unreactive P [mg]	0.09	(0.01)	7	0.1	(0.02)	7	0.1	(0.01)	7	0.1	(0.02)	7	0.2	(0.03)	7	0.3	(0.04)	7	0.2	(0.03)	7	0.3	(0.02)	7
Total P [mg]	0.14	(0.03)	7	0.2	(0.02)	7	0.1	(0.01)	7	0.2	(0.03)	7	0.2	(0.03)	7	0.3	(0.08)	7	0.2	(0.03)	7	0.4	(0.04)	7
NO_3^-–N [mg]	0.79	(0.54)	7	2.4	(2.27)	7	13.5	(3.83)	7	12.5	(2.97)	7	ND		–	ND		–	32.7	2.8	7	29.7	(3.17)	7
NH_4^+–N [mg]	0.14	–	1	0.5	(0.07)	7	0.2	(0.02)	4	0.9	(0.16)	7	0.3	(0.08)	5	0.7	(0.44)	5	0.4	(0.10)	5	1.3	(0.46)	7
DON [mg]	10.4	(1.05)	7	7.5	(0.78)	7	5.9	(1.10)	7	6.7	(0.87)	7	15.5	(1.57)	7	19.6	(0.70)	7	15.4	(2.72)	7	21	(2.03)	7
Total N [mg]	11.22	(1.08)	7	10.3	(2.46)	7	19.5	(3.53)	7	20.1	(3.31)	7	15.8	(1.54)	7	20.2	(0.92)	7	48.4	(4.30)	7	52	(5.23)	7
N_2O-fluxes																								
N_2O-flux [ng m^{-2} s^{-1}]	9.06	(2.66)	6	20.9	(7.11)	6	94.1	(39.09)	6	274	(61.40)	6	1.9	(1.09)	6	2	(0.93)	6	200	(35.25)	6	283	(33.58)	6
Plant parameters																								
Total biomass [g]	46.1	(3.70)	7	36.7	(2.26)	7	43.8	(4.03)	7	36.9	(1.39)	7	34.4	(2.00)	7	38.7	(2.92)	7	36.4	(4.48)	7	37.8	(2.49)	7
Plant N [mg]	1088	(43.10)	7	908	(42.92)	7	1003	(24.67)	7	951	(51.85)	7	498	(16.29)	7	544	(41.22)	7	514	(39.91)	7	567	(32.72)	7
Plant P [mg]	101	(7.01)	7	79.8	(6.02)	7	91.4	(5.11)	7	78.8	(5.15)	7	84.7	(5.45)	7	73.1	(5.55)	7	77.7	(4.83)	7	77.6	(3.18)	7
Plant N:P ratio	11	(0.72)	7	11.6	(0.71)	7	11.1	(0.50)	7	12.2	(0.69)	7	6	(0.35)	7	7.5	(0.47)	7	6.6	(0.30)	7	7.3	(0.34)	7
Soil parameters																								
Mineral NO_3^-–N [mg]	14.13	(2.89)	5	13.6	(3.90)	5	14.8	(2.65)	5	11.2	(1.71)	5	ND		–	ND		–	8.3	(1.16)	7	8.4	(0.72)	7
Mineral NH_4^+–N [mg]	9.93	(0.43)	4	8.9	(1.18)	4	9.2	(0.15)	3	9.3	(0.24)	4	28.8	(5.08)	7	19.6	(1.10)	7	7.3	(0.93)	5	7.4	(1.01)	6
Total soil N [g]	11.54	(0.66)	7	13.9	(2.40)	6	10.9	(0.92)	7	9.7	(0.75)	7	10.2	(0.26)	7	9.4	(0.27)	7	10.2	(0.33)	7	11.1	(0.47)	7
Available soil P [mg]	6.93	(0.69)	7	6.2	(0.49)	7	5.6	(0.41)	7	6.5	(0.33)	7	5.5	(0.44)	7	4.8	(0.37)	7	5.6	(0.52)	7	6.1	(0.76)	7
Microbial biomass																								
Microbial C [mg]	1835	(45.79)	7	1436	(137.90)	7	1677	(76.01)	7	1475	(113.80)	7	1455	(64.96)	7	1237	(113.00)	7	1646	(75.60)	7	1433	(79.58)	7
Microbial N [mg]	298	(9.00)	7	220	(19.39)	7	272	(15.06)	7	240	(20.14)	7	165	(8.18)	7	147	(13.98)	7	190	(8.34)	7	167	(8.00)	7
AMF root colonization																								
HC (%)	50.43	(7.00)	7	0	(0.00)	7	64.1	(5.07)	7	0	(0.00)	7	12	(2.36)	7	0	(0.00)	7	12.7	1.61	7	0	0	7
AC (%)	5.57	(1.90)	7	0	(0.00)	7	8.29	(1.19)	7	0	(0.00)	7	0.57	(0.20)	7	0	(0.00)	7	0.14	0.14	7	0	0	7
VC (%)	14	(1.43)	7	0	(0.00)	7	20	(2.88)	7	0	(0.00)	7	6	(1.57)	7	0	(0.00)	7	6	1.18	7	0	0	7

Table 2
Significance levels of 4-way ANOVAS analyzing the effects of Soil-type, N-fertilizer and AMF and all interactions on the different P compounds in leachate as shown in Fig. 1 (***, $P < 0.001$; **, $P < 0.01$;*, $P < 0.05$). For reactive P, no ANOVA was carried out because >30% of the values was below the detection limit. See Table S2 for detailed statistical output.

Source of variation	TDP	Unreactive P
Block	*	***
Soil	***	
N-fertilizer		
AMF	***	**
Soil × N-fertilizer		
Soil × AMF		
N-fertilizer × AMF		

Table 3
Significance levels of 4-way ANOVAS analyzing the effects of Soil-type, N-fertilizer and AMF and all interactions on the different N compounds in leachate as shown in Fig. 2 and on N_2O fluxes as shown in Fig. 3 (***, $P < 0.001$; **, $P < 0.01$;*, $P < 0.05$; ·, $p < 0.1$). See Table S2 for detailed statistical output.

Source of variation	NH_4–N	NO_3–N	DON	N_2O-flux
Block	**	***	*	
Soil		***	***	
N-fertilizer		***		***
AMF	**		.	***
Soil × N-fertilizer				**
Soil × AMF			**	.
N-fertilizer × AMF				**

Leaching of DON was higher in the heath than in the pasture soil. In the heath soil on average 24% less DON was leached in the AMF treatment (Tukey HSD, $P = 0.006$) but this was not the case for the pasture soil (Tukey HSD, $P = 0.87$) (Fig. 2c) as reflected by a significant interaction of AMF with soil type (Table 3).

TDN leaching was higher with NO_3^- than with NH_4^+ fertilization but not affected by AMF. The highest TDN leaching was observed in the heath soil receiving NO_3^- fertilizer resulting in a significant interaction of soil type with N fertilizer (Tables 1 and S2).

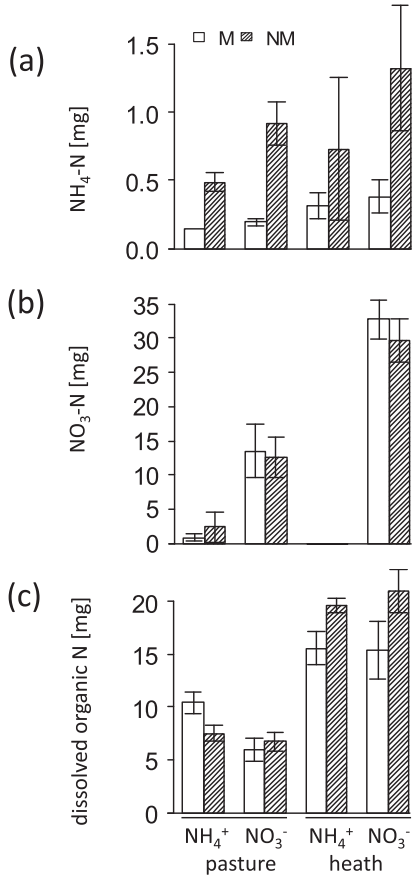

Fig. 2. Leaching losses of NH_4^+–N (a), NO_3^-–N (b) and dissolved organic N (c) from grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars). Error bars indicate ±1 SE ($n = 7$, for NH_4^+–N, n is partially lower because several data points were below the detection limit, in the heath soil fertilized with NH_4^+, no NO_3^-–N was detected in the leachates, see Table 1).

3.2. N_2O fluxes

In both soils, N_2O fluxes 24 h after fertilization were significantly reduced by AMF by 47.1% with NO_3^- fertilizer (Tukey HSD, $P = 0.004$). With NH_4^+ fertilizer, N_2O fluxes were much lower and not affected by AMF (Tukey HSD, $P = 0.937$) (Fig. 3), as reflected by a significant interaction of AMF with N fertilizer type (Table 3).

3.3. Plant biomass and nutrient contents

There was a significant interaction between the effects of soil type and AMF on plant biomass and N contents (Table 4). While in the pasture soil, plant biomass and N contents were increased by 22 and 13% with AMF (Tukey HSD, $P = 0.013$ and $P = 0.016$), respectively, there was no effect of AMF on plant biomass and N contents in the heath soil (Tukey HSD, $P = 0.67$ and $P = 0.54$) (Fig. 4a, b). Plant N contents were much higher in the pasture than in the heath soil (Fig. 4b).

Plant P contents were also higher in the pasture than in the heath soil (Fig. 4c) and averaged across both soil types and fertilizer treatments, plant P contents were significantly increased with AMF (+15%).

The plant N:P ratio was significantly higher in the pasture than in the heath soil and was constantly reduced by 18% with AMF (Tables 1 and S2).

3.4. Soil nutrient contents

Available soil P at the end of the experiment was significantly higher in the pasture soil compared to the heath soil (Tables 1 and S2). Soil mineral NO_3^- –N contents were lower in the heath than in the pasture soil and were not affected by AMF. In the heath soil fertilized with NH_4^+, no soil mineral NO_3^-N could be detected but

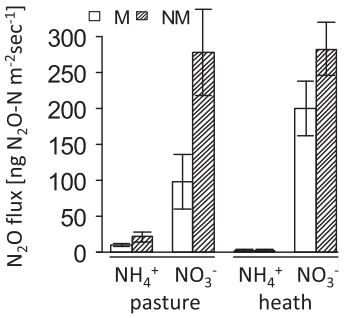

Fig. 3. N_2O fluxes in grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars) measured 24 h after the N fertilizer had been applied. Error bars indicate ±1 SE ($n = 6$).

mineral NH_4^+–N contents were distinctly higher compared to the other soil-fertilizer combinations (Tukey HSD<0.001) (Table 1). This was reflected by a significant interaction of soil type with N fertilizer (TableS2).

3.5. AMF root colonization and microbial biomass

Root length colonized by AMF was 57.3 and 12.4% for the pasture and the heath soil, respectively. No AMF colonization was detected in the NM treatments (Table 1).

Microbial biomass C and N contents were overall increased by 19% in the M compared to the NM treatments (Table 1) and were higher in the pasture soil compared to the heath soil (Tables 1 and S2). The microbial biomass C:N ratio was significantly increased in the heath soil compared to the pasture soil (Tables 1 and S2).

3.6. P and N mass balance

We compared the initially available amounts of P and N in soil plus the amounts added with fertilization, with the respective amounts at the end of the experiment and amounts removed by plants, leachate and N immobilized in microbial biomass.

Of the P initially available in soil (soil P + fertilizer P), between 0.67 and 1.92% had been lost through leaching. About 3.7 times the amount of initially available and fertilized P was found in the plant soil system at the end of the experiment (Table S3). This indicates that most P detected at the end had been mobilized from initially non-available soil P resources. On average 17.5% more P had additionally been mobilized from initially non-available soil P resources in the presence of AMF ($F_{1,\ 42} = 9.81$; $P = 0.003$) (Table S3).

Of the mineral N initially available in soil (soil N + fertilizer N), between 5.7 and 29.8% had been lost through leaching. About 4.7 times the amount of mineral N initially present and N fertilized was found in the plant soil system at the end of the experiment (Table S4). This indicates that most N detected had been mobilized from initially organic soil N resources. On average, 17.3% more N had additionally been mobilized from organic soil N resources in the presence of AMF in the pasture soil (AMF:soil interaction: $F_{1,42} = 11.63$; $P = 0.001$; Tukey HSD, $P < 0.001$), while the amount of additionally mobilized N was slightly lower (−4.8%) in presence of AMF in the heath soil (Tukey HSD, $P = 0.9$; Table S4).

4. Discussion

This is the first study providing a comprehensive assessment of the influence of AMF on N and P cycling in a single experiment, including effects on leaching losses and N_2O emissions. We show for the first time that leaching of dissolved organic N and unreactive P compounds can be reduced in the presence of AMF.

Other studies showed reduced reactive P leaching losses, but a contribution of AMF to the reduction of unreactive P leaching had so far not been shown. This finding is important, because in our study and other studies investigating P leaching (Ulén, 1999; Neumann et al., 2012), a considerable fraction of total P leaching occurred in unreactive forms. A reduction of reactive P leaching by AMF can be explained by enhanced uptake of P from soil solution due to exploitation of a bigger soil volume by AMF rooting systems compared to non-mycorrhizal plant roots (Jakobsen et al., 1992; Jansa et al., 2005). A reduction of unreactive P leaching can be related to an either direct or indirect increase of mineralization of organic P compounds in the presence of AMF and subsequent uptake of the inorganic products (Jayachandran et al., 1992; Koide and Kabir, 2000). Also, the utilization of insoluble inorganic P compounds by AMF has been shown (Bolan et al., 1987) and probably contributed to the reduction of unreactive P leaching.

In the presence of AMF, the mobilization of initially non-available soil P resources was significantly enhanced compared to the non-mycorrhizal treatments indicating an overall increase in P cycling by AMF. As the mobilization of P from soil resources was strongly correlated to plant P contents, this effect is likely to be indirectly caused via AMF mediated improvements in plant nutrition.

Total P leaching losses depended strongly on soil type and were much higher in the heath soil compared to the pasture soil. The heath soil had a much higher sand and organic matter content than the pasture soil (Table S1), both being properties often reducing P sorption capacity of soils (Weaver et al., 1988; Atalay, 2001; Daly et al., 2001). The calcareous pasture soil, hence, probably had a higher ability to fix P and, consequently, P leaching losses were lower.

While AMF are believed to have no saprotrophic abilities (Smith and Smith, 2011b), they have been shown to utilize nutrients derived from organic matter (Feng et al., 2003; Hodge and Fitter, 2010) and may also indirectly enhance organic matter mineralization (Hodge et al., 2001; Atul-Nayyar et al., 2009; Cheng et al., 2012). Some studies also suggest direct uptake of organic N compounds; (Hawkins et al., 2000; Whiteside et al., 2009, 2012) from soil. Hence, the reduction in DON leaching in the heath soil could result from enhanced DON mineralization, resulting in mineral N release, or from direct uptake of DON in the presence of AMF.

It is well established that AMF can take up NH_4^+ from soil and transfer it to their host plants (Frey and Schuepp, 1993; Johansen et al., 1993; Mäder et al., 2000). Moreover, it has been shown that AMF preferentially take up NH_4^+ rather than NO_3^- (Govindarajulu et al., 2005; Tanaka and Yano, 2005). Hence, the observed overall reduction in NH_4^+ leaching through AMF could result from enhanced NH_4^+ immobilization from the soil by AMF rooting systems. This would reduce the amount of NH_4^+ in the soil prone to leaching.

In the acid heath soil fertilized with NH_4^+, we successfully created NH_4^+ dominated conditions as indicated by the absence of NO_3^- in the soil and leachates (Fig. 2b and Table 1). Under these conditions, we expected the strongest reduction of N leaching losses by AMF as plants were suggested to rely more on AMF for N-acquisition when NH_4^+ is the dominating N form (Johansen et al., 1993; Hamel, 2004). In contrast to our expectations, we did not observe pronounced effects of AMF on NH_4^+ leaching losses and also plant N contents were not enhanced in that treatment. The high amounts of mineral NH_4^+ in the soil at the end of the experiment (Table 1) either indicate that NH_4^+ was not the preferred form for biological N uptake under these conditions or that NH_4^+ uptake and translocation by AMF is a slow process (e.g. the majority of NH_4^+ fertilizer (88.6%) was applied, 1 day before harvesting the experiment).

Microbial biomass N contents were constantly higher in the M treatments. Earlier work showed that AMF can immobilize substantial amounts of N in their hyphal biomass (Hodge and Fitter, 2010). AMF hyphae could have served as an N sink in the M treatments, hence, reducing N leaching. Another possibility is that the presence of AMF promoted microbial communities more efficiently immobilizing N.

In the pasture soil, plant biomass and N contents were increased by AMF, while they tended to be reduced in the heath soil. Increased plant N uptake in the M treatments might, thus, have contributed to the significantly reduced NH_4^+ leaching in the pasture soil. Effects on leaching losses in the heath soil can, however, not be explained by increased plant N uptake.

The strongly reduced root colonization by AMF in the heath soil compared to the pasture soil is in line with the literature reporting reduced AMF root colonization under low pH conditions (van Aarle

Table 4
Significance levels of 4-way ANOVAS analyzing the effects of Soil-type, N-fertilizer and AMF and all interactions on plant biomass and nutrient contents as shown in Fig. 4 (***, $P < 0.001$; **, $P < 0.01$;*, $P < 0.05$). See Table S2 for detailed statistical output.

Source of variation	Biomass	P Content	N content
Block	***	*	
Soil	*	**	***
N-fertilizer			
AMF		**	
Soil × N-fertilizer			
Soil × AMF	**		**
N-fertilizer × AMF			

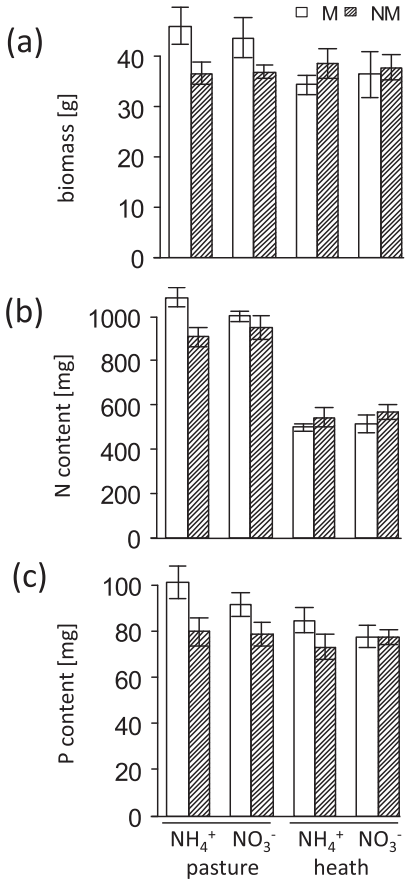

Fig. 4. Plant biomass (a) and plant N (b) and P contents (c) in grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars). Error bars indicate ±1 SE ($n = 7$).

et al., 2002; Goransson et al., 2008). Several of the observed AMF effects in this study were, however, more pronounced in the acid heath soil. This indicates that even when AMF abundance in the roots is low, they can still exert a significant influence on nutrient cycling processes.

Plant N:P ratios have been proposed as an indicator of nutrient limitation for plants (Koerselman and Meuleman, 1996). The biomass N:P ratios in this experiment were all below 14, indicating N limited conditions. However, N limitation seems to have been more severe in the heath soil with an average plant N:P ratio of 6.9 compared to an average plant N:P ratio of 11.5 in the pasture soil. It has been proposed that nutrient stoichiometry can determine whether the AMF symbiosis turns out to be mutualistic or antagonistic (Johnson, 2010). In line with this, AMF had a stronger impact on plant biomass in the pasture soil which had a lower relative P-availability compared to the heath soil. Thus, the differences in the AMF effects on plant growth between the soil types could result from difference in nutrient availability and nutrient ratios between the soils.

Furthermore, both soils strongly differed in their C:N ratios, the heath soil showing a C:N ratio of 24.6 which is remarkably higher than the C:N ratio of 7.4 found for the pasture soil.

N availability has been reported to be reduced and competition between plants and the soil microbial community for N to be enhanced when the C:N ratio is high. The competitive ability of the soil microbial community to acquire N is believed to be superior to plants under these conditions (Kaye and Hart, 1997).

Microbial biomass N content overall was higher with AMF, while in the pasture soil, plant N contents were also increased. In the heath soil, plant N contents were much lower and they were still slightly lower in the presence of AMF. These result suggest, that the microbial biomass was overall capable to improve its' N nutrition in the presence of AMF. Under conditions of relatively high N availability in the pasture soil, plant N nutrition also benefitted from the presence of AMF. In the heath soil, with a relatively lower N availability, plants did not benefit from AMF in terms of N nutrition, indicating a stronger competitive ability for N of the microbial biomass under these conditions. This agrees with the assumption that AMF contribute to plant N supply only under conditions where N is available in amounts sufficient to satisfy AMF demands and additionally allow AMF N transfer to the plant hosts (Fitter et al., 2011).

Until now, very few studies addressed a potential effect of AMF on emissions of the greenhouse gas N_2O. While one study found no effects of AMF on N_2O emissions (Cavagnaro et al., 2012), this study confirms recent results showing that AMF can regulate N_2O emissions from soil (Bender et al., 2014; Lazcano et al., 2014).

As NO_3^- is the main substrate for producing N_2O, it is to be expected that we observed higher N_2O emissions under NO_3^- fertilization and negligible emissions in the heath soil fertilized with NH_4^+ where no NO_3^- was detected (Table 1). Note that, the reduction of N_2O fluxes measured 24 h after fertilization can only give an indication of whether AMF reduced N_2O emissions, as these data represent a snapshot of N_2O emissions in time and do not allow inferences about total N_2O losses associated with the amount of fertilizer applied. Still, the significantly reduced N_2O fluxes over both soil types provide, in addition to two previous experiments (see Bender et al., 2014) and the study by Lazcano et al. (2014), a strong indication that AMF affect N_2O emissions under a wide range of conditions, including acidic soils fertilized with NO_3^-.

5. Conclusions

The results presented here show the influence of AMF on N and P cycling, including effects on leaching losses and N_2O emissions in two distinct soil environments. We provide first evidence that the leaching of dissolved organic N and unreactive P compounds can be reduced in presence of AMF. This is important because these compounds can comprise significant fractions of total leaching losses in several ecosystems (Schoenau and Bettany, 1987; Ulén, 1999; Smolander et al., 2001; Ghani et al., 2010). Thus, our findings imply that leaching of organic and unreactive nutrient compounds must not be ignored when testing AMF effects on nutrient leaching.

While P losses through leaching were reduced in presence of AMF, simultaneously the amount of available P being cycled through our model grasslands was increased. These results suggest

that AMF can increase the P use-efficiency in plant-soil systems by enhancing nutrient mobilization while reducing losses.

For N, the results were more complex. In the pasture soil, the amount of N cycled through the plant-soil system was significantly enhanced in presence of AMF, but total N leaching losses were not affected. In the heath soil, AMF did not enhance the amount of cycled N, and the presence of AMF had no significant effect on plant N contents. However, there was a reduction in N leaching losses in presence of AMF under these conditions. Taken together these results indicate that a potential reduction of N leaching is uncoupled from effects on plant N uptake and that the interference of AMF with the N cycle is context dependent.

The effects of AMF on nutrient transformation processes in the soil are not well understood, especially for N. There is an urgent need to conduct process based studies, to fully understand how AMF affect nutrient cycling and how they could be managed to exploit their potential to promote sustainable nutrient cycles.

Acknowledgments

The authors thank Luise Köhl and Nora Aellen for their great support in the greenhouse, Mark Rollog for his contributions in the planning of the experiment, Albrecht Neftel and Markus Jocher for providing material and know-how for the N_2O measurements, Hans Stünzi, Hans-Ruedi Bosshart, Erika Hinnen, Agush Loshi and Brigitte Weisshaupt for support with nutrient analyses, Hans-Ruedi Oberholzer and Andrea Bonvicini for support with Microbial biomass measurements and two anonymous reviewers for valuable comments on the manuscript. This study was funded by the Swiss National Science Foundation (SNSF), grant no.125428 awarded to MvdH.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.10.016.

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