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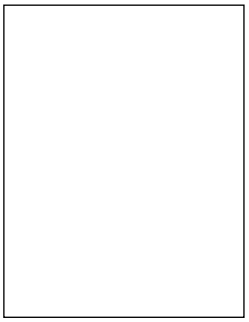

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A comparison of different preservation methods for nitrogen isotopes in soil extractable NO_3^-

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ABSTRACT

The nitrogen isotope ratio ($\delta^{15}N$) of soil extractable NO_3^- plays a pivotal role in the study of nitrogen biogeochemical circulation in ecosystems. However, the NO_3^- content and its isotope composition of soil samples are unstable, making sample storage critical for preserving the nitrogen isotope composition of extracted soil nitrates. Nevertheless, studies on the appropriate selection of storage methods after soil sampling are scarce. In this investigation, we compared two commonly used methods of storing soil nitrate samples and investigated the stability of nitrogen isotopes in soil nitrates. The results demonstrated that no significant changes in the NO_3^- concentration and $\delta^{15}N$ value occurred in the samples stored at -18°C. However, the soil NO_3^- content markedly increased and NO_3-$\delta^{15}N$ significantly changed after air-drying storage. Meanwhile, we also found that NO_3^- and its $\delta^{15}N$ were well preserved in the filtered soil extracts after one month. In contrast, the NO_3^- concentration gradually decreased and the ^{15}N in NO_3^- was gradually enriched in the bactericidal agent-containing soil mixture solution during the storage period. Overall, our results indicated that nitrogen isotopes in NO_3^- could be effectively preserved in frozen-stored soil samples or filtered soil extracts. In addition, for field investigations conducted in remote areas and continued for a long time period (and lacking a refrigerant supply), soil extraction/filtration using a $CaSO_4$-saturated solution may be a superior preparation and storage method for analyzing soil NO_3^- nitrogen isotopes.

Key Words: nitrate radicals, nitrogen isotopes, soil storage, ion exchange, nitrogen isotope tracing

INTRODUCTION

Stable isotope analysis is a powerful tool for the analysis of soil nitrogen transport and transformation (Mathieu *et al*., 2007). The ratio of $^{15}N/^{14}N$ obtained from a comparison of nitrogen isotope values in soil samples can be used to determine the transport process and origin of the element N. Various biochemical processes of nitrogen can lead to the fractionation of stable nitrogen isotopes, *e.g.*, nitrification and denitrification (Aulakh *et al*., 1992; Sigman *et al*., 2001). Therefore, $\delta^{15}N$ can be used to trace nitrogen transport and transformation in these biological processes. Compared with other analytic methods such as NO_3^- and NH_4^+ tests that can also be applied to investigate these biogeochemical processes (Ross and Hales, 2003; Islam *et al*., 2008; Rock *et al*., 2011; Smith and Kellman, 2011), $\delta^{15}N$ tracing possesses irreplaceable advantages due to its high sensitivity.

In previous research, $\delta^{15}N$ in soil NO_3^- has been used to explore a variety of biogeochemical processes in ecosystems, including soil nitrogen transformations and microbial activities. Several studies on tracing plant nitrogen sources have explored the relationship between plant nitrogen and soil NO_3-$\delta^{15}N$ (Comstock, 2001; Houlton *et al*., 2007; Wang *et al*., 2013). However, soil inorganic nitrogen might undergo substantial changes during the sample storage period prior to analysis (Turner and Romero, 2009). Reportedly, the storage method can affect the inorganic nitrogen content, including NO_3^- and NH_4^+, during transportation from the sampling sites to the laboratory (Birch, 1960; Mulvaney, 1996). In addition, stable nitrogen isotopes in the soil NO_3^- of the samples can significantly change after sample collection (Hales and Ross, 2008). Moreover, because most of the field investigation sites are far from the laboratory, the soil samples must be appropriately stored before NO_3-$\delta^{15}N$ analysis, which inevitably leads to the changes mentioned above. Therefore, the preservation of the NO_3^- content and the accompanying $\delta^{15}N$ is a tremendous challenge for research involving natural soil samples.

Freezing and air-drying are two of the most commonly recommended soil storage methods. Research has shown that the nitrogen isotope composition of soil can be preserved for 56 weeks under frozen conditions (Fogg *et al*., 1998). Freezing has been recommended by many researchers for NO_3^- preservation (*e.g.*, Koba *et al.*, 2010; Wang *et al*., 2013). Meanwhile, some researchers have used an air-drying method to store samples (Hyodo *et al.*, 2009). However, the storage-associated changes in nitrogen isotope values in the soil NO_3^- have yet to be fully elucidated. Studies of nitrogen isotope preservation in soil extracts are also scarce. Therefore, the exploration of storage methods for soil nitrate samples in the field is of great significance.

In this study, we investigated the effects of various storage methods on the nitrogen isotope values of soil NO_3^-. Our objectives were as follows: (1) to compare the possible effects of the existing storage methods for soil nitrate samples on nitrate concentration and the nitrogen isotope composition; and (2) to further explore storage methods of soil nitrate

samples for nitrogen isotope analysis that are suitable for the field (particularly in remote areas and for long-term storage) by combining ion exchange laboratory methods (Hu and Liu, 2014).

MATERIALS AND METHODS

Samples

Two types of soil samples from pines and shrubs were used in this study (Table I). Both were yellow-brown soils, which were collected from the Institute of Earth Environment, Chinese Academy of Sciences, during the period of 2012 to 2013. At each sample site, approximately 6 kg of surface soil (0~10 cm) was collected and sieved (<2 mm) to remove large granules, stones, dead leaves, and roots. Each treated sample was immediately divided into two portions for parallel tests.

Subsequently, the soil samples were used in the two experiments, as follows:

(1) Soil storage: To investigate the nitrate content and isotope composition of the soils stored under freezing or air-drying conditions, we collected four soil samples during the period from November 2012 to February 2013, which were numbered as Soil I, Soil II, Soil III, and Soil IV (Fig. I). Approximately 300 g of fresh soil from each sample was subject to extraction. Then, the acquired supernatant was immediately used to detect the NO_3^- concentration and NO_3-$\delta^{15}N$ of fresh soil. The rest of the soil sample was divided into two portions, with one portion stored by freezing and the other portion stored by natural air-drying. Subsequently, 300 g of the stored soil was obtained for the analysis of NO_3^- and NO_3-$\delta^{15}N$ values at each time point of 1 week, 2 weeks, and 4 weeks.

(2) Sample storage after soil extraction: To explore the effect of storage methods on soil extracts, two soil samples, numbered as soil V and soil VI, were collected in April 2013. Approximately 300 g of fresh soil from each sample was subject to extraction. The acquired supernatant was immediately used to detect the NO_3^- content and NO_3-$\delta^{15}N$ of fresh soil. The rest of the sample was also soaked using a $CaSO_4$-saturated solution, and the obtained soil solution was stored using three different methods: a) the soil solution was filtered, and the acquired supernatant solution was then stored for one month; b) after a bactericidal agent (HgCl) was added, the soil mixture solution was stored for one month; c) the soil solution was stored for one day without the addition of HgCl to verify the effect of the bactericidal agent on the soil mixture solution. Regardless of the storage methods, all solutions were stored at room temperature (23 ± 3^oC) without exposure to direct sunlight (Fig. I.) Finally, 300 g of the stored soil was obtained for the analysis of NO_3^- and NO_3-$\delta^{15}N$ values at each time point of 1 week, 2 weeks, and 4 weeks. Deionized water was used in all experimental processes and to monitor changes in the background level of nitrogen isotopes during the entire processes of ion exchange and extraction.

TABLE I

NO_3^- concentration and $\delta^{15}N$ values of samples stored under various conditions. The results for fresh soil were obtained immediately after sampling to avoid disturbances. Other results were obtained after different storage periods (1 day, 1 week, 2 weeks or 4 weeks). $\delta^{15}N$ values are shown as the mean ±standard deviation.

Sample	Date	Ecosystem	Storage methods	NO_3^- concentration					$\delta^{15}N$				
				fresh	1 day	1 week	2 weeks	4 weeks	fresh	1 day	1 week	2 weeks	4 weeks
				mgN•kg-1					‰				
Soil I	2012.11	pines	Freezing	55.1±0.8		54.7±1.6	57.6±3.0	66.0±3.5	3.9±0.1		3.8±0.1	3.5±0.2	3.7±0.3
			Air-drying	55.1±0.1		70.7±1.5	70.8±1.7	81.8±3.0	3.9±0.1		3.8±0.1	3.7±0.3	3.5±0.1
Soil II	2013.2	pines	Freezing	240.4±0.3		231.4±7.4	251.2±0.8		2.4±0.1		2.4±0.1	2.2±0.1	
			Air-drying	240.4±0.3		256.1±3.9	284.7±5.3		2.4±0.1		2.5±0.1	2.5±0.2	
Soil III	2012.11	shrubs	Freezing	0.9±0.1		1.0±0.1	1.1±0.2		-3.1±0.1		-3.3±0.1	-3.1±0.1	
			Air-drying	0.9±0.1		2.7±0.1	2.8±0.1		-3.1±0.1		-2.4±0.1	-1.7±0.2	
Soil IV	2013.2	shrubs	Freezing	2.8±0.1		3.2±0.1	3.1±0.1		0.7±0.1		0.2±0.1	0.6±0.1	
			Air-drying	2.8±0.1		6.3±0.1	6.5±0.1		0.7±0.1		0.1±0.1	0.6±0.2	
Soil V	2013.4	pines	supernatant	602.4±19.4		571.0±8.1	606.8±13.2	593.4±12.1	2.8±0.1		2.4±0.1	2.6±0.1	2.5±0.1
			extracts with soil		564.2±0.1					1.8±0.1			
			extracts with soil and microbial biocide		599.1±2.3	586.2±2.1	557.9±8.1	545.3±6.1		2.9±0.1	2.9±0.1	3.7±0.1	4.9±0.1
Soil VI	2013.4	shrubs	supernatant	2.7±0.1		3.0±0.1	3.0±0.1	3.1±0.1	-2.7±0.1		-2.3±0.2	-2.4±0.2	-2.8±＜0.1
			extracts with soil		1.8±0.1					-0.6±0.2			
			extracts with soil and microbial biocide		2.5±0.1	0.9±0.4	n.a.	n.a.		-2.3±0.1	2.9±0.1	3.7±0.1	3.5±0.1

Sample analysis

NO_3 was extracted using a $CaSO_4$-saturated solution (approximately 21.5 mg/L $CaSO_4$) at room temperature (Hood-Nowotny et al., 2011). The ratio of soil to $CaSO_4$ solution was approximately 1:1 (v/v). 300 g soil samples and 300 ml of $CaSO_4$ solution were placed together in a 600-ml plastic bottle and shaken (180-200rpm) for 1 hour. The solution was then centrifuged for 30 min at 2500 rpm. The mixture was filtered using a Buchner funnel with Whatman GF/F filter paper (this step was omitted for samples in which the extracts were preserved with soil or soil and HgCl in experiment 2). The concentration of extracted NO_3^- was then determined using an ICS-1000 ion chromatography system (DIONEX, Sunnyvale, California USA) with a relative standard deviation (RSD) < 5%.

To determine the $\delta^{15}N$ values of the extracted NO_3^-, an ion exchange method was used to purify the extracted NO_3^- (Hu and Liu, 2014). Based on the concentration of the extracted NO_3^-, we calculated how much extracted soil solution was needed to ensure that there was less than 8 mg NO_3^-. The quantitative solution was passed through a Bio-Rad (Hercules, California) AG1-X8 anion exchange resin column with 200-400 mesh and an exchange capacity of 1.2 $cmolc \cdot kg^{-1} \cdot ml^{-1}$. This column has a relatively high affinity for nitrate (Silva et al., 2000), and absorbs nitrate onto the resin. The customized column (40 cm high, 8 mm diameter) was equipped for the analysis, and each column was loaded with 7 ml of the AG1-X8 anion resin. A total of 110 ml of 0.5 $mol \cdot L^{-1}$ HCl was then used to elute NO_3^- from the anion exchange resin. We discarded the first 60 ml and collected the last 50 ml to avoid interference with other ions (e.g., SO_4^-). A peristaltic pump supplied a suitable flow rate to ensure complete anion exchange.

To remove Cl^- and transform NO_3^- to $AgNO_3$ for MS analysis, silver oxide (Ag_2O) was added to the eluate of ion exchange resin. The Ag_2O (Ag_2O content ≥ 99.7%) was washed with deionized water without NO_3^- to remove any contaminant NO_3^-. Approximately 3.5 - 4 g of Ag_2O was used per eluate. The solution was stirred until the pH reached 5.5 - 6, as confirmed by pH paper. The solution was then filtered through a Whatman #1 filter to remove the precipitate from the eluate. Finally, the solution was freeze dried to collect $AgNO_3$ particles.

$AgNO_3$ was placed in 4.5×6 mm silver capsules, with each capsule containing at least 360 μg NO_3. Nitrogen isotope ratios were determined using a CE FLASH 1112 elemental analyzer (EA) connected via a continuous flow interface to a Finnigan MAT Delta Plus mass spectrometer (EA-IRMS) at the Institute of Earth Environment, Chinese Academy of Sciences (CAS) in Xi'an. All of the $\delta^{15}N$ values were reported in per mille (‰) relative to the atmospheric N_2 isotope standard. Duplicate or tripartite analyses for the extracted nitrate $\delta^{15}N$ of each subsample (depending on its extractable NO_3^- concentration) were conducted after the chemical process to ensure the accuracy of the results.

We used a KNO_3 reference material ($\delta^{15}N$ = +6.27‰) and two international isotope reference materials (IAEA-N3, $\delta^{15}N$ = +4.70‰ and USGS-25, $\delta^{15}N$ = -30.4‰) to control the analytical accuracy of EA-IRMS. Repeated analyses of laboratory soil standards with confirmed $\delta^{15}N$ values were performed daily to ensure instrument accuracy. The standard deviation of the repeated analyses

of the standards (KNO_3, IAEA-N3, USGS-25 and laboratory soil standards) is smaller than ±0.15‰. The deviation for the isotope results in the entire experimental process, which included nitrate extraction, ion exchange, $AgNO_3$ production, and subsequent nitrogen isotope ratio measurement, was smaller than ±0.3‰. Due to the small range of error for some data points, error bars for these samples are smaller than the symbol size if not visible in the figures.

Statistical analysis

Standard deviations were used to examine the differences between replicate analyses. One-way ANOVA was used to compare every preservation methods for each soil sample after the preservation period. Paired t-tests were used to examine the difference in preserving effect for each sample among different methods. All statistical analyses were performed using SPSS 20.0 for Windows.

RESULTS

Soil storage

The NO_3 and NO_3-$\delta^{15}N$ values for the soil samples under various storage conditions are presented in Table I. The NO_3^- concentration in the soil samples that were stored frozen was not significantly different from the NO_3^- concentration immediately measured in the fresh soil samples ($P>0.05$). For example, for Soil II and Soil III, the NO_3^- concentrations were 240.4 $mgN \bullet kg^{-1}$ and 0.9 $mgN \bullet kg^{-1}$, respectively, in the fresh soil, and 251.2 $mgN \bullet kg^{-1}$ and 1.1 $mgN \bullet kg^{-1}$, respectively, in the soil that had been stored in the frozen condition for 2 weeks (Fig. II). These results indicated that the soil that was stored frozen had a similar NO_3^- concentration to that of the fresh soil.

The $\delta^{15}N$ value changed slightly in the soil that was stored frozen. For example, for Soil I, the NO_3-$\delta^{15}N$ values were 3.9‰ and 3.7‰ in the fresh soil and in the soil that had been stored frozen for 4 weeks, respectively; for Soil III, the NO_3-$\delta^{15}N$ value was -3.1‰ in the fresh soil and remained -3.1‰ after the soil had been stored frozen for 4 weeks.

The change in the NO_3-$\delta^{15}N$ value during the frozen storage period was not statistically significant for Soil I or Soil III ($P>0.05$) (Table II). Despite the finding that the difference in the NO_3-$\delta^{15}N$ value between the fresh soil and the frozen-stored soil of Soil II and Soil IV was statistically significant ($P<0.05$), we believe that the NO_3-$\delta^{15}N$ value did not substantially change because its relative difference was less than 0.3‰. Therefore, our results indicated that soil storage by freezing was able to satisfactorily preserve the NO_3^- and NO_3-$\delta^{15}N$ content of the soil. (Fig. II)

TABLE II

The one-way ANOVA *P* values for each method for all of the soil samples during the entire preservation period.

Sample	Storage methods	P (NO_3^- concentration)	P ($\delta^{15}N$)
Soil I	Freezing	0.068	0.391
	Air-drying	0.001	0.096
Soil II	Freezing	0.092	0.017
	Air-drying	0.002	0.722
Soil III	Freezing	0.772	0.875
	Air-drying	0.000	0.248
Soil IV	Freezing	0.072	0.035
	Air-drying	0.000	0.021
Soil V	supernatant	0.264	0.095
	extracts with soil and microbial biocide	0.037	0.000
Soil VI	supernatant	0.075	0.069
	extracts with soil and microbial biocide	0.010	0.000

Regardless of the difference in the NO_3^- concentration among the soil samples, the four soil samples demonstrated a remarkable increase in NO_3^- concentration during the air-drying process (*i.e.*, an increase from 55.1 mgN•kg^{-1} to 81.8 mgN•kg^{-1} for Soil I and from 2.8 mgN•kg^{-1} to 6.5 mgN•kg^{-1} for Soil IV). The NO_3^- concentration changed significantly in the soil stored by air-drying ($P<0.05$) (Table I).

In addition, the NO_3-$\delta^{15}N$ value did not remain stable in all soil samples during the natural air-drying process. Although no remarkable change in the NO_3-$\delta^{15}N$ value was observed for Soil I, Soil II, or Soil III stored by air-drying ($P>0.05$), the relative difference between the fresh soil and the air-dried soil was greater than 0.3‰ for Soil I and Soil III. For Soil I, the NO_3-$\delta^{15}N$ value was 3.9‰ in the fresh soil and decreased to 3.5‰ after the soil was stored for four weeks by air-drying; for Soil III, the NO_3-$\delta^{15}N$ value was -3.1‰ in the fresh soil and -1.7‰ after two weeks of soil storage by air-drying. Therefore, combined with the changes in NO_3^- concentration, it is reasonable to conclude that compared with frozen storage, air-dried storage exhibits inferior performance in preserving soil NO_3^-.

Sample storage after soil extraction

The two different samples, Soil V and Soil VI, were found to have a significant change in the

NO_3^- concentration within 24 hours in the bactericidal agent-free soil solution; the NO_3^- concentration dropped from 602 mgN•kg^{-1} to 564 mgN•kg^{-1} in Soil V and from 2.7 mgN•kg^{-1} to 1.8 mgN•kg^{-1} in Soil VI within 24 hours. In the bactericidal agent-containing soil solution, the NO_3^- concentration was well preserved after 24 hours of storage; the NO_3^- concentration was 602 mgN•kg^{-1} originally and was 599 mgN•kg^{-1} after 24 hours of storage in the bactericidal agent-containing soil solution of Soil V; the NO_3^- concentration was 2.8 mgN•kg^{-1} originally and was 2.9 mgN•kg^{-1} after 24 hours of storage in the bactericidal agent-containing soil solution of Soil VI. However, thereafter, the NO_3^- concentration notably decreased over time (P<0.05) and became undetectable after two weeks of storage (Table I). This decrease was accompanied by a change in the NO_3-δ^{15}N value (Fig. III). Along with the decreased NO_3^- concentration, the NO_3-δ^{15}N value became positive in all soil samples. For example, in the bactericidal agent-containing soil mixture solution of Soil V, the NO_3^- concentration decreased from 602 mgN•kg^{-1} to 545 mgN•kg^{-1} with an increase in the NO_3-δ^{15}N value from 2.8‰ to 4.9‰.

In the soil samples, the NO_3^- content and its δ^{15}N value were well preserved for at least one month in the supernatant solution after removing the soil particles by filtration (Fig. III). For Soil V, the NO_3^- concentration and the NO_3-δ^{15}N value were 602 mgN•kg^{-1} and 2.8‰, respectively, in the fresh soil and 593 mgN•kg^{-1} and 2.5‰, respectively, in the supernatant after one month of storage. For Soil VI, the NO_3^- concentration and the NO_3-δ^{15}N value were 2.7 mgN•kg^{-1} and -2.7‰, respectively, in the fresh soil and 3.1 mgN•kg^{-1} and -2.8‰, respectively, in the supernatant solution after one month of storage. These findings indicate that the NO_3^- content and NO_3-δ^{15}N value remained stable in the supernatant solution for one month (P>0.05).

The statistical significance of the differences between the different storage methods for each soil sample was evaluated using P values (Table III). The results demonstrated that the concentration of NO_3^- significantly varied among different storage methods for Soil I, Soil III, Soil IV, and Soil VI (P<0.05). The δ^{15}N value exhibited a remarkable difference among different storage methods for Soil III, Soil V, and Soil VI (P<0.05). These results indicate that the changes in the NO_3^- content or the NO_3-δ^{15}N in soil samples vary substantially with different sample storage methods.

TABLE III

The paired t-test P values for the difference between different preservation methods for each soil sample.

Sample	P (NO_3^- concentration)	P (δ^{15}N)
Soil I	0.046	0.204
Soil II	0.148	0.107
Soil III	0.148	0.005
Soil IV	0.199	0.040
Soil V	0.119	0.001
Soil VI	0.001	0.011

Discussion

The effect of air-drying on NO_3- $\delta^{15}N$ preservation:

Air-drying is a commonly used storage method. A previous study revealed that after air-dried storage, the NO_3^- content remarkably increased in three soil samples collected from central Panama (Turner and Romero, 2009). Our study also demonstrated that air-dried storage significantly affected the NO_3^- content in the soil extracts. Almost all of the soils that were stored by natural air-drying underwent changes in the NO_3^- concentration and NO_3-$\delta^{15}N$ value (Fig. II). Despite the insignificant change in the NO_3-$\delta^{15}N$ value in a portion of the soil samples, the remarkable change in the NO_3^- concentration in these samples implies that the air-drying storage method cannot satisfactorily preserve NO_3^- in soil.

Commonly, changes in soil NO_3 concentration may be caused by a change in the rate of soil nitrification. The soil nitrification rate is usually determined by the supply of nitrification substrates, the size and activity of nitrifying populations, and the mineralization rate of NO_3^- (Grenon *et al*., 2004). Researchers have reported that soil biomass can markedly change under natural air-drying conditions (Stenberg *et al*., 1998; Martí *et al*., 2012); the soil microbial composition can change to a certain extent, which may affect the soil nitrification rate during the air-drying process. Consequently, the soil nitrogen cycle can be altered, leading to a change in the NO_3^- content in the soil. Thus, the increasing NO_3 concentration in our results may be caused by an increased soil nitrification rate. Therefore, air-drying might not be an optimal method for sample storage for soil NO_3^- and NO_3-$\delta^{15}N$ analysis (Ross *et al*., 1980; Pulleman *et al*., 1999).

The effect of sample freezing on NO_3- $\delta^{15}N$ preservation:

Previous studies have recommended freezing as a suitable method for soil sample storage (Dalias *et al*., 2002; Mimmo *et al*., 2008; Martí *et al*., 2012). Our results also verified that the NO_3^- content and NO_3-$\delta^{15}N$ were well preserved in all natural soil samples stored frozen at -18°C for a short period. In the samples with different soil NO_3^- concentrations, freezing at -18°C can impede the transport and transformation of soil NO_3^- during short-term storage (Fig. II). Research has shown that frozen samples have the same bio-composition as that of fresh soil samples (Martí *et al*., 2012). This similarity suggests that the key to terminating the N transformation in soil is the termination of microbial activity. Freezing can effectively reduce microbial activities in soil, thus preserving NO_3^- and NO_3-$\delta^{15}N$ effectively during the storage period. Therefore, the storage of frozen samples might be a method suitable for the preservation of NO_3-$\delta^{15}N$ in the field.

Although the frozen storage method is reliable, it has some limitations when used in field experiments. In the field, this method requires refrigerants such as dry ice, which is difficult to obtain. Because field investigations often last for several weeks in the summer, the difficulties in the storage

and supply of refrigerants cause difficulties in soil sample storage. To address this problem, we have tried to explore whether soil extracts could be used to preserve soil NO_3^- in the field.

The effect of the soil extraction/filtration storage on NO_3- $\delta^{15}N$ preservation:

To clarify whether eliminating the effect of microbes is conducive to the preservation of NO_3^- in soil extracts, we conducted a comparative experiment using the filtered soil extract solution and bactericidal agent (HgCl)-containing soil solution.

In the experiment on the two different soil samples (Soil V and Soil VI), the soil extract solution was sterilized and investigated first because this method was the easiest to perform. The results revealed that the bactericidal agent could only keep the NO_3^- content and NO_3-$\delta^{15}N$ stable for 24 hours, whereas the NO_3^- in samples changed significantly in the soil solution without bactericidal agents after 24 hours under the same conditions. After being stored for one week, the NO_3^- content and NO_3-$\delta^{15}N$ in bactericidal agent-containing solution was remarkably changed. During this process, the NO_3-$\delta^{15}N$ value changed in the positive direction, with a decrease in the NO_3^- concentration (Fig. III). Commonly, it is believed that biological activity such as nitrification and denitrification causes enrichment of ^{15}N in substrates (Templer *et al*. 2007), which implies that the bactericidal agent failed to effectively inhibit the microbial consumption of NO_3^- in the soil mixture solution, which caused ^{15}N enrichment. Our finding proves that adding bactericidal agents to the soil mixture solution does not satisfactorily preserve the NO_3^- content of the soil sample.

Nevertheless, after one month, the NO_3^- content and NO_3-$\delta^{15}N$ remained the same in the filtered supernatant solution as in the fresh soil (Fig. III), indicating that soil extract storage after filtration has a satisfactory NO_3-$\delta^{15}N$-preserving performance. Under regular experimental conditions, a solution filtered with GF/F filter paper is considered to be free of microbial interference (Laanen *et al.*, 2011; Dong *et al.*, 2014). As discussed above, microbial activity is a pivotal factor that influences the short-term preservation of NO_3^- in samples. Therefore, the NO_3^- contents and NO_3-$\delta^{15}N$ values can be simply and effectively preserved in a filtered supernatant solution that is stored at room temperature (23±3°C) without exposure to direct light.

According to our findings, immediately extracting soil samples and storing the acquired supernatant solution at the sampling sites may be a superior method for preserving NO_3^- and NO_3-$\delta^{15}N$ in addition to the frozen storage method. Because NO_3-$\delta^{15}N$ can remain well-preserved in a supernatant solution that is sealed and stored at room temperature (23±3°C) for more than one month, this extraction/filtration storage method can be a simpler and more reliable method for field sampling and experiments.

The effect of soil nitrate content on NO_3- $\delta^{15}N$ preservation

Yvonne Oelmann *et al.* (2007) stressed the importance of substrates in nitrification reactions. Our results also demonstrated that the change in the $\delta^{15}N$ value in the stored soil was substantially

affected by the NO_3 content. In high-NO_3^- concentration soils (Soil I and Soil II), the NO_3^- concentration and NO_3-$\delta^{15}N$ were more stable during storage compared with other low-NO_3 concentration soils (Soil III and Soil IV) (Fig. II). As discussed above, although the freezing method or extraction method can be used to store soil nitrate samples, these methods can only reduce the amount of microbes and suppress microbial activity to a certain extent. But, microbial activity still caused minor changes in nitrogen isotopes in soil nitrates. According to our results, these minor contributions can be ignored for soil samples with a high concentration of NO_3^-, but might be relatively large in samples with a very low concentration of NO_3^-. This finding indicates that even if an effective storage method is used, NO_3 and its $\delta^{15}N$ might still inevitably experience an unstoppable transformation, which is more pronounced in low-NO_3 concentration soil than in high-NO_3^- concentration soil. Therefore, greater attention should be paid to sample storage for the analysis of low-NO_3^- concentration soil. For example, samples with low-NO_3^- concentration should be analyzed as soon as possible and the preservation time should be reduced. Controlling the background of the analysis process is also significant for low-NO_3^- concentration soil.

CONCLUSIONS

Several existing storage methods (freezing, air-drying and two extract-preserving methods) for soil nitrate samples were compared in this study. Based on the experimental results, we draw the following conclusions. First, the natural air-drying storage method is simple to operate but can cause changes in the soil nitrate content and isotope composition. Second, the frozen-storage method is reliable, making it a suitable tool when freezing conditions can be achieved. Third, the storage method by which the soil sample is extracted and then filtered can ensure the stability of nitrogen isotopes in soil nitrates. In addition, this method has the advantage of being applicable in field investigations, particularly in the field research of nitrogen isotopes in soil nitrates, for which a frozen condition cannot be achieved and a long period of sample storage is required.

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Captions

Table I. NO_3^- concentration and $\delta^{15}N$ values of samples stored under various conditions. The results for fresh soil were obtained immediately after sampling to avoid disturbances. Other results were obtained after different storage periods (1 day, 1 week, 2 weeks or 4 weeks). $\delta^{15}N$ values are shown as the mean ±standard deviation.

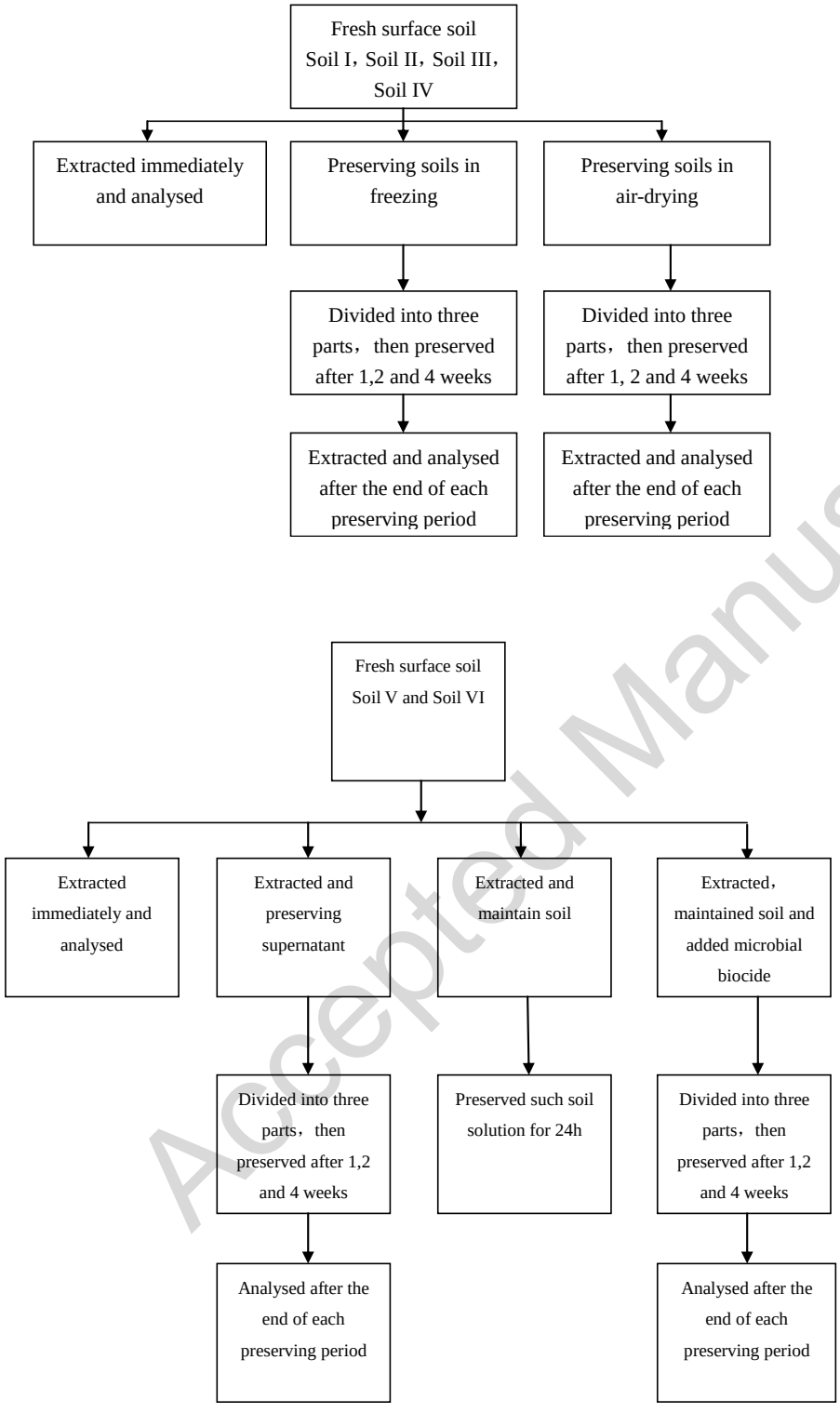
Table II. The one-way ANOVA *P* values for each method for soil samples during the entire preservation period.

Table III. The paired t-test P values for the difference between different preservation methods for each soil sample.

Figure I. The sample separation method for each preservation method.

Figure II. The storage effects on extractable NO_3^- concentration and $\delta^{15}N$ ‰ from four soil samples. The samples were extracted using a $CaSO_4$-saturated solution at room temperature. Samples were stored at -18°C or in air for 1 week, 2 weeks, or 1 month. The standard deviation for $\delta^{15}N$ ‰ duplicate analysis was less than ±0.3‰ according to laboratory standards. Error bars are smaller than the symbol size if not visible.

Figure III. The storage effects on NO_3^- concentration and $\delta^{15}N$ ‰ in NO_3^- extracted from two samples using three different extraction methods: 1) preserving the supernatant of extracts, 2) preserving extracts with soil and 3) preserving extracts with soil and a microbial biocide. The NO_3 was extracted using a $CaSO_4$-saturated solution at room temperature. The standard deviation for $\delta^{15}N$ ‰ duplicate analysis was less than ±0.3‰ according to laboratory standards. Error bars are smaller than the symbol size if not visible.



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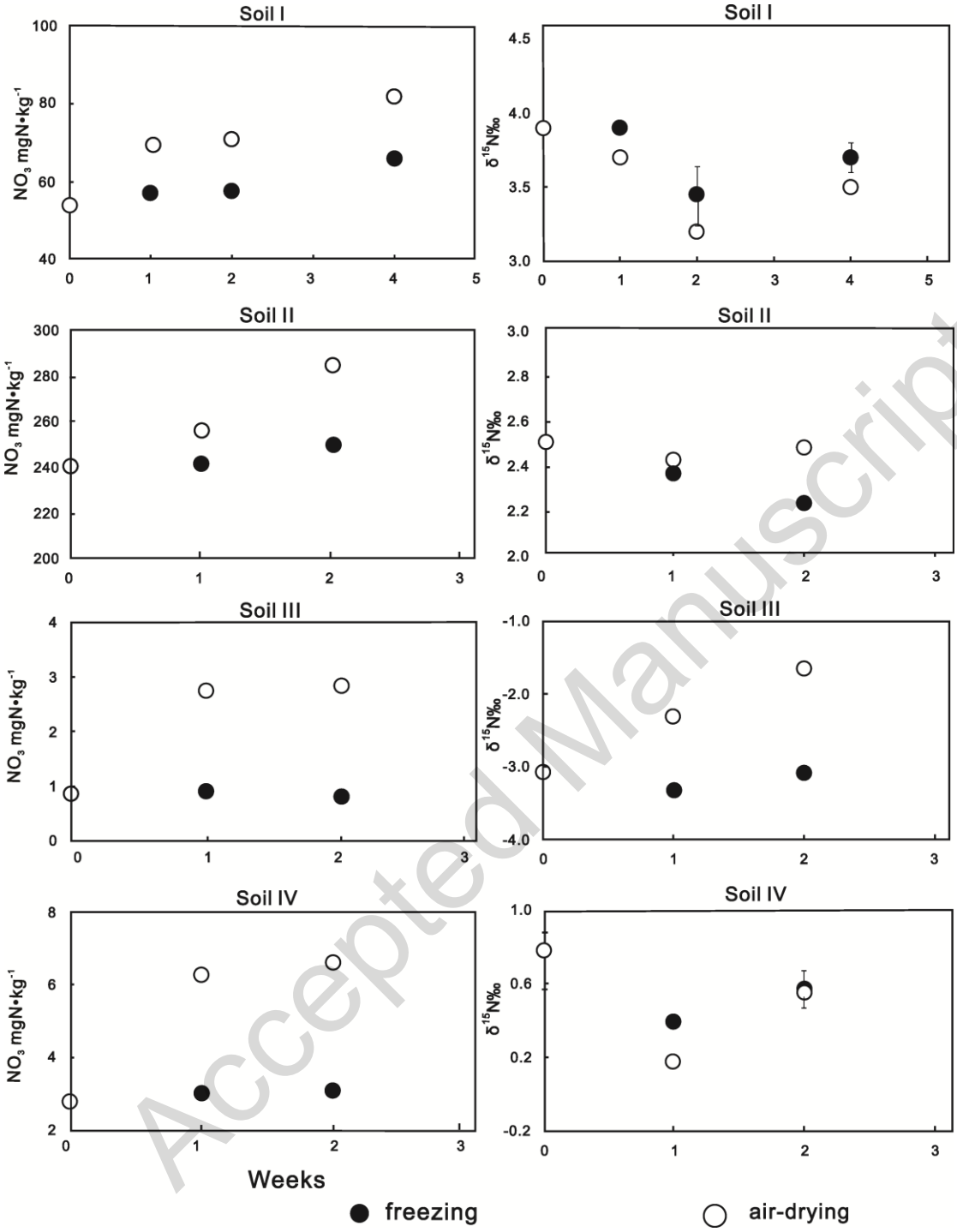

Figure 2. The storage effects on extractable NO_3^- concentration and $\delta^{15}N$ ‰ for four soil samples. The samples were extracted using a $CaSO_4$-saturated solution at room temperature. Samples were stored at -18°C or in air for 1 week, 2 weeks, or 1 month. The standard deviation for $\delta^{15}N$ ‰ duplicate analysis was less than ±0.3‰ according to laboratory standards. Error bars are smaller than the symbol size if not visible.

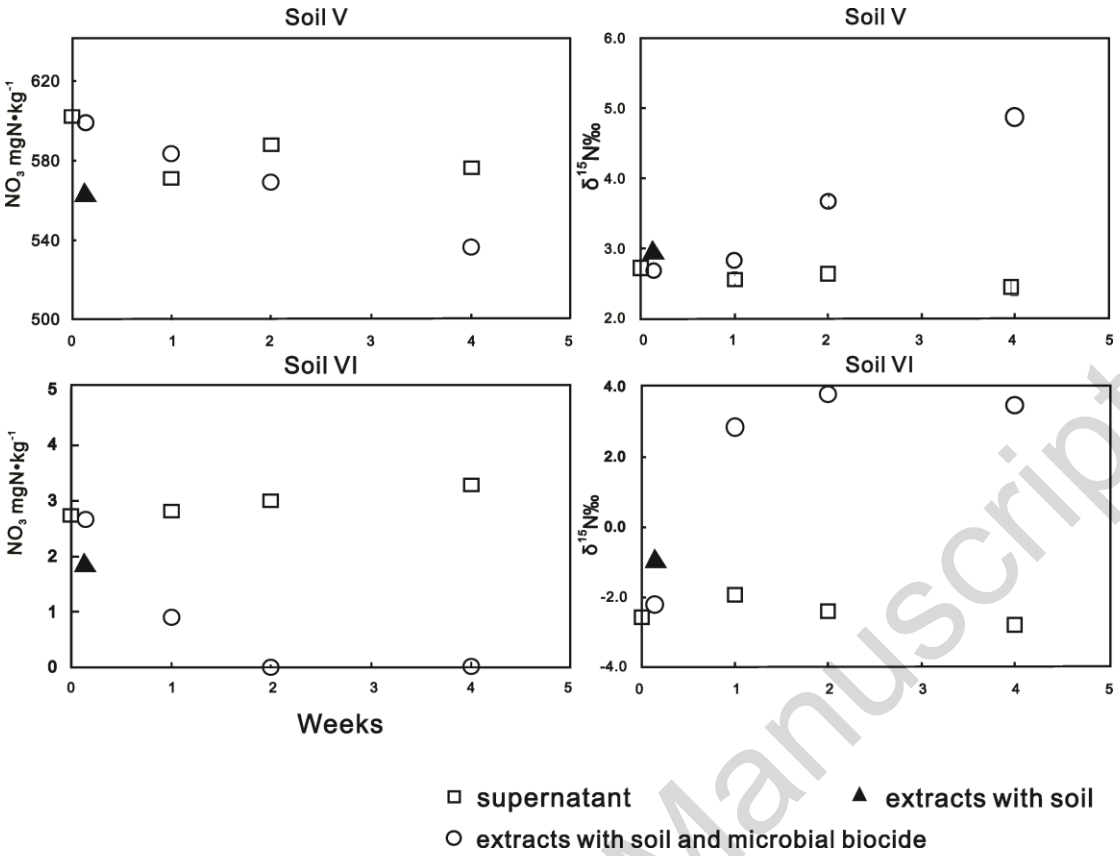

Figure 3. The storage effects on NO_3^- concentration and $\delta^{15}N$ ‰ in NO_3^- extracted from two samples using three different extraction methods: 1) preserving the supernatant of extracts, 2) preserving extracts with soil and 3) preserving extracts with soil and a microbial biocide. The NO_3 was extracted using a $CaSO_4$-saturated solution at room temperature. The standard deviation for $\delta^{15}N$ ‰ duplicate analysis was less than ±0.3‰ according to laboratory standards. Error bars are smaller than symbol size if not visible.