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Improved Extraction Method of Soil Nitrite

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Abstract: Soil nitrite (NO_2^-) is an important reactive intermediate in many nitrogen transformation processes, but it is unstable under acidic conditions. Canonical extraction method of soil NO_2^- with potassium chloride (KCl) solution greatly underestimates its concentration. In order to reflect the concentration more accurately, we optimized the extraction method of soil NO_2^- in this study. Moreover, soil ammonium (NH_4^+) and nitrate (NO_3^-) were also systematically investigated to achieve efficient extraction of soil inorganic nitrogen. The results showed that un-buffered KCl significantly underestimated soil NO_2^- concentration compared to DIW. The highest recovery of NO_2^- was obtained by extracting with DIW at 10 min of oscillation for three soils. Compared with DIW, the concentration of NH_4^+ and NO_3^- in soil extracted from KCl solution increased significantly. Furthermore, the soil inorganic nitrogen content of extracts stored at 4°C for one day were closer to directly measurements of fresh samples than the other storage methods. Overall, the recommend analysis method for soil NO_2^- was extracted by DIW, oscillated for 10 min, and filtered by a 0.45 μm filter, and soil NH_4^+ and NO_3^- were extracted with KCl solution and oscillated for 30 min. The extract should be stored at 4°C and analyzed within 24 hours.

Keywords: soil nitrite; deionized water; potassium chloride; oscillation time; storage duration

1. Introduction

Nitrite (NO_2^-) is a key intermediate in several nitrogen transformation processes [nitrification, denitrification, nitrifier denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and chemo-denitrification] [1–5] and is also closely associated with gaseous nitrogen emissions as an independently existing nitrogen reservoir [6]. Due to its extremely rapid metabolism, it is considered as an instantaneous product and is often neglected in previous soil nitrogen (N) cycle studies [7–9]. However, NO_2^- accumulation occurs when some environmental factors lead to the decoupling of ammonia oxidation from nitrite oxidation [10,11]. For example, the application of urea or ammonium-based fertilizer may cause instantaneous accumulation of NO_2^- during nitrification [8,12,13], and higher pH values may exacerbate this process [14]. Soil NO_2^- accumulation may cause a number of environmental problems, for example, NO_2^- reacts with other substances to form gases such as nitrous acid (HONO), nitric oxide (NO), nitrogen dioxide (NO_2), and nitrous oxide (N_2O), affecting the oxidizing capacity of the atmosphere, air quality and human health [15–17]. Nowadays, the source and importance of NO_2^- in water and soil are getting more and more attention. Accurate and rapid determination of soil NO_2^- will contribute to a deeper understanding of the transformation process of nitrogen in soil.

Typically, 2 mol L^{-1} KCl solution is used to extract NO_2^- from soil, which is also a classical method for extracting soil ammonium (NH_4^+) and nitrate (NO_3^-) [18]. During the extraction of NO_2^-, there are generally no biological or chemical reactions that cause changes in its concentration or isotopic composition [19]. However, KCl causes NO_2^- degradation under acidic and neutral conditions, greatly underestimating the NO_2^- concentration in soil [14,18]. Studies have shown that only 22–40% of NO_2^- was recovered from soils with pH between 4.8–5.4 [18,20]. Nevertheless, extraction of soil NO_2^- with 2 mol L^{-1} KCl solution is still a recommended method to be used in soil analysis manuals and international standards [21–23].

New method has been proposed to improve the low extraction efficiency of NO_2^-. The recovery of NO_2^- in acidic soil was higher when it was extracted by deionized water (DIW) with a small amount (4 g) of soil, but the suspended solids in the DIW extracts may affect the absorbance measurements during NO_2^- analysis [18]. To maximize the recovery of soil NO_2^-, the pH of extracting solution with calcium hydroxide [$Ca(OH)_2$] [24], calcium carbonate ($CaCO_3$) [25], or potassium hydroxide (KOH) [19] was adjusted. For example, Stevens and Laughlin (1995) [19] adjusted the pH of the extracting solution to 8.0 with 2 mol L^{-1} KOH and a soil/extractant ratio of 1:1, which resulted in a significant increase in NO_2^- recovery. However, this method is only applicable to a large number of soil samples (200 g), and deviation from this parameter may lead to dispersion of silt and clay particles and/or dissolution of organic matter [26], making it difficult to obtain a clear solution for analysis [18]. This adjustment process is cumbersome and increases the experimental time. In addition, higher pH tends to cause volatilization losses of NH_3 [27], which potentially decreases NH_4^+ recovery if applied the same extraction method.

In recent years, a large number of studies have been conducted on the factors affecting the determination of soil NH_4^+ and NO_3^-, including the extraction method [28], the concentration of the extractant [28], the storage method [29], and the oscillation time [28], etc. However, the effects of these factors on soil NO_2^- concentration have not been systematically reported. Therefore, this study aims to improve the effective extraction and the accuracy of soil NO_2^- determination, and to provide a basis for realizing the efficient extraction of NO_2^-, NO_3^-, and NH_4^+.

2. Materials and Methods

2.1. Soil Sampling

The soils used here (Fluvo-aquic soil, Mollisol, and Ultisol) were previously studied by Song et al. (2023) [17] and have been described in detail. Briefly, Fluvo-aquic soil was collected from Quzhou County, North China Plain (36°52′N, 115°10′E), with pH of 7.98, TN and C/N of 0.11% and 9.23 respectively. The climate is a temperate monsoon climate, with an average annual temperature and precipitation of 13.2°C and 494 mm, respectively. The crops planted are wheat and maize rotation. Mollisol was collected from Lishu County in Northeast China (43°37′N, 124°36′E), with pH of 5.51, TN and C/N of 0.13% and 10.13, respectively. The climate is a temperate monsoon climate, with an average annual temperature and precipitation of 6.5°C and 650 mm, respectively. The planting crop is maize. Ultisol was collected from Jinjing City in Central South China (28°38′N, 113°19′E), with pH of 5.35, TN and C/N of 0.11% and 9.72, respectively. The climate is a subtropical monsoon climate, with an average annual temperature and precipitation of 17.5°C and 1330 mm, respectively. The crops planted are oilseed rape and maize. Five sampling points were randomly selected for each site and mixed evenly, with a depth of 0–20 cm. After removing roots, rocks, seeds, and other debris, the soil was air-dried, and after grinding, the soil was kept at room temperature through a 2 mm sieve for further use.

2.2. Experimental Design

We first compared inorganic N concentrations extracted by different extraction methods (DIW, un-buffered 2 mol L^{-1} KCl, or pH-buffered 2 mol L^{-1} KCl) to assess whether increasing the pH of the extracting solution would affect soil NO_2^- concentrations. Three groups of treatments were set up in the experiment, as follows: (1) Only DIW; (2) un-buffered 2 mol L^{-1} KCl; (3) pH = 7.5, 6.5, and 6.0 phosphate buffer solution (PBS) + 2 mol L^{-1} KCl (1:4) were added to the Fluvo-aquic soil, and pH = 8.4, 8.0, 7.5, and 7.0 phosphate buffer solution + 2 mol L^{-1} KCl (1:4) were added to the Mollisol, and Ultisol, respectively. Different pH of PBS were prepared by mixing 1/15 mol L^{-1} KH_2PO_4 and Na_2HPO_4 in different proportions. The soil-liquid ratio of all the above treatments was 1:5 (weight/volume, w/v), followed by shaking at 200 rpm and 25°C for 30 min, centrifuged at 9000 rpm for 15 min, and filtration first through 9cm medium speed filter paper (aperture 15-20μm) and then by 0.45μm aperture filter. The pH and inorganic nitrogen content of the extracting solution were simultaneously measured by a pH meter (Mettler Toledo FE28, Switzerland) and a SmartChem automatic chemical analyzer 450 (AMS-Alliance, Italy), respectively.

We further investigated the effect of different storage methods and duration on soil inorganic nitrogen concentration. Air-dried soils (5 g, n = 3) were extracted in 25 mL un-buffered 2 mol L^{-1} KCl or DIW (filtered through 0.45 μm). The extraction methods were as described above. The difference is that one set of extracting solution was taken for direct determination of soil inorganic nitrogen concentration, while the others were stored at −20°C for 1 day, and at 4°C for 1, 3, and 6 days, respectively, before determination.

Finally, we studied the effect of oscillation duration on soil NO_2^- recovery. NO_2^- standard solution was added to evaluate the recovery of NO_2^- in the extracts (3 mg N kg^{-1} soil, extracted with DIW and pH-buffered 2 mol L^{-1} KCl). The extraction method was as described above. The difference is that the oscillation was 10 min and 30 min, respectively, and the extraction solution was placed at 4°C for storage and analyzed within 24 h. Three replicates were set for all the above treatments. Soil without adding standard solution was used as a control. The recovery of NO_2^- was calculated based on the difference measured in soil with and without adding standard solution:

$$Recovery = \frac{N_b - N_a}{N_o} \times 100\%$$

where N_b and N_a represent the NO_2^- concentration extracted from the added standard solution and control soil, respectively; N_o represents the NO_2^- concentration in the standard solution.

2.3. Statistical Analyses

All test data were preliminarily sorted and in-depth calculated by Microsoft Excel 2019. SPSS Statistics 26.0 (IBM, USA) was used for one-way ANOVA or two-factor ANOVA to determine the differences in soil inorganic nitrogen concentrations between treatments (t and LSD test, $P<0.05$). When necessary, data were transformed to meet the assumption of normality.

3. Results

3.1. DIW Extraction Can Achieve Higher Soil NO_2^- Content

The pH of the un-buffered KCl extracts was 7.68, 4.16, and 4.65 for the Fluvo-aquic soil, Mollisol, and Ultisol, respectively, which was significantly lower than the pH of the DIW extracts (Figure 1). After adding phosphate buffer solution, pH of the extracts were significantly decreased in Fluvo-aquic soil and increased in Mollisol and Ultisol. The pH was 7.14, 6.17, and 5.96 for the pH-buffered KCl extracts in Fluvo-aquic soil, while that were maintained at 6.0–7.5 in Mollisol and Ultisol.

The variation of inorganic nitrogen in different treatments are shown in Figure 2. The NO_2^- content was significantly higher in Fluvo-aquic soil (1.18–2.06 mg N kg^{-1}) than in Mollisol (0.02–0.03 mg N kg^{-1}) and Ultisol (0.02–0.08 mg N kg^{-1}). Extraction with DIW can achieve higher NO_2^- content than un-buffered KCl solution in all three soils. The extracted NO_2^- concentrations were mostly increased after adding of phosphate buffer solution with different pH, but which were still lower than that in the DIW extractions. However, soil NH_4^+ concentration was reduced by 25% (Fluvo-aquic soil), 35% (Mollisol), and 60% (Ultisol) when extracted by DIW compared with un-buffered KCl, respectively. Soil NO_3^- concentrations were significantly higher in un-buffered KCl than DIW extracts of Fluvo-aquic soils, while the opposite was true for Mollisol and Ultisol.

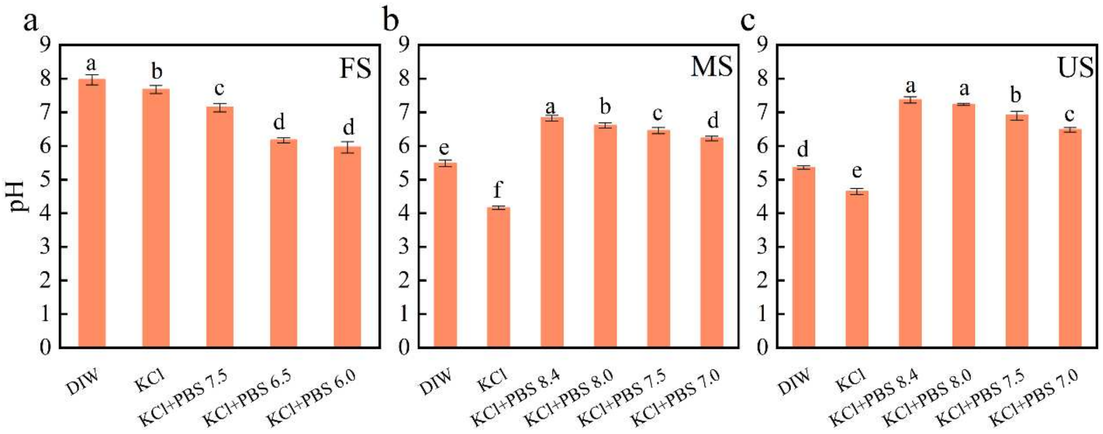

Figure 1. pH variation of soil extracts from different treatments. DIW, deionized water; KCl, 2 mol L^{-1} KCl; PBS 8.4, 8.0, 7.5, 7.0, 6.5, and 6.0 denote phosphate buffer solution with the pH of 8.4, 8.0, 7.5, 7.0, 6.5, and 6.0, respectively. FS: Fluvo-aquic soil; MS: Mollisol; US: Ultisol. Different lowercase letters mean significant difference at $P < 0.05$.

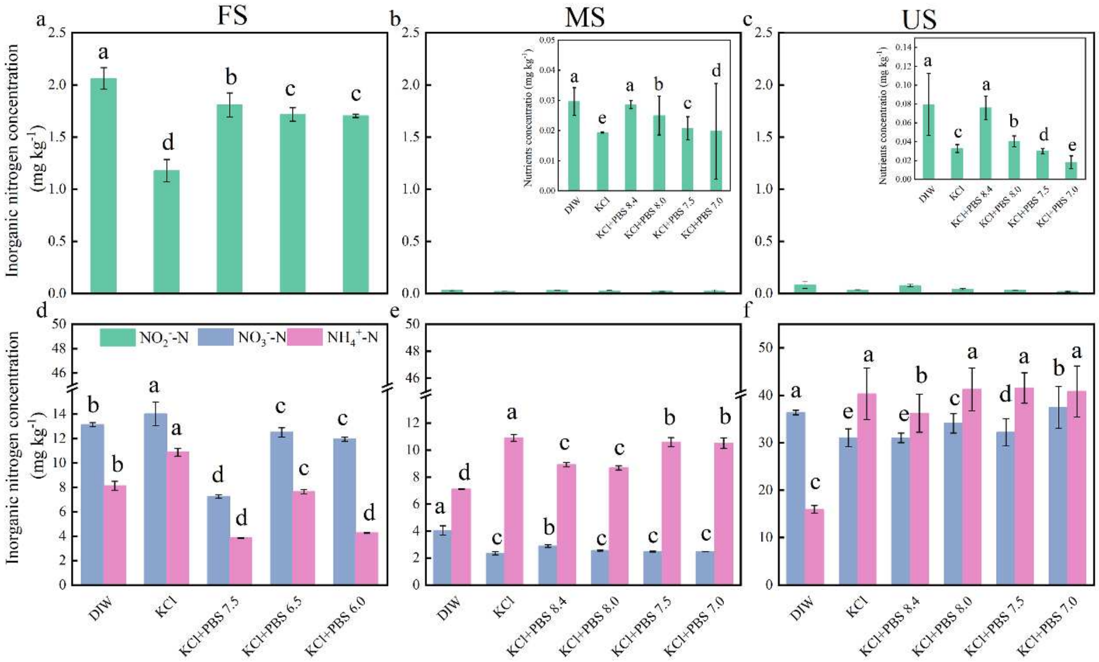

Figure 2. Variation of inorganic nitrogen concentration in different treated soils. DIW, deionized water; KCl, 2 mol L^{-1} KCl; PBS 8.4, 8.0, 7.5, 7.0, 6.5, and 6.0 denote phosphate buffer solution with pH of 8.4, 8.0, 7.5, 7.0, 6.5, and 6.0, respectively; FS: Fluvo-aquic soil; MS: Mollisol; US: Ultisol. Different lowercase letters mean significant difference at $P < 0.05$.

3.2. Storage Increased NO_2^- and NH_4^+ Content

Storage methods significantly affected the concentration of inorganic nitrogen in the three soils (Figure 3). Soil NO_2^- content was closer to directly measured content of fresh samples when stored at 4°C than −20°C, regardless of extraction with DIW or un-buffered KCl. However, there was a tendency of increase with longer storage time ($P < 0.05$). Compared with storage 1 d at 4°C, storage 6 d at 4°C increased soil NO_2^- content by 3.4 (Fluvo-aquic soil), 1.6 (Mollisol), and 1.8 folds (Ultisol), respectively. Soil NO_3^- concentration extracted by un-buffered KCl decreased with the storage time (4°C), while NH_4^+ was the opposite. Soil NH_4^+ concentration increased by 51% (Fluvo-aquic soil), 59% (Mollisol), and 20% (Ultisol) after stored for 6 d compared to 1 d at 4°C. We also found no significant difference between stored for 1 d at 4°C and directly measured for DIW extracted NO_2^- and un-buffered KCl extracted NH_4^+ concentrations ($P > 0.05$).

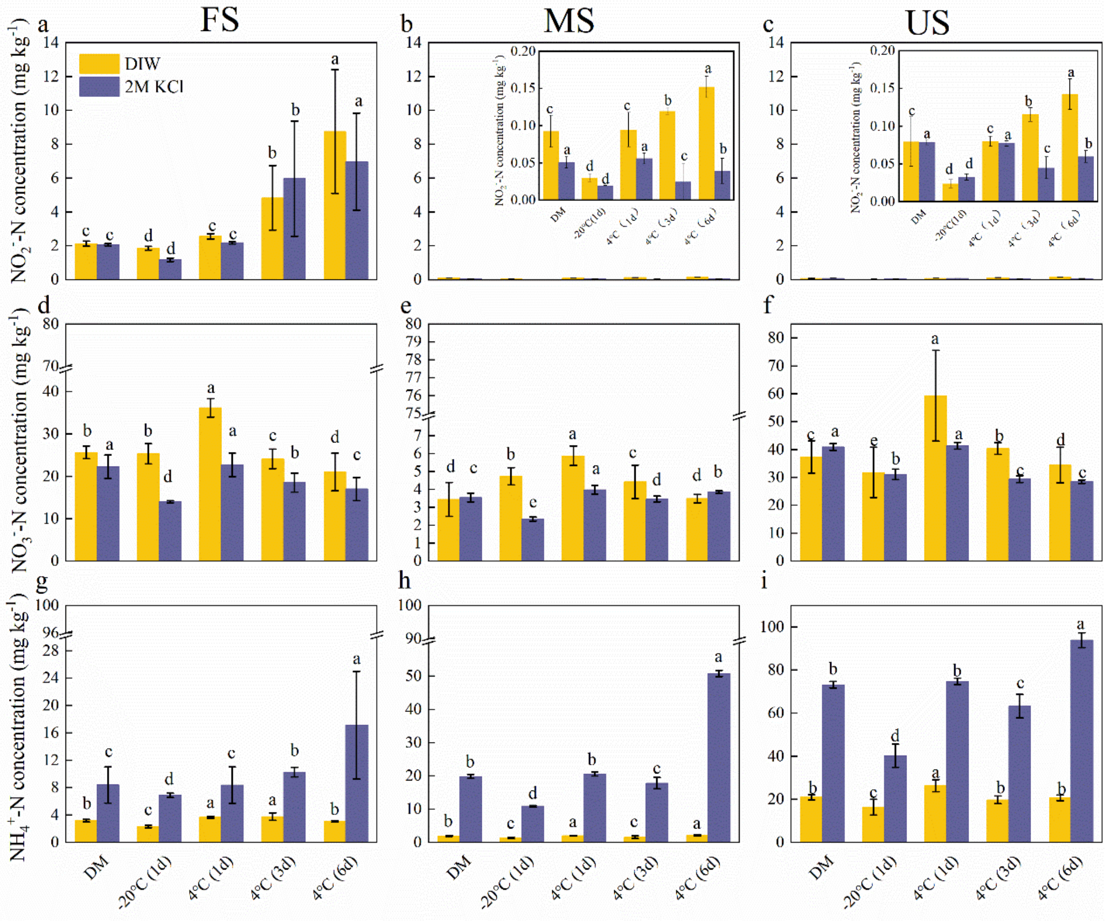

Figure 3. Changes in inorganic nitrogen concentration in extracts from different storage methods. DM, directly measured; −20°C (1d), stored at −20°C for 1 day; 4°C (1d), (3d), and (6d) mean stored at 4°C for 1, 3, and 6 days, respectively; FS: Fluvo-aquic soil; MS: Mollisol; US: Ultisol. Different lowercase letters mean significant difference at $P < 0.05$.

3.3. *Effect of Oscillation Time on NO_2^- Recovery*

Oscillation time had a significant effect on NO_2^- recovery in acidic soils (Mollisol and Ultisol) rather than alkaline soil (Fluvo-aquic soil) (Figure 4). When oscillating for 10 min, the NO_2^- recovery rates of the three soils were 85–87% (Fluvo-aquic soil), 92–95% (Mollisol), and 91.39–91.72% (Ultisol), respectively, in the two extraction methods (DIW and pH-buffered KCl). No significant difference was found between each treatment ($P > 0.05$). With the increase of oscillation time, the recovery of NO_2^- in acidic soil decreased, especially in the DIW treatment. The NO_2^- recovery was also affected by the pH of extraction solution in acidic soils. After oscillation 30 min, the NO_2^- recovery rates of the pH-buffered treatments were 88% (Mollisol) and 90% (Ultisol), respectively, which were significantly higher than those of the DIW treatments ($P < 0.05$). The results of two-way ANOVA showed that there was a significant interaction effect between pH and oscillation time on the recovery of NO_2^- in acidic soils, but not in alkaline soil ($P > 0.05$). In summary, extraction with DIW and oscillating for 10 min could be suitable for both acidic and alkaline soils.

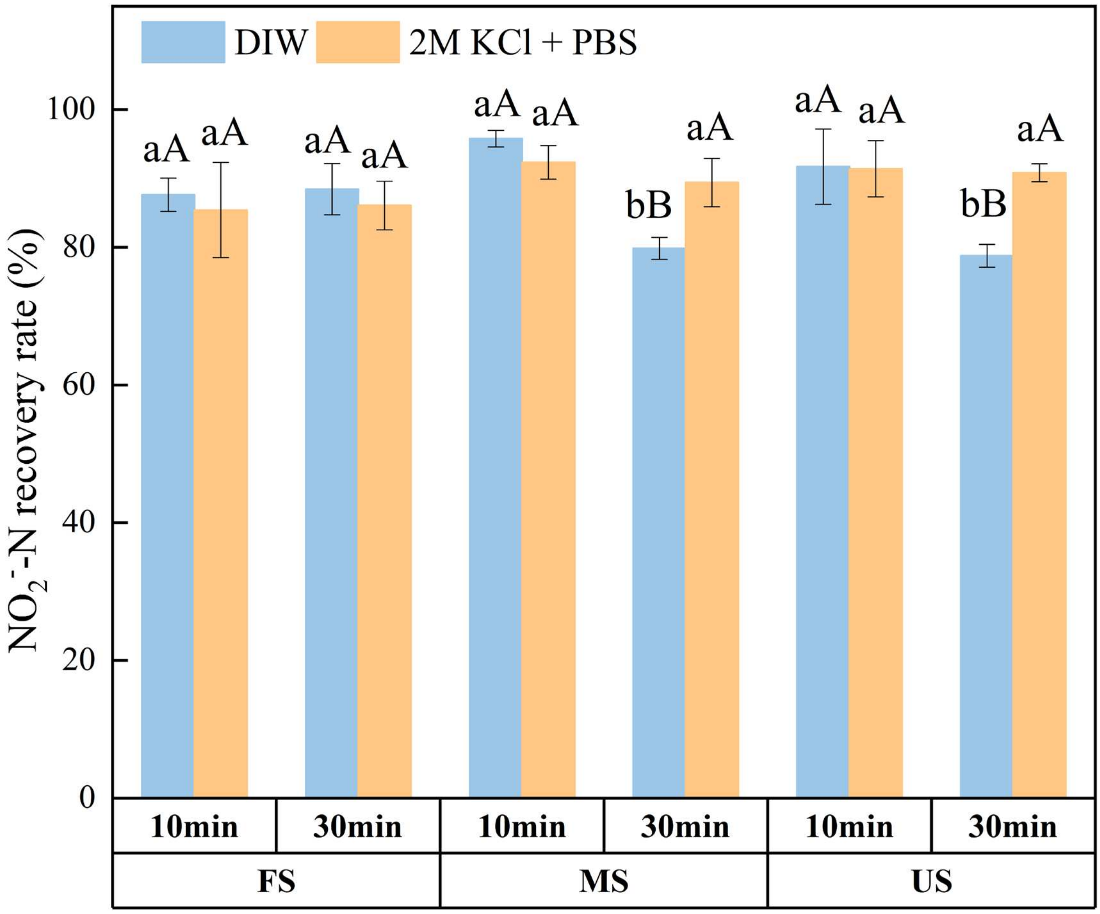

Figure 4. Soil NO_2^- recovery rate with different oscillation time. DIW, deionized water; 2 M KCl + PBS, 2 mol L^{-1} KCl and PBS of different pH: pH 7.5 of PBS is added to the Fluvo-aquic soil, and pH 8.4 of PBS is added to Mollisol and Ultisol; FS: Fluvo-aquic soil; MS: Mollisol; US: Ultisol. Different lowercase letters mean significant difference between the DIW and KCl + PBS treatments at $P < 0.05$. Different uppercase letters mean significant difference between different oscillation times at $P < 0.05$.

4. Discussion

4.1. Soil NO_2^- Concentration

Compared with DIW, extracting with un-buffered KCl solution significantly reduced the pH of extracts in Mollisol and Ultisol (Figure 1b–c), which may be due to the release of protons by K^+ adsorption, an increase in ionic strength, and the hydrolysis of extractable aluminum. In acidic environments, NO_2^- protonates into HNO_2 (pK_a=3.3, 25°C), which subsequently decomposes to gaseous nitrogen products such as HONO, NO, or NO_2, and can explain the loss of NO_2^- in acidic environments [17,18,30]. These results were confirmed by lower NO_2^- concentrations (35%–59%) in the un-buffered KCl extract than that of DIW (Figure 2a–c). In addition, chemical fixation of NO_2^- by insoluble soil organic matter [20,31], reaction with metal oxides [32], or rapid conversion to NO_3^- [25,31] may also be responsible for the low NO_2^- concentration in the un-buffered KCl extract. To maximize the recovery of soil NO_2^-, the pH of the extracting solution was adjusted with $Ca(OH)_2$ [24], $CaCO_3$ [25], or KOH [19]. Indeed, the pH of the pH-buffered KCl extracts increased significantly (Figure 1b–c), and the NO_2^- concentration also increased significantly (Figure 2b–c). The extracted NO_2^- by pH 8.4 buffer solution was even comparable with that of DIW in Mollisol and Ultisol. It was found that the recovery of NO_2^- was about 69% when the extracts pH was 4.8–6.0, and as high as 95% when the pH was adjusted to 7.8 [32]. In conclusion, extracted by un-buffered KCl could greatly underestimate soil NO_2^- concentrations.

Although DIW is capable of extracting higher concentrations of NO_2^-, it produces a brown extract that interferes with colorimetric analysis (Figure 5). Homyak et al. (2015) [18] found that the concentration of NO_2^- in DIW extract measured by colorimetric analysis was 35% higher than that by ion chromatography. However, conventional filters did not remove suspended particles, and the color reagent flocculates with the suspended solids, resulting in higher absorbance readings and overestimation of the NO_2^- concentration. The measured results were consistent after filtration by 0.45 μm filter [18].

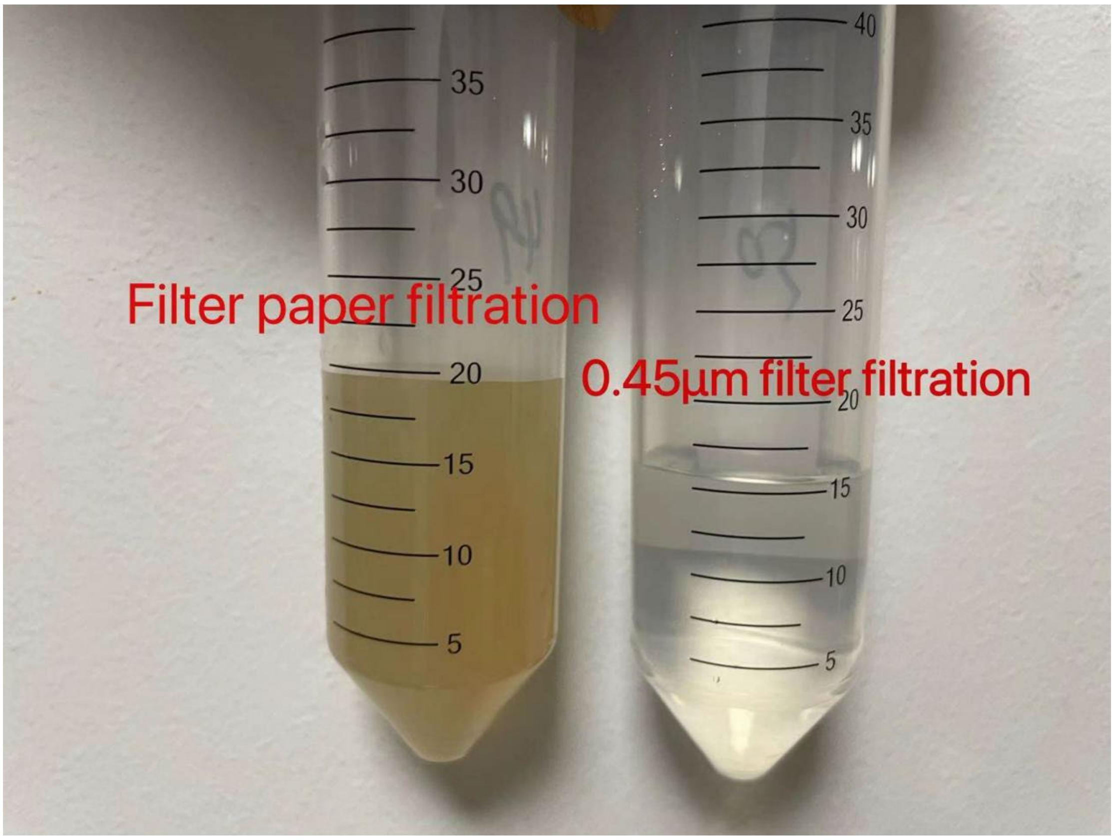

Figure 5. Soil NO_2^- was extracted with deionized water to produce brown extract, which was filtered by a 0.45 μm filter to obtain clear extract.

Oscillation time was also a key factor affecting soil NO_2^- recovery. Acidic soil NO_2^- recovery decreased significantly with increasing oscillation time (Figure 4). It was shown that NO_2^- recovery at 10 min of oscillation was 86%, which was three times higher than that at 30 min of oscillation [19]. Our study showed that the NO_2^- recovery of acidic Mollisol and Ultisol extracted by DIW was greater than 90% at 10 min of oscillation. Higher NO_2^- recovery were obtained by keeping the extraction process at a higher pH when oscillating for 30 min.

During the analysis process, the extracts may not be determined immediately and stored for a period of time because of various reasons. However, few studies have considered the effect of the storage duration on soil NO_2^- concentration. We found that NO_2^- concentration of samples stored at 4°C was closer to the directly measured value of fresh sample, rather than that of −20°C (Figure 3a–c). The reason may be due to NO_2^- degradation caused by freezing and thawing. In addition, the NO_2^- concentration gradually increased with the increase of storage time (4°C). Therefore, it is recommend that NO_2^- should be measured as soon as possible after extraction, to obtain more accurate value.

4.2. Soil NH_4^+ and NO_3^- Concentrations

Compared with un-buffered KCl, extraction with DIW significantly underestimated NH_4^+ concentration in the three soils, and NO_3^- concentration in the Fluvo-aquic soil (Figure 2d–f). Tu et al. (2021) [28] also found that measured concentrations of NH_4^+ and NO_3^- in KCl extracts were 1.6–2.6 and 1.1–1.8 folds higher than those of DIW, respectively. Lim et al. (2018) [14] showed that DIW extracted NO_3^- concentrations were only 50–60% of those extracted by 2 mol L^{-1} KCl. Due to the significant ion exchange capacity of soil [14], soil colloids are usually negatively charged and can adsorb NH_4^+, while high concentrations of K^+ can displace NH_4^+ adsorbed on soil colloids. However, the negatively charged NO_3^- ions will not be adsorbed by the soil, and "freely" exists in soil solution.

Although most of the literature suggests that the oscillation time for extracting NH_4^+ and NO_3^- is 60 min [28,33], some studies believe that the concentration of NO_3^- does not change significantly after 15–30 min of oscillation [34,35]. NH_4^+ and NO_3^- concentrations were relatively stable at the late stage of oscillation (30–60 min), which indicate that 30 min of oscillation is long enough to extract soil NH_4^+ and NO_3^- [35]. For soils with very high mineral nitrogen content, the oscillation time may be extended to 60 min. In this study, NH_4^+ and NO_3^- were extracted with an oscillation time of 30 min.

Storage conditions significantly affected soil NH_4^+ and NO_3^- concentrations. We found that soil NH_4^+ or NO_3^- concentrations were close to the directly measured values of fresh samples when un-buffered KCl or DIW extracts were stored at 4°C, which were also higher than that at −20°C (Figure 3d–i). NH_4^+ and NO_3^- concentrations in extracts stored at −20°C were only 50–60% and 60–80% of those at 4°C, respectively. This may be due to the degradation of NH_4^+ and NO_3^- due to freezing or thawing process. However, a gradual increase in NH_4^+ concentration and decrease in NO_3^- concentration with soil sample extracts were stored at 4°C (Figure 3d–i), which were also confirmed by the results from Li et al. (2012) [33]. Thus, we recommend the extracted solution could be stored at 4°C and analyzed within 24 h.

5. Conclusions

The soil NO_2^- extraction method was optimized in order to more accurately reflect the true concentration of NO_2^- in soil. Un-buffered KCl significantly underestimated soil NO_2^- concentration compared to DIW. Further studies showed that the oscillation time and preservation method significantly affected the NO_2^- concentration. The highest NO_2^- recoveries for the three soil NO_2^- extracted by DIW were obtained at 10 min of oscillation. Soil inorganic nitrogen content in extracts stored at 4°C for one day was closer to direct measurements of fresh samples than other preservation methods. Overall, the optimal conditions for determination of soil NO_2^- was using DIW as the extractant, oscillating for 10 min, and passing through 0.45 μm filter. Soil NH_4^+ and NO_3^- were extracted using conventional KCl solution. Both extracts should be measured immediately, otherwise they should be stored at 4°C and analyzed within 24 h. Our study achieved efficient extraction of soil NO_2^-, NO_3^-, and NH_4^+, respectively, and further optimization is needed if we want to achieve their efficient extraction at the same time.

Author Contributions: Yaqi Song: Data analysis and visualization, writing the manuscript. Dianming Wu: Conception, funding acquisition, methodology, review and editing. Peter Dörsch: Review and editing. Lanting Yue: Measurement. Lingling Deng: Measurement. Chengsong Liao: Supervision. Zhimin Sha: Supervision, editing. Wenxu Dong: Funding acquisition, supervision. Yuanchun Yu: Funding acquisition, supervision.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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