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Multiple effects of nitrate amendment on the transport, transformation and bioavailability of antimony in a paddy soil-rice plant system

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ABSTRACT

Nitrate (NO_3^-) is known to be actively involved in the processes of mineralization and heavy metal transformation; however, it is unclear whether and how it affects the bioavailability of antimony (Sb) in paddy soils and subsequent Sb accumulation in rice. Here, the effects of NO_3^- on Sb transformation in soil-rice system were investigated with pot experiments over the entire growth period. Results demonstrated that NO_3^- reduced Sb accumulation in brown rice by 15.6% compared to that in the control. After amendment with NO_3^- , the Sb content in rice plants increased initially and then gradually decreased (in roots by 46.1%). During the first 15 days, the soil pH increased, the oxidation of Sb(III) and sulfides was promoted, but the reduction of iron oxide minerals was inhibited, resulting in the release of adsorbed and organic-bound Sb from soil. The microbial arsenite-oxidizing marker gene *aoxB* played an important role in Sb(III) oxidation. From days 15 to 45, after NO_3^- was partially consumed, the soil pH decreased, and the reductive dissolution of Fe(III)-bearing minerals was enhanced; consequently, iron oxide-bound Sb was transformed into adsorbed and dissolved Sb species. After day 45, NO_3^- was completely reduced, Sb(V) was evidently reduced to Sb(III), and green rust was generated gradually. Thus, the available Sb decreased due to its enhanced affinity for iron oxides. Moreover, NO_3^- inhibited the reductive dissolution of iron minerals, which ultimately caused low Sb availability. Therefore, NO_3^- can chemically and biologically reduce the Sb availability in paddy soils and alleviate Sb accumulation in

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rice. This study provides a potential strategy for decreasing Sb accumulation in rice in the Sb-contaminated sites.

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Introduction

Antimony (Sb) contamination has attracted increasing attention recently, as it is clastogenic, carcinogenic and highly ecotoxic, resulting in high risks of food contamination and to human health (Sundar and Chakravarty, 2010). Sb-containing additives are widely used in manufacturing, transportation and daily life products, including brake lining, alloys, ceramics, glass and batteries; thus, Sb ores are frequently mined and smelted due to the high demand (Amarasiriwardena and Wu, 2011; Shtangeeva et al., 2011). Consequently, Sb pollutants generated by mining activities enter soils via surface runoff and farmland irrigation, resulting in the accumulation of Sb in food crops and eventually threatening human health and safety (Flynn et al., 2003; Pierart et al., 2015; Fu et al., 2016). Xikuangshan, located in Hunan Province of South China, is the world's largest antimony mine (Wu et al., 2011) and has been active since 1897 (Liu et al., 2010). Therefore, the Sb pollution in surrounding agricultural soil is severe, with an Sb content of up to 2159 mg/kg (Wang et al., 2010), which is much higher than the average abundance in the earth's crust (0.75 mg/kg) (Hu and Gao, 2008). In addition, in a polluted mining area of Hunan Province, one of the main rice production areas in China, the Sb content in rice grains is high at 160 to 930 $\mu\text{g/kg}$ (Ren et al., 2014). Therefore, it is essential to simultaneously reduce the risk of Sb accumulation in rice and maintain high rice production in Sb-contaminated paddy soils.

Owing to its high solubility and phytoavailability, nitrate (NO_3^-) is widely used as a nitrogen fertilizer in paddy fields (Sebilo et al., 2013), including potassium nitrate, calcium nitrate and ammonium nitrate (Han et al., 2016; Kim et al., 2017). In addition, nitrate has been used to remediate heavy metal-contaminated soils because (1) the soil pH increases during the denitrification of nitrate (van Rijn et al., 2006), resulting in enhanced hydrolysis and precipitation of heavy metal cations such as Cd^{2+} , Pb^{2+} , and Zn^{2+} (Zeng et al., 2011; Zhao et al., 2012); (2) nitrate can stimulate soil bacteria to oxidize As(III) to As(V) (Zhang et al., 2017), which can be immobilized by soil minerals (Goldberg, 2002; Dixit and Hering, 2003); (3) microbially mediated nitrate reduction coupled with Fe(II) oxidation (Smith et al., 2017; Li et al., 2018; Liu et al., 2019a) can inhibit the reductive dissolution of iron-bearing minerals in soil and enhance the coprecipitation of heavy metals in soil minerals (Wang et al., 2019).

Despite the above proposed pathways of heavy metal transformations in soil, the effects of nitrate amendment on the behavior of Sb in soil-plant systems are poorly understood. For remediation of the Sb in the contaminated paddy soil around the Xikuangshan Mine, the addition of nitrate may reduce Sb release by inhibiting the reductive dissolution of iron minerals (Hockmann et al., 2014). Furthermore, Fe(II) oxidation could be enhanced by coupling with nitrate reduction, which will incorporate Sb into Fe(III) precipitates. The above processes could immobilize Sb and decrease Sb accumulation in rice. Moreover, nitrate is able to inhibit the microbially-mediated reduction of SO_4^{2-} to sulfide ($\text{S}_2^{2-}/\text{S}^{2-}$) by sulfur reducing bacteria (SRB) (Soares, 2002; Hubert and Voordouw, 2007) and thus diminish Sb immobilization (Wilson et al., 2004), because the insoluble stibnite (Sb_2S_3) was directly formed via the precipitation of sulfides and Sb(III) (Han et al., 2018), and Sb can also be co-precipitated or adsorbed by iron sulfides (FeS/FeS_2) (Wilson et al., 2007). Due to the high concentrations of iron and sulfate in the acid mine drainage (AMD), the geochemical behaviors of heavy metal could be influenced by the iron and sulfur cycles (Karimian et al., 2018). For instance, the newly-formed iron minerals from Fe(II) oxidation had strong adsorption capacities for Sb(III) and Sb(V) (Guo et al., 2014); the reduction of Fe(III)-bearing minerals could lead to the release of heavy metals (Cooper et al., 2006); and the reduction of sulfate to sulfide caused the complex-precipitation of heavy metals by thioal-

cohol (Janyasuthiwong et al., 2015). Meanwhile, the NO_3^- has been reported to promote the oxidation of the reduced forms of Fe and S (Klueglein et al., 2014; Marzocchi et al., 2014), resulting in the migration and transformation of Sb.

Nitrate as an electron acceptor could stimulate Sb(III)-oxidizing bacteria (Terry et al., 2015) and enhance the oxidation of Sb(III) to more mobile Sb(V) products, resulting in a high migration ability of Sb in soil (Vithanage et al., 2013). The high pH resulting from the denitrification of nitrate was also favorable for increasing Sb mobility (Klitzke and Lang, 2009). Although it was reported that NO_3^- ultimately decreased Sb accumulation in rice plants at the mature rice stage (Wang et al., 2019), nitrate amendment had both positive and negative effects on Sb behaviors in soil-plant systems. However, due to the lack of experimental evidences on the Sb transformation in soil-rice system, the multiple effects of nitrate on Sb partitioning in soil and Sb accumulation in rice over an entire rice growth cycle are still poorly understood.

In this study, pot experiments were designed with nitrate-amendment treatments and a control. During the entire lifecycle of rice, rice plants, pore water and rhizosphere soils were collected, and the Sb/N/Fe/S species, soil pH and oxidation-reduction potential (ORP), and functional genes were measured at different time intervals. The main objectives were (1) to demonstrate the effects of nitrate on Sb transformation in soils and accumulation in rice, (2) to illustrate the potential roles of coexisting elements (N, Fe, and S) and microbial community, and (3) to reveal the underlying key processes for nitrate-induced Sb mobilization and immobilization. The findings could be used to precisely evaluate the positive and negative effects of nitrate on Sb transformation and accumulation in rice, which could be beneficial for obtaining a safe and effective nitrate amendment strategy for Sb-contaminated rice paddy fields.

1. Materials and methods

1.1. Soil sampling and analysis

Surface soils (0–25 cm) were collected randomly at three locations in a Sb-contaminated paddy field (111°27'E, 27°42'N) around Xikuangshan Mine in Lengshuijiang City, Hunan Province, China (Appendix A Fig. S1), in March 2017, and then the collected soils from the three locations were mixed thoroughly and air-dried naturally before pot experiment. The analysis methods and test results for the pH and contents of organic carbon (SOC), dissolved SO_4^{2-} (Dis- SO_4^{2-}), total Fe (TFe), total Sb (TSb), and other major components in the contaminated paddy soils are described in Supplementary Data (Appendix A Section S1).

1.2. Pot experiment design

Pot experiments were conducted in a climate-controlled greenhouse. The air-dried soils were sieved through a 2-mm sieve to ensure homogeneity. NO_3^- (20 mmol/kg in soil) (AR Tianjin Kemio Chemical Reagent, China) and fertilizers of 0.21 g urea (AR Guangzhou Chemical Reagent, China), 0.455 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (AR Guangzhou Chemical Reagent, China) and 0.036 g KH_2PO_4 (AR Guangzhou Chemical Reagent, China) per kg dried soil were finely dispersed in 870 g of air-dried soil by stirring, then mixed with 12.13 kg of air-dried soil, and finally the soils (13 kg) were immediately transferred into 15 L pots with 4 replicates of each treatment. The soils were flooded sufficiently with tap water (2–3 cm above the soil surface) and soaked for 24 hr.

Huanghuazhan rice (*Oryza sativa* L. subsp. *Indica*) seeds were obtained from the seed bank of South China Agricultural University,

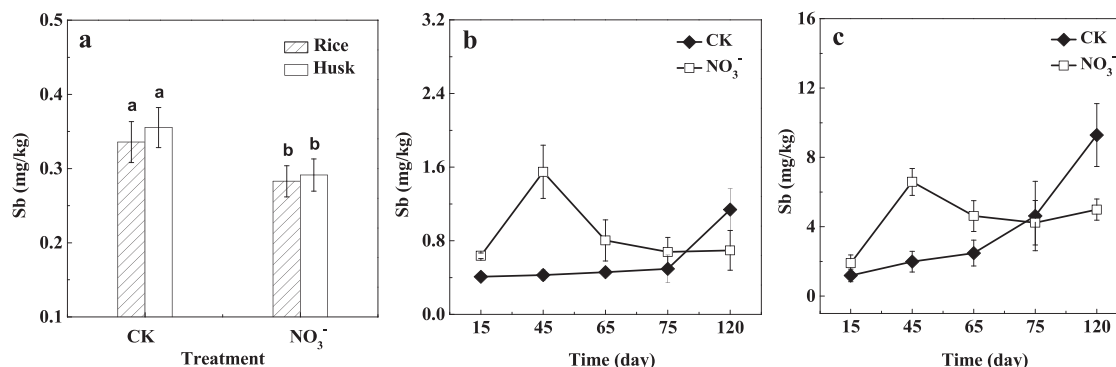


Fig. 1 – Sb accumulations in (a) rice and husks, (b) parts above roots and (c) roots in the NO_3^- treatment during the entire growth period; where the letters of a and b in Fig. 1a are significant difference labels. The data are the means \pm SDs ($n = 3$).

Guangzhou. Seeds were disinfected using 75% ethanol (AR Shanghai Meryer Chemical Reagent, China) and 3% hydrogen peroxide (AR Sigma-Aldrich, USA) and germinated on moist absorbent paper in the dark at 28 °C for a week, and then the rice buds were transferred to nutrient solution for half a month with a relative humidity of 60%–70%. The rice seedlings were transplanted into each pot with six plants per pot. To maintain a water depth of 2–3 cm above the soil surface, the pots were irrigated with tap water daily during the entire rice lifecycle. The soil pH and ORP were determined in situ by a portable digital pH/ORP analyzer (HQ11d Hach, USA). Pore water samples were collected using Rhizon soil moisture samplers (MOM19.21.21 Rhizon, Holland) on days 1, 3, 5, 7, 10 and 15 and then once every 10 days until day 115. The plants and rhizosphere soils were collected on days 15, 45, 65, 75 and 120 to represent the seedling, tillering, jointing, heading, and maturation stages, respectively, and the rhizosphere soil was also sampled on day 5 to monitor any rapid change in soil properties after flooding.

1.3. Analytical methods

All the samples of soils, pore water, plants and rice were collected for chemical and biological analysis. The detailed methods of sample preparation and measurements were described in Appendix A Section S2. Specifically, the chemical analyses included the concentrations of Dis-Fe(II) (dissolved Fe(II))/TFe (dissolved total Fe) and Dis-Sb(III)/Sb(V)/TSb in pore water; the Sb contents in different tissues of rice plants; the Sb partitioning in rhizosphere soils; the dissolved sulfate (Dis-SO_4^{2-}), acid-volatile sulfur (AVS), HCl-extracted Fe(II) (HCl-Fe(II)) and oxalate-extracted Fe (Oxalate-Fe) in rhizosphere soils. The biological analyses included quantification and clone library of *aoxB* genes (encoding the As(III) oxidase catalytic subunit). All experimental data are shown as the means \pm SDs ($n = 3$), and statistical analyses of the experimental data were performed with SPSS® 22.0 (SPSS, USA) software. The statistical significance of the differences between different treatments were determined by analysis of variance with $P < 0.05$.

2. Results

2.1. Effects of NO_3^- on Sb accumulation in plants

As shown in Fig. 1a, the addition of NO_3^- significantly reduced the Sb contents in brown rice and husk by 15.6% and 17.1%, respectively, compared to those in the control. As shown in Fig. 1b and c, the Sb accumulation in plants (roots and parts above roots) on day 120 was significantly decreased by 38.8% in parts above roots and by 46.1% in roots compared with that in the control. However, the variation in the Sb accumulation in rice plants differed with and without nitrate. The Sb content in the control increased over time during the entire growth period. However, the Sb content in the NO_3^- treatment

on day 45 increased to 1.55 and 6.58 mg/kg in the parts above root and in the roots, respectively, then decreased, eventually becoming lower than the contents in the control at the mature stage.

2.2. Effects of NO_3^- on Sb species in pore water

As shown in Fig. 2a, the Dis- NO_3^- concentration in the NO_3^- treatment continuously declined over time, and the NO_3^- concentration (95.7 mg/L) on day 45 was even lower than the maximum concentration of Dis- NO_3^- (136.4 mg/L) in the control, indicating that microbial denitrification was stimulated by the addition of nitrate. The results in Fig. 2b show that the dissolved total Sb in pore water (Dis-TSb) in the control increased to the peak concentration (1.21 mg/L) on day 15 and then decreased gradually to 0.01 mg/L on day 115. After amending with nitrate, Dis-TSb increased with the maximum concentration (2.06 mg/L) on day 15, indicating that nitrate could substantially enhance the mobility of Sb in soils. The Dis-TSb in pore water included dissolved Sb(V) (Dis-Sb(V)) and dissolved Sb(III) (Dis-Sb(III)). The concentrations and kinetics of Dis-Sb(V) in Fig. 2c were very similar to those of Dis-TSb, while the concentration of Dis-TSb in control was 2.5 times that of Dis-Sb(III) on day 25, demonstrating that Dis-Sb(V) was the major form of Sb in the pore water. In NO_3^- treatment, Dis-Sb(III) increased before day 5 but decreased then dramatically to 0.03 mg/L on day 35. On day 15, compared to the control, the Dis-Sb(III) concentration with NO_3^- decreased by 0.43 mg/L, indicating the occurrence of Sb(III) oxidation. There was no significant correlation between the Dis-TSb concentrations and Sb contents in rice plants (Appendix A Fig. S2a and b), suggesting that the Sb accumulation in rice plants was not determined by the dissolved Sb in pore water.

2.3. Effects of NO_3^- on Sb partitioning in soils

The total Sb (TSb) in rhizosphere soil was extracted with different extractants ($(\text{NH}_4)_2\text{SO}_4$, $\text{NH}_4\text{H}_2\text{PO}_4$, oxalate, and oxalate + ascorbic acid). In the control, both $(\text{NH}_4)_2\text{SO}_4$ -TSb and $\text{NH}_4\text{H}_2\text{PO}_4$ -TSb increased over the entire growth period (Fig. 3a and b), while oxalate-TSb and oxalate + ascorbic acid-TSb decreased gradually (Fig. 3c and d). With NO_3^- treatment, $(\text{NH}_4)_2\text{SO}_4$ -TSb and $\text{NH}_4\text{H}_2\text{PO}_4$ -TSb increased before day 45 and then decreased gradually, eventually reaching levels 22.3% and 31.4% lower than those in the control on day 120. Oxalate-TSb decreased substantially before day 45 and then reached a plateau.

The variation in oxalate + ascorbic acid-TSb with nitrate was almost the same as that without nitrate, but the TSb content was always higher than that in the control. $(\text{NH}_4)_2\text{SO}_4$ -TSb and $\text{NH}_4\text{H}_2\text{PO}_4$ -TSb were consistent with the Sb content in rice plants (Fig. 1b and c), and the correlation analysis demonstrated that $(\text{NH}_4)_2\text{SO}_4$ -TSb and $\text{NH}_4\text{H}_2\text{PO}_4$ -TSb were linearly correlated with the Sb content in rice plants (Appendix A Fig. S2c–f). Since the exchangeable/adsorbed forms of soil heavy metals are generally regarded as the main indicator of heavy metal accumulation in crops (Ren et al., 2014), these

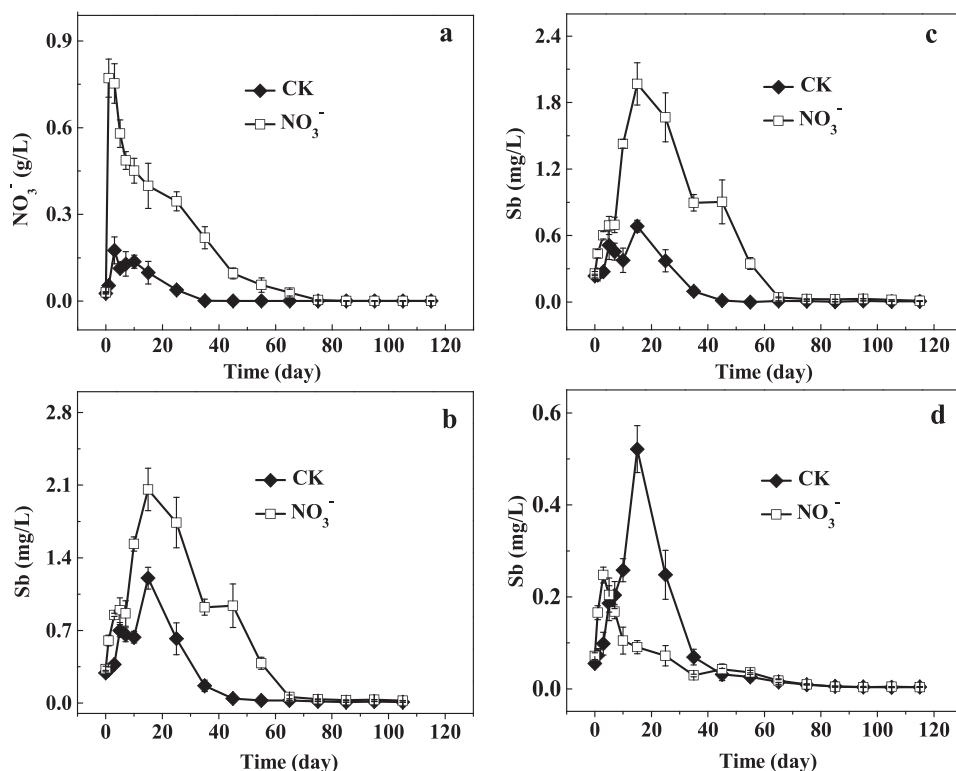


Fig. 2 – Concentrations of (a) Dis- NO_3^- , (b) Dis-TSb, (c) Dis-Sb(V), and (d) Dis-Sb(III) in pore water in the NO_3^- treatment during the entire rice lifecycle. The data are the means \pm SDs ($n = 3$).

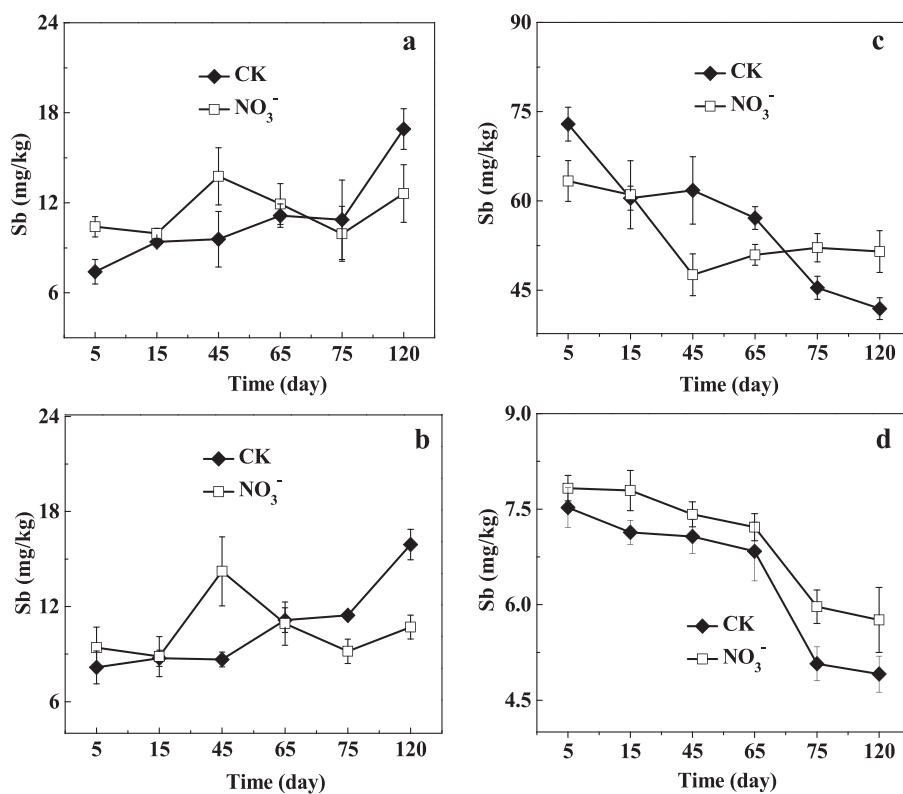


Fig. 3 – Contents of (a) $(\text{NH}_4)_2\text{SO}_4$ -TSb, (b) $\text{NH}_4\text{H}_2\text{PO}_4$ -TSb, (c) oxalate-TSb and (d) oxalate + ascorbic acid-TSb extracted from rhizosphere soils in the NO_3^- treatment over the entire rice lifecycle. The data are the means \pm SDs ($n = 3$).

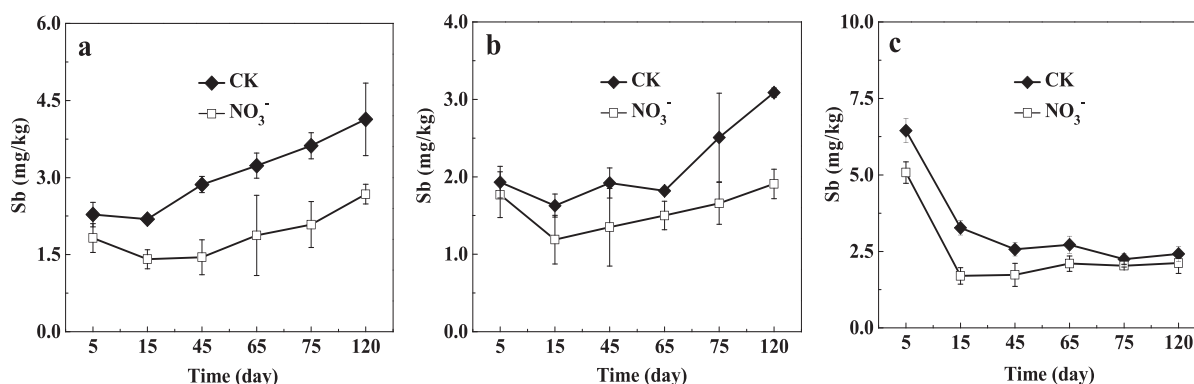


Fig. 4 – Contents of (a) $(\text{NH}_4)_2\text{SO}_4\text{-Sb(III)}$, (b) $\text{NH}_4\text{H}_2\text{PO}_4\text{-Sb(III)}$ and (c) oxalate-Sb(III) extracted from rhizosphere soils in the NO_3^- treatment over the entire rice lifecycle. The data are the means \pm SDs ($n = 3$).

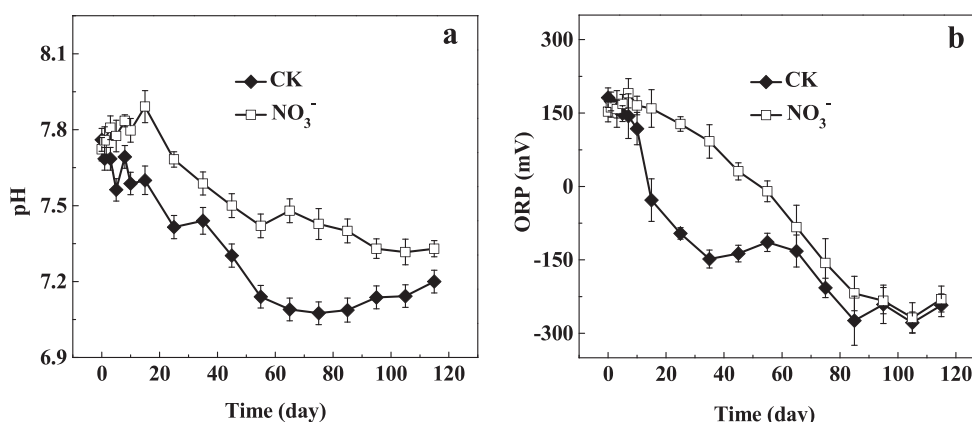


Fig. 5 – pH (a) and ORP (b) in rhizosphere soils under NO_3^- treatment over the entire rice lifecycle. The data are the means \pm SDs ($n = 3$).

results indicated that $(\text{NH}_4)_2\text{SO}_4\text{-TSb}$ and $\text{NH}_4\text{H}_2\text{PO}_4\text{-TSb}$ could well reflect the bioavailability of Sb to rice plants.

As shown in Fig. 4, during the growth period, $(\text{NH}_4)_2\text{SO}_4\text{-Sb(III)}$ and $\text{NH}_4\text{H}_2\text{PO}_4\text{-Sb(III)}$ increased gradually, while oxalate-Sb(III) decreased substantially in the first 15 days and then reached a plateau. Compared with the control treatment, the addition of NO_3^- substantially reduced all three forms of extracted Sb(III). Specifically, $(\text{NH}_4)_2\text{SO}_4\text{-}$, $\text{NH}_4\text{H}_2\text{PO}_4\text{-}$ and oxalate-Sb(III) eventually decreased by 35.2%, 38.2% and 12.5%, respectively. Hence, the presence of NO_3^- could definitely promote the oxidation of various Sb(III) species in soil.

2.4. Effects of NO_3^- on soil pH/ORP and Fe/S transformation

During the entire growth period, both pH and ORP decreased (Fig. 5). For example, the pH decreased from the initial value of 7.8 to the lowest value of ca. 7.1, and the soil ORP decreased from 160 mV to the most negative value of ca. -300 mV. The addition of NO_3^- significantly elevated the soil pH and ORP. Specifically, the highest pH caused by the NO_3^- treatment was 7.89 on day 15, and the maximum difference in ORP between the control and NO_3^- treatments was 240.3 mV on day 35.

From Fig. 6, the four forms of total iron and Fe(II), including Dis-TFe and Dis-Fe(II) in pore water and HCl-Fe(II) and oxalate-TFe extracted from rhizosphere soils, increased over the entire growth period, indicating a biogeochemical process in which iron-bearing minerals were continuously reduced, dissolved and converted to amorphous forms in flooded paddy soil. The addition of nitrate substantially reduced the four forms of total iron and Fe(II). For instance, Dis-

TFe and Dis-Fe(II) on day 115 in the NO_3^- treatment decreased by 37.1 and 29.7 mg/L, respectively. Compared with those in the control, the HCl-Fe(II) and oxalate-TFe on day 65 in the NO_3^- treatment decreased by 89.5% and 24.3%, respectively. These results confirmed that the addition of NO_3^- dramatically decreased the dissolution of iron-bearing minerals in soils by inhibiting Fe(III) reduction and promoting Fe(II) oxidation.

In addition, NO_3^- also decreased the acid-volatile sulfur (AVS)-S contents (Fig. 7). At the mature stage (day 120), NO_3^- decreased the content of AVS-S by 42.5% compared to that in the control. This could suppress the attenuation of $\text{SO}_4^{2-}\text{-S}$, resulting in weak reduction of SO_4^{2-} to $\text{S}^{2-}/\text{S}_2^{2-}$. Furthermore, NO_3^- decreased the H_2O_2 -extracted organic-bound Sb, as shown in Appendix A Fig. S3a. At the tillering stage (day 45), NO_3^- caused the maximum decrease in organic-bound Sb of 41.0%. Additionally, the correlation analysis demonstrated that the H_2O_2 -Sb contents were linearly correlated with the AVS-S contents (Appendix A Fig. S3b), which is similar to previous reports that soil sulfide is an important factor affecting the organic-bound forms of heavy metals (Jong and Parry, 2004).

2.5. Effects of NO_3^- on functional microorganisms and genes

Anaerobic Sb(III) oxidation in soil is generally considered a microbially mediated biogeochemical process (Li et al., 2016), and NO_3^- could serve as an electron acceptor (Achnich et al., 1995). Despite the lack of investigation on the characteristic genes of microbial Sb(III) oxidation, the As(III)-oxidizing gene (*aioA*) was observed to play an important role in Sb(III) oxidation (Nguyen et al., 2017);

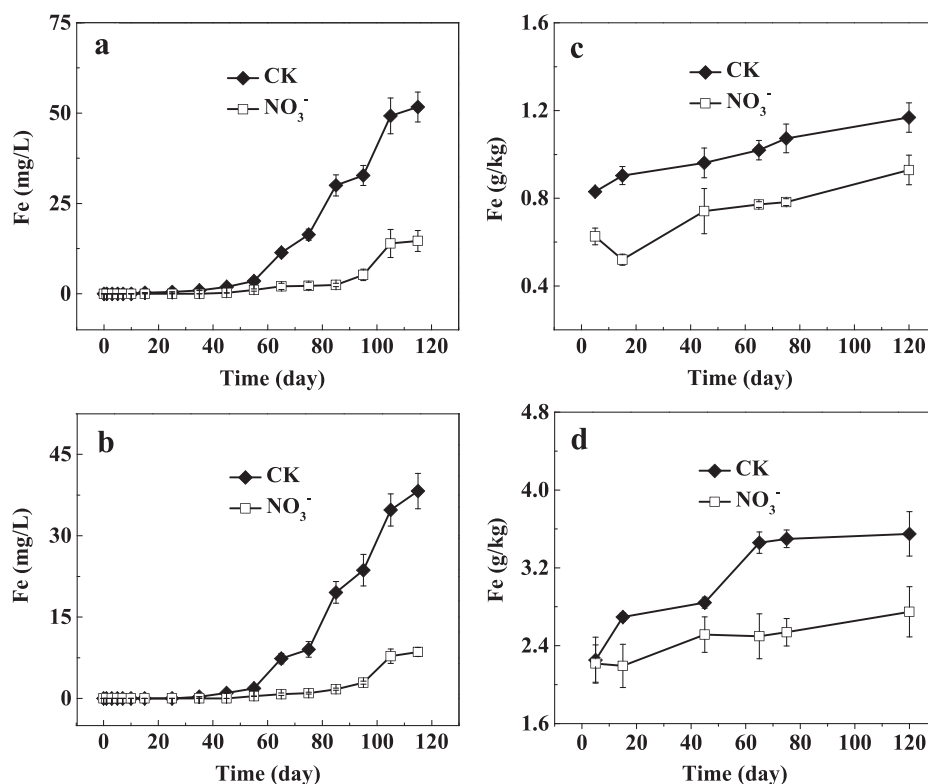


Fig. 6 – Contents of (a) Dis-TFe, (b) Dis-Fe(II) in pore water and (c) HCl-Fe(II), (d) oxalate-TFe extracted from rhizosphere soils from the NO₃⁻ treatment over the entire rice lifecycle. The data are the means \pm SDs ($n = 3$).

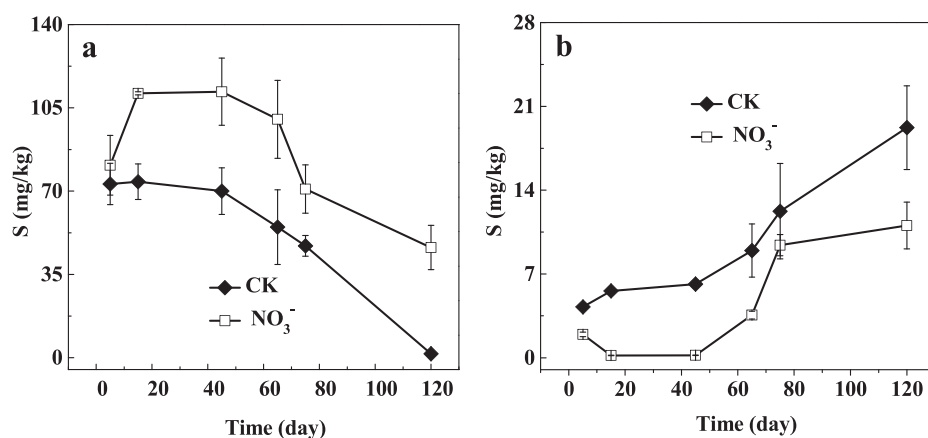


Fig. 7 – Contents of S-SO₄²⁻ (a) and S-AVS (b) extracted from rhizosphere soils from the NO₃⁻ treatment during the entire rice lifecycle. The data are the means \pm SDs ($n = 3$).

thus, the *aoxB* gene for As(III) oxidation (Heinrich-Salmeron et al., 2011; Hu et al., 2015) was considered a suspected functional gene for microbial Sb(III) oxidation. The results in Fig. 8a show that the copy numbers of the *aoxB* gene increased over time in the control and NO₃⁻ treatments, and NO₃⁻ clearly increased the *aoxB* copy numbers by 54.8% to 92.7% compared with those in the control. From Fig. 8b, the major microbial community members containing *aoxB* genes included *Curvibacter*, *Kaistia*, *Burkholderia*, and *Aminobacter* spp., with relative abundances $\geq 5\%$. On days 45 and 75, the relative abundances of *Curvibacter* and *Kaistia* spp. decreased with the addition of nitrate, but the relative abundances of *Burkholderia* and *Aminobacter* spp. were enhanced by NO₃⁻.

The Simpson index and principal component analysis (PCA) results in Appendix A Fig. S4 demonstrated that the α -diversity of

Sb(III)-oxidizing microorganisms decreased with nitrate amendment before complete nitrate reduction on day 75, and the Sb(III)-oxidizing microbial community in the nitrate treatment group was significantly different from that in the control group. Moreover, we constructed a phylogenetic tree from the *aoxB* gene sequences with the top 10 OTUs in terms of relative abundance in the control and NO₃⁻ treatments (Appendix A Fig. S5) and found that these top 10 OTUs were closely related to well-known As(III) oxidizers, such as *Betaproteobacteria* bacterium RIFCSPLOWO2_02_FULL_62_17, *Ralstonia* syzygii R24, and *Limnobacter* sp. 83, suggesting that the detected microbial community members containing *aoxB* genes should be able to oxidize As(III).

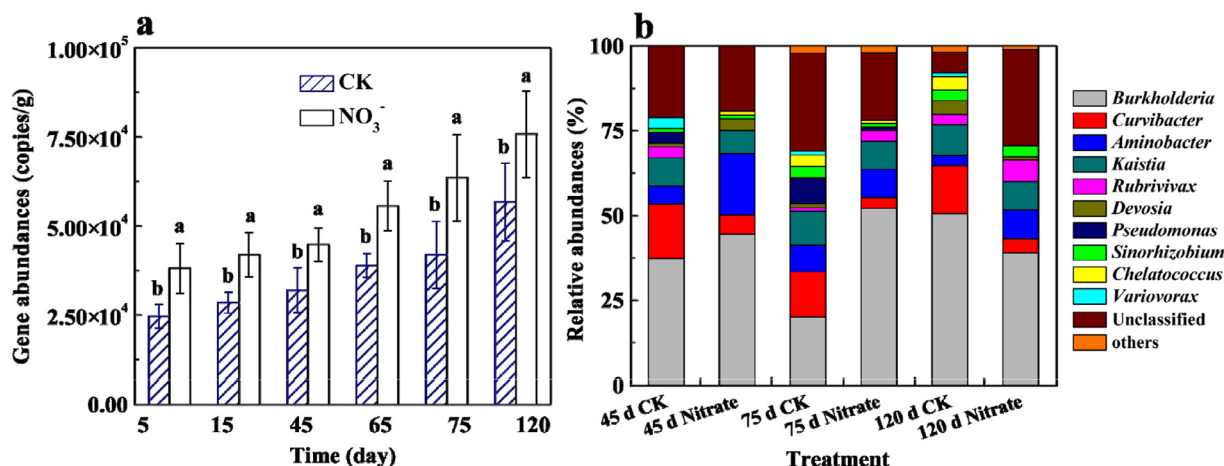


Fig. 8 – Abundances (a) and diversities (b) of Sb(III) oxidation-related functional microorganisms in the rhizosphere soils under NO_3^- treatment over the entire rice lifecycle; where the letters of a and b in Fig. 8a are significant difference labels. The data are the means \pm SDs ($n = 3$). 45 d CK, 75 d CK and 120 d CK mean the rhizosphere soils in the control sampled on day 45, 75 and 120; 45 d Nitrate, 75 d Nitrate and 120 d Nitrate mean the rhizosphere soils in NO_3^- treatment sampled on day 45, 75 and 120.

3. Discussion

3.1. Possible reactions in the paddy soil-rice plant system with Sb and nitrate

The Sb accumulation in rice is mainly determined by the available Sb in soils, so it is necessary to clearly illustrate the underlying processes that could influence Sb availability. Anoxic conditions were maintained in the flooded paddy soil, so the available Sb might have been influenced by anaerobic reactions, such as reductive dissolution of Fe(III)-bearing minerals, biological sulfate reduction, and subsequent pH/ORP variations, which might have directly influenced the Sb speciation in soils. After amendment with nitrate, microbial denitrification occurred, which could be coupled with Fe(II) oxidation (Liu et al., 2019b) and might also inhibit Fe(III) reduction and sulfate reduction, so Sb transformation might interact with the cycling of Fe and S, resulting in changes in Sb availability and subsequent Sb accumulation in rice. Specifically, Sb mobilization could occur via the bioreduction of Fe-Sb-bearing minerals, the oxidation of sulfide and Sb(III), and the elevation in pH, while Sb immobilization could be attributed to the oxidation of Fe(II) coupled with nitrate reduction, the inhibitory effect of nitrate on iron bioreduction, and the enhanced generation of green rust by sulfide oxidation. Hence, multiple effects should be considered as factors influencing the Sb transformation and accumulation in rice, and these effects could positively or negatively influence Sb availability in soils and accumulation in rice. Since the Sb bioavailability with nitrate amendment changed substantially over the entire growth period, the dominant mechanisms are discussed separately according to the occurrence of biotic-abiotic reactions at different growth stages.

3.2. Mechanisms of NO_3^- enhancing Sb bioavailability before the tillering stage

During the initial period from day 5 to 15 before the tillering stage, NO_3^- could be gradually reduced via denitrification. Since the redox potential of nitrate is much more positive than that of iron, nitrate reduction was more preferential than iron reduction, resulting in the reductive dissolution of iron-bearing minerals in soil not being obvious at this stage. Sb(III) was biologically oxidized to more mobile Sb(V) (Guo et al., 2014) when NO_3^- served as an electron acceptor in the anaerobic flooded soil (Nguyen et al., 2017). The AVS decreased due to biological oxidation of sulfides coupled with nitrate reduc-

tion (Hayakawa et al., 2013; Marzocchi et al., 2014), resulting in Sb release from organic-bound forms. Because protons are consumed via denitrification (van Rijn et al., 2006), the soil pH increased after nitrate addition, and the more active Sb(V) could be released at high pH because Sb(V) generally exists as electronegative $\text{Sb}(\text{OH})_6^-$ in soil (Herath et al., 2017). Therefore, the maximum Dis-TSb in pore water appeared in the NO_3^- treatment on day 15. From day 15 to 45, the obvious iron reduction in the presence of nitrate at day 45 was 30 days later than that in the control (day 15). Although the low ORP was favorable for iron reduction, the inhibitory effect of nitrate on the bioreduction of Fe-Sb-bearing minerals could have resulted in the low mobilization of Sb in soils. The soil pH decreased, and the rate of Sb(III) oxidation to Sb(V) slowed; thus, the second peak in Dis-TSb should be mainly from released reduced Fe(III)-bearing minerals. Moreover, the decrease in soil pH was favorable for Sb adsorption, resulting in an increase in adsorbed TSb in soils and a decrease in dissolved Sb in pore water.

3.3. Mechanisms of NO_3^- reducing Sb bioavailability in the later stage

After day 45, the decrease of dissolved NO_3^- concentration in the NO_3^- treatment caused the disappearance of microbially mediated Fe(II)/Sb(III) oxidation coupled with nitrate reduction, and subsequently resulted in the decrease of Sb bioavailability. With the decrease in soil ORP in the NO_3^- treatment after day 45, the microbial reduction of Sb(V) (Nguyen and Lee, 2014) could be enhanced, and an increase in $(\text{NH}_4)_2\text{SO}_4$ -/ $\text{NH}_4\text{H}_2\text{PO}_4$ -oxalate-Sb(III) was observed. High sulfate and high pH were found in the NO_3^- treatment, so the newly formed Fe(II) and sulfate under this high pH might have generated green rust ($\text{Fe}_4^{\text{II}}\text{Fe}_2^{\text{III}}(\text{OH})_{12}\cdot\text{SO}_4\cdot 3\text{H}_2\text{O}$) (Ona-Nguema et al., 2002), which can chemically reduce Sb(V) to Sb(III) (Mitsunobu et al., 2008; Li et al., 2016). Since Sb(III) is more easily fixed by iron minerals than Sb(V) (Guo et al., 2014; He et al., 2015; Qi and Pichler, 2017), the chemical and biological reduction of Sb(V) to Sb(III) can enhance Sb immobilization by active iron minerals (mainly amorphous and poorly crystallized iron oxides) in soil, and consequently, the bioavailability of Sb was gradually decreased in the later stage of rice growth (after day 45). Moreover, due to the inhibition by NO_3^- of the reduction and dissolution of Fe(III)-bearing minerals in the first 45 days, more iron minerals were retained to re-immobilize more Sb, so the NO_3^- ultimately resulted in lower contents of TSb in both adsorbed forms and in rice plants than those in the control.

3.4. Mechanisms of microbially mediated Sb transformation in the NO_3^- treatment

Although the observed oxidation of Sb(III) with NO_3^- in soil was considered a microbially driven biochemical process (Nguyen and Lee, 2015; Li et al., 2016), the knowledge of functional microorganisms capable of oxidizing Sb(III) is still limited, and the functional genes involved in Sb(III)-oxidizing biochemical processes are unclear. Fortunately, the following information is known: (1) As and Sb have similar ORPs (Wilson et al., 2010); (2) Functional microorganisms may use similar metabolic pathways for As and Sb transformations (Sun et al., 2017); (3) Some bacteria, such as *Agrobacterium tumefaciens* (Lehr et al., 2007) and *Hydrogenophaga taeniospiralis* strain IDSBO-1, can drive the microbial oxidation of Sb(III) and As(III) (Terry et al., 2015); (4) The arsenite oxidase gene *aoxA* also potentially plays an important role in anaerobic Sb(III) oxidation (Li et al., 2019). The δ -Sb(V) (total Sb(V) content extracted by $(\text{NH}_4)_2\text{SO}_4/\text{NH}_4\text{H}_2\text{PO}_4/\text{oxalate}$ in the NO_3^- treatment minus that in the control) and δ -Gene *aoxB* (*aoxB* abundance in the NO_3^- treatment minus that in the control) had a significant positive correlation (Appendix A Fig. S6b), suggesting that the arsenite oxidase marker gene *aoxB* was indeed related to the oxidation of Sb(III) to Sb(V).

Furthermore, *Burkholderia*, *Curvibacter*, *Aminobacter* and *Kaistia* were identified as the main functional genera of the potential Sb-oxidizing microbial community with the *aoxB* gene. *Burkholderia* genera are major As(III) oxidizers (Slyemi et al., 2007), and Sb(III) oxidation has also been attributed to *Burkholderia* spp., which are Betaproteobacteria *Curvibacter* spp., important Fe(II) oxidizers in the groundwater environment (Gülay et al., 2018; Tong et al., 2019), have been reported to be genetically similar to Sb(III)-oxidizing microbes (Terry et al., 2015). The genus *Aminobacter* is widely distributed in terrestrial environments (McDonald, 2005) and can also oxidize Sb(III) (Shi et al., 2013). *Kaistia* is putatively As-resistant (Zecchin et al., 2019), but its relationship with As(III)/Sb(III) oxidation has not been reported. Since NO_3^- increased the relative abundance of the genera *Burkholderia* and *Aminobacter*, which were reported to have the abilities to reduce NO_3^- or use NO_3^- as a respiratory electron acceptor (Labbe, 2004; Levy et al., 2008; Kondo et al., 2009; Rusch et al., 2015), NO_3^- reduction may be coupled with Sb(III) oxidation, even though the molecular mechanism was still suspended.

3.5. Environmental and practical implications

In this study, NO_3^- eventually decreased the mobility of Sb and reduced the Sb accumulation in rice plants, so NO_3^- can be considered an environmentally friendly amendment for simultaneous fertilization and remediation of Sb-contaminated paddy soils. Since the nitrate-triggered Sb immobilization was tightly related to the iron-bearing minerals, the nitrate fertilizers would be better applied in iron-rich paddy soils. However, the risks of applying NO_3^- should also not be ignored, as it can raise the soil pH and oxidize Sb(III) to Sb(V) to enhance the mobility of Sb during the middle stages of rice growth. The excessive application of NO_3^- may cause high soil pH and long-term inhibition of the biochemical reduction of Sb(V) and lead to an increase in Sb bioavailability. Based on this finding, the strategy of using nitrate fertilizers in paddy soil should be carefully conducted in the practical application, so for future studies, it would be necessary to investigate the appropriate stage for applying nitrate during the whole growth period. Furthermore, it should be noted that when applying NO_3^- in alkaline Sb-contaminated soil because (1) the NO_3^- can elevate soil pH via denitrification, which could further enhance the mobility of Sb and (2) the main Sb form (Sb(V)) would be more easily activated by OH^- in alkaline soil. Accordingly, since the pH (7.75 ± 0.15) in this experimental soil was higher than that (6.8 ± 0.1) in the soil investigated by Wang et al. (2019), the remedial effect of NO_3^- on Sb bioavailability in our study is slightly weaker than that in the study conducted by Wang et al. (2019). Therefore, the application of nitrate is a potential strategy for decreasing Sb accumulation in rice with consideration of important parameters, including the soil pH, nitrate concentration, and growth stage.

4. Conclusions

The amendment of nitrate alleviated the Sb accumulation in brown rice eventually, though the Sb bioavailability increased in the initial period. The denitrification of NO_3^- enhanced Sb mobility by stimulating the oxidation of Sb(III)/sulfides and elevating the soil pH. After NO_3^- was consumed, Sb(V) was reduced to Sb(III) by functional microbes and newly generated green rust, so the Sb bioavailability decreased due to the strong affinity between Sb and iron oxides. Due to the inhibition of Fe(III) reduction by NO_3^- , less Sb was released from Fe-Sb-bearing minerals, eventually resulting in lower Sb accumulation in the NO_3^- treatment than in the control. Therefore, the multiple effects of nitrate on the Sb bioavailability have been clarified comprehensively over the entire lifecycle of rice, and it would be essentially investigating the Sb speciation in soils and rice plants by using the advanced characterization techniques in the future studies. The study provides new insights in understanding Sb biogeochemical behavior in paddy fields and could be used to develop a practical strategy for reducing Sb contamination in soil-rice system with environmental-friendly nitrate fertilizers.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.07.009.

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