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Enhancing nitrobenzene biodegradation in aquatic systems: Feasibility of using plain soil as an inoculant and effects of adding ascorbic acid and peptone

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HIGHLIGHTS

- Soil is feasible to inoculate the system for enhanced nitrobenzene biodegradation.
- Both reducer and substrate are critical for stimulating nitrobenzene biodegradation.
- The reducer would offer electron donors and stimulate/inhibit biomass growth.
- Nitrobenzene degradation follows pseudo first-order kinetics as a function of biomass and reducer concentration.
- Nitrobenzene was transformed to aniline and finally mineralized in the system.

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ABSTRACT

Nitrobenzene (NB) is recalcitrant to microbial biodegradation due to the electron-deficient character of the nitro group (NO^{2-}). Prior work has found that the reductant could enhance NB biodegradation by providing excess electron donors. However, the existing theory couldn't explain the increase-and-decrease pattern of the NB biodegradation rate with an increase in a reductant concentration. Our results suggest that the reductant affects NB biodegradation by two mechanisms: the available electron donors and the stimulation or inhibition of biomass growth, which are linked by a pseudo-first-order reaction kinetics. In addition, the results showed that directly inoculating the plain soil into the aquatic system and then allowing the synergistic effect of the organic reductant (ascorbic acid) and the substrate (peptone) enhance NB biodegradation. Employing the new method, 200 mg L^{-1} NB was transformed in 72 h. GC–MS analysis detected two novel intermediate metabolites, indicating that NB was degraded into aniline and further transformed into acetanilide and 9-octadecenamide before its mineralization. This study sheds light on how to exploit the synergistic effects of the availability of excess electron donors and biomass growth by controlling the reductant and a substrate in the right concentration range (e.g., ascorbic acid < 0.8 mgL^{-1} + peptone).

1. Introduction

Nitrobenzene (NB), as a model nitroaromatic compound, has been causing increased water pollution in the world because of its extensive use in chemical syntheses of drugs, herbicides, dyes, and other industrial chemicals for many years (Liu et al., 2012). Due to the electron-deficient character of NB's nitro-group, it is difficult for indigenous bacteria to degrade NB directly (Wang et al., 2011; Huang et al., 2012). Therefore, bioremediation of NB is often carried out by inoculation of pure culture or mixed microbial communities (Megharaj et al., 2011). In previous studies, activated sludge was widely used to inoculate the reactor system for enhancing NB biodegradation (Wang et al., 2014; Zhang et al., 2015). However, activated sludge usually contains a large number of pollutants including persistent organic pollutants and heavy metals (Dalzell et al., 2002; Xu et al., 2017), which may affect the performance of NB reduction in the system inoculated with activated sludge.

Recently, Li et al. (2017a, 2017b) established a novel approach to

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stimulate biodegradation of soil contaminated with 4-chloronitrobenzene (4NCB) or NB. The method is to inoculate the soil reactor with pure culture and add an organic reductant, e.g., ascorbic acid (= a naturally occurring form of vitamin C, VC) and an organic nitrogen source (peptone). Completely removal of 1 g-4NCB kg^{-1} soil was observed within 8 days in the soil batch reactors. It is unknown whether this method (i.e., using pure culture as inoculant + adding the reductant and substrate for synergistic stimulation of bioremediation, which is called Li's approach hereafter) can be extended by using a plain soil, instead the strains isolated from the soil, to inoculate the remediation system. By intuition, due to a large number of microorganisms in the soil, there must exist soil microorganisms with NB biodegradation ability (Li et al., 2017a). Therefore, we hypothesize that Li's approach could be used by inoculating the soil into the system for enhancing NB-biodegradation in aquatic systems. To test this hypothesis, it is important to test different reductants and to screen the best one for effectively enhancing NB biodegradation ability.

A large number of previous studies demonstrated that reductants s could enhance the degradation rate of nitroaromatic compounds (Klupinski et al., 2004; Hofstetter et al., 2006; Naka et al., 2006; Colón et al., 2008; Chen et al., 2016). It was reported that the enhanced NB reduction was dependent on the availability of excess electron donors, and there were linear free energy relationships for the biotic and abiotic reduction of nitroaromatic compounds (Luan et al., 2010, 2015). At present, it is widely accepted that the reductant effect on enhancement of nitroaromatic compounds biodegradation is mainly due to the electron donating property of the reductants. However, sufficient information is not available on whether the availability of excess electron donors is the only mechanism for reductants to enhance biodegradation. Such knowledge is critical as the reductant in the environment could have a variety of chemical properties and structures under different conditions, which, in total, may trigger different mechanisms for NB biodegradation.

In light of the above analysis, we conducted the present study with the following objectives: 1) to verify whether inoculation of a plain soil into the aquatic system could effectively enhance biotreatment of NB-contaminated water by Li's approach; 2) to investigate the effects of reductant on NB biodegradation with or without substrate in mineral salt medium (MSM) (i.e., to discover the other possible mechanism for the reductant to affect NB biodegradation); and 3) to disclose the possible NB biodegradation pathway involved in the systems used in this study.

2. Materials and methods

2.1. Soil and strain

Three distinct types of arable soil were obtained from different sites for cultivation of corn, vegetable, or meadow of Chongqing, P. R. China (Table 1). All samples were taken from the upper 5 cm of the site and stored in glass bottles at 4 °C for no more than 2 days before processing. Table 1 shows the properties of the three kinds of soil samples.

The soil samples were used as the inoculant in the majority of the tests in this study (see below). In some (control) tests, the pure culture of bacterial strain, *staphylococcus carnosus* lihwang S12 (NCBI accession no. KM093862) (called strain S12 thereafter) was also used as the inoculant for the comparison purpose. Strain S12 was isolated from the same soil in previous studies and routinely maintained in Luria-Bertani medium (LB) (Li et al., 2017b). In this study, the pure culture of strain S12 was grown in LB broth at 30 °C for 24 h and then centrifuged at 6000 rev. min^{-1} (rpm) for 10 min; the settled bacteria strain was washed twice for full resuspension and the used as the inoculant in some (control) tests.

2.2. Experimental design

Table 2 shows experimental design of the batch tests (sets 1 to 5). All these batch tests were conducted using 250 mL batch reactors (glass bottles) filled with 50 mL of reaction solution and placed in a rotary shaker (DDHZ-300, Taicang Experiment Equipment Factory, Jiangsu, China) at 28 °C and 180 rpm. As the first stage, sets 1 to 3 were tested to screen the optimal reductant with or without the addition of a substrate (1% peptone, w/w). In sets 2 and 3, all of the 50 mL reaction solutions contained 200 mgL^{-1} NB, 1 gL^{-1} the soil (i.e., 0.05 g soil in 50 ml test solution) and 0.4 gL^{-1} reductant. In the second stage (tests related to sets 4 and 5), effects of reductant concentration [e.g., ascorbic acid (VC) = 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 gL^{-1})] were determined on NB biodegradation and the value of OD_{600} in MSM with (set 4) or without (set 5) peptone. In sets 4 and 5, the 50 mL of reaction solution in 250 mL bottles contained 200 mgL^{-1} NB and 0.05 g soil (or 1 ml of MSM with suspended pure strain S12).

Two justifications for designing the tests shown in Table 2 should be noted here. First, our previous studies indicate that the addition of a nitrogen source (e.g., peptone) serves as the obligatory substrate to enhance nitroaromatic removal by increasing the strains' biomass [9]. Adding an additional carbon source had a negligible impact on the strains' growth and removal of 4NCB or NB [9, 10]. Thus, peptone was used as the sole additional substrate (to provide nitrogen source) in the study. Second, biodegradation of nitroaromtic compounds could be enhanced with an increase in reductant concentration. Luan et al. (2015) have demonstrated that the rate enhancement of NB biodegradation was dependent on the availability of excess electron donors (reductant). However, information is not available on whether and how an increase in organic reductant concentration (e.g., VC) would enhance NB biodegradation in the system inoculated with plain soil. The experimental design of the optimal peptone concentration was as follows: 1) all of the 50-mL reaction solutions contained 200 mgL^{-1} NB and 1 gL^{-1} the soil (i.e., 0.05 g soil in 50-mL test solution); 2) effects of reductant concentration (e.g., peptone = 0.1, 0.5, 2, 5, 10, and 20 gL^{-1}) on the NB residue concentration and the *OD* after 24 h were determined.

2.3. Analytical methods

For tests of sets 1 to 5, the densities of the indigenous

Table 1
Soil characteristics.

Parameters	Soil 1	Soil 2	Soil 3
pH	7.6	7.2	7.7
Moisture content	16%	17%	14%
Total organic carbon	2.3%	2.1%	2.5%
Total nitrogen	0.094%	0.131%	0.119%
Site condition/location	The vegetable garden of Chongqing	The cornfield of Chongqing	The meadow soil of Chongqing

Table 2
Experimental sets and conditions.

	Peptone	Reductant	Nitrobenzene	Experimental purpose
Set 1	−	−	+	testing nitrobenzene biodegradation by indigenous microorganisms from soils 1, 2, or 3; results were used as a benchmark (control test)
Set 2	−	+	+	testing nitrobenzene biodegradation by indigenous microorganisms from soils 1, 2, or 3 under the influence of adding one of the 4 reductants
Set 3	+	+	+	testing nitrobenzene biodegradation by indigenous microorganisms from soils 1, 2, or 3 under the influence of adding one of the 4 reductants and one substrate (peptone)
Set 4	−	+	+	testing nitrobenzene biodegradation by indigenous microorganisms from the soil 1 under the influence of reductant concentration
Set 5	+	+	+	testing nitrobenzene biodegradation by strain S12 (as a control test) and associated OD_{600} or by indigenous microorganisms from the soil 1 under the influence of reductant concentration and one substrate (peptone)

Note: All of the 50 mL reaction solutions contained: 1) 0.05 g soil for sets 1 to 5 or 1 ml of mineral salt medium (MSM) with re-suspended strain S12 for the control test of set 5; and 2) 50 ml MSM. + or - represents with or without the addition of substance. In set 2, reductant represents 0.01 g $FeSO_4$ or 0.01 g vitamin C (VC) (= ascorbic acid, $C_6H_8O_6$, a naturally occurring form of VC) or 0.01 g mannitol ($C_6H_{14}O_6$) or 0.01 g glucose ($C_6H_{12}O_6$)·In set 3, substrate represents 1% peptone (W/W), and reductant represents 0.02% $FeSO_4$ or 0.02% VC or 0.02% mannitol or 0.02% glucose (W/W). In Sets 4 and 5, substrate represents 1% peptone (W/W), and reductant concentrations (in gL^{-1}) were 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 VC (W/W). The concentration of nitrobenzene was 200 mg L^{-1} for all sets 1−5. Peptone was purchased from Aoboxing Bio-tech Co., Ltd. (Beijing, China).

microorganisms (OD_{600}) and the NB concentrations were measured after 72 h. To analyze the NB and aniline degradation pathway, 0.5 g soil was inoculated in 50 ml MSM with 0.02% VC, 1% peptone, and 200 mgL^{-1} NB or 200 mgL^{-1} aniline to make the reaction solution, which was then placed into the 250 ml bottles for cultivation (in the rotary shaker at 28 °C and 180 rpm). After 0, 24, 36, 48, and 96 h of cultivation, 50 ml culture was centrifuged at 5000 rpm for 20 min, and the supernatant was transferred to a 125-ml separating funnel for extraction of the metabolites for three times. The extracts were condensed to 1 ml via a rotary evaporator, transferred to 1-ml Agilent bottles, and then stored at 4 °C for GC/MS analysis. The concentrations of NB were measured using high-performance liquid chromatography (HPLC) (Agilent 1260, Wilmington, DE, USA) equipped with an Agilent Extend-C18 column (150 mm × 4.6 mm). The analyses were performed with a flow of methanol/water (v/v, 7:3) at a rate of 1.0 ml min^{-1}. The bacterial cell density was measured as OD_{600} (UV1000 model; Shanghai Tianmei Scientific Instruments Co., Ltd., Shanghai, China). The identification of the degradation intermediates of NB or aniline and was conducted using gas chromatograph/mass spectrometry (GC/MS 7890 B, Agilent, USA). The GC/MS was configured with an Agilent HP-5 MS column (30 m × 0.25 mm i. d. × 0.25 μm). Helium (purity 99.999%) was employed as the carrier gas with a constant column flow of 1.0 ml min^{-1} (split: 10:1). The oven temperature gradient program was: 37 °C for 2 min; 37 °C–180 °C (10 °C min^{-1}), 180 °C–250 °C (30 °C min^{-1}), and 250 °C for 2 min. The temperatures for the injector and ion source were 250 °C and 230 °C, respectively. Mass scan range was 50−550 amu. Each experiment of all the tests (including tests in sets 1 to 5 and the pathway/extraction tests) was replicated four times (n = 4). The statistical analyses were carried out using Origin 7.5 for Windows. The presented results were mean values ± standard deviations from four independent samples.

3. Results and discussion

3.1. Effect of reductant and peptone on nitrobenzene biodegradation in MSM

As shown in Fig. 1A, addition of a reductant had no obvious effect on stimulating NB biodegradation by the three soil microbial communities. In the presence of manntiol, the soil microbial communities had the best NB removal as compared with the other reductants (VC, D-glucose, or Fe^{2+}). The residual concentration of NB was 170.2, 176.1, or 165.4 mgL^{-1} after 72 h in the reactors filled with soils 1, 2, and 3, respectively. With the addition of D-glucose, the residual concentration of NB wsd 180.6, 181.3, or 178.6 mgL^{-1} in the reactors filled with soils 1, 2, and 3, respectively, which were slightly lower than that in the control tests (Fig. 1A). This result is inconsistent with previously studies that the addition of the reductant could enhance biodegradation of NB and 4NCB by strain (Luan et al., 2010; Li et al., 2017a, 2017b), but is consistent with a previous report that 4NCB biodegradation by soil microbial communities was not affected by the addition of the reductant (Under review).

As show in Fig. 1B, supplement with peptone, the addition of all reductants except for glucose showed a synergistic effect on NB biodegradation by the soil microbial communities. In the presence of peptone, the residual NB concentration in the controls was 122.3, 132.5, or 119.2 mgL^{-1}, respectively. With peptone and D-glucose, the residue NB concentration was 134.3, 127.9, or 124 mgL^{-1} NB in the reactors filled with soils 1, 2, and 3, respectively, almost the same as that in the peptone only reactor (controls). The other two reductants ($FeSO_4$ and manntiol) with peptone had the synergistic effect on the NB biodegradation. With the additional $FeSO_4$, the residue NB concentration was 59.2, 50.2, or 62.3 mgL^{-1} in the reactors filled with soils 1, 2, and 3, respectively. In the presence of mannitol and peptone, the NB residual was 101.2, 98.7, or 93.2 mgL^{-1} NB in the reactors filled with soils 1, 2, and 3, respectively. With reductant VC and peptone, the NB removal in reactors filled with soils 1, 2, or 3) could more than 92%, with no apparent difference. Thus, soil 1 was selected as sole sample in the next step of the experiment.

Results shown in Fig. 1 directly prove that Li's approach can be extended to the inoculation of plain soil for effective NB biodegradation in contaminated water and indicate that the reductant (VC), the nitrogen source (peptone), and their combination would significantly affect NB bioremediation.

3.2. Effect of reductant concentration

Fig. 2 A shows that the residual concentration of NB decreased from 188.2 ± 4.7 to 174.9 ± 4.8 mgL^{-1} at 72 h with an increase in VC concentration (from 0 to 0.8 gL^{-1}), but increased from 174.9 ± 4.8 to 184.4 ± 3.9 mgL^{-1} with a further increase in VC concentration from 0.8 to 2.0 g/L. Fig. 2B shows that in the presence of peptone, the residual concentration of NB sharply decreased from 153.4 ± 9.53 to 1.95 ± 2.1 mgL^{-1} and then increased to 185.3 ± 17.7 mgL^{-1} as the VC

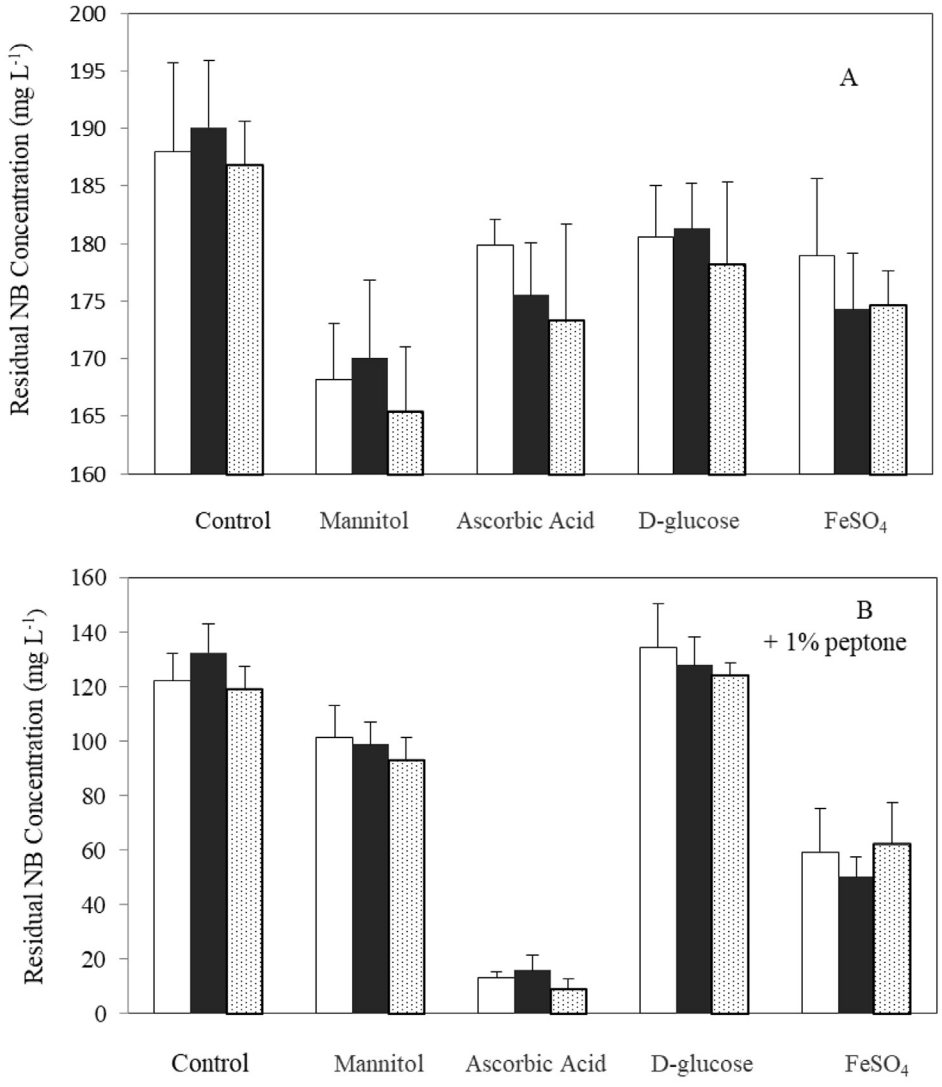


Fig. 1. Effects of reductants (Control, D-glucose, Mannitol, VC, and Fe^{2+}) on nitrobenzene biodegradation (200 mgL^{-1}) after 72 h. (A) Inoculated with plain soils 1(□), 2(■), or 3 (▨) but without peptone (sets 1 and 2 in Table 2). (B) Inoculated with plain soils 1, 2, or 3 and with peptone (set 3).

concentration changed from 0 to 3 g/L. Fig. 2C shows that, in the presence of peptone, reductant concentration affected the NB biodegradation by pure culture strain S12. When the reductant (VC) concentration increased from 0 to 0.8 g/L, the residues NB concentration decreased from 137.8 ± 8.1 to 91.5 ± 0.3 mgL^{-1}, and then increased to 121.8 ± 5.1 mgL^{-1} with an increase in the VC concentration from 0.8 to 2 gL^{-1}. For the first time, this study demonstrated that the rise and fall of NB biodegradation was affected by an increase in reductant (VC) concentration, which could not be explained readily by the present theory. Our results in Fig. 2 B and 2C show the same trend of NB biodegradation regardless whether the inoculation was a pure culture strain or plain soil (with microbial communities). Thus, inoculation with pure or mixed microbial population is not the reason for increased reductant concentration to cause a later-on decline of NB biodegradation.

It is very interesting to ask the question 'why and how the rise and fall of NB biodegradation is linked with the VC concentration?'. Liang et al. (2016) indicates that increasing VC concentration would result in improved NB degradation in purified water, but no answer was given about whether increasing VC concentration would affect NB biodegradation by microorganisms. Luan et al. (2015) disclosed that the NB′ biodegradation rate of strain CN32 could increase with an increase in reductant (clay−Fe (II)) concentration, and they proposed that the availability of the excess electron donors would lead to an increase in NB biodegradation. Similarly, the increasing reductant concentration could enhance the biodegradation of nitroaromatic compounds by the availability of excess electron donors (Luan et al., 2010). For instance, And and Grundl (2000) showed that the reduction rate of 4-chloronitrobenzene increased with an increase in $FeOH^{+}$ concentrations. Fig. 2 indicates that at a lower level of the VC concentration, our results are consistent with Luan et al. (2015). However, as the VC concentration further increased, NB biodegradation would be reduced because VC would inhibit biomass growth at a higher concentration (Hernandezpatlan et al., 2017; Aashima et al., 2018; Galanakis et al., 2018; Lu et al., 2018), which is a new mechanism discovered in this study.

To further confirm this new mechanism, biomass concentration (OD_{600}) was measured as a function of VC dose. Fig. 3 shows that an increase in VC concentration from 0 to 2 gL^{-1} resulted in a decrease in microbial biomass of both pure strain S12 and soil microorganisms; the OD_{600} values of soil microorganisms decreased from

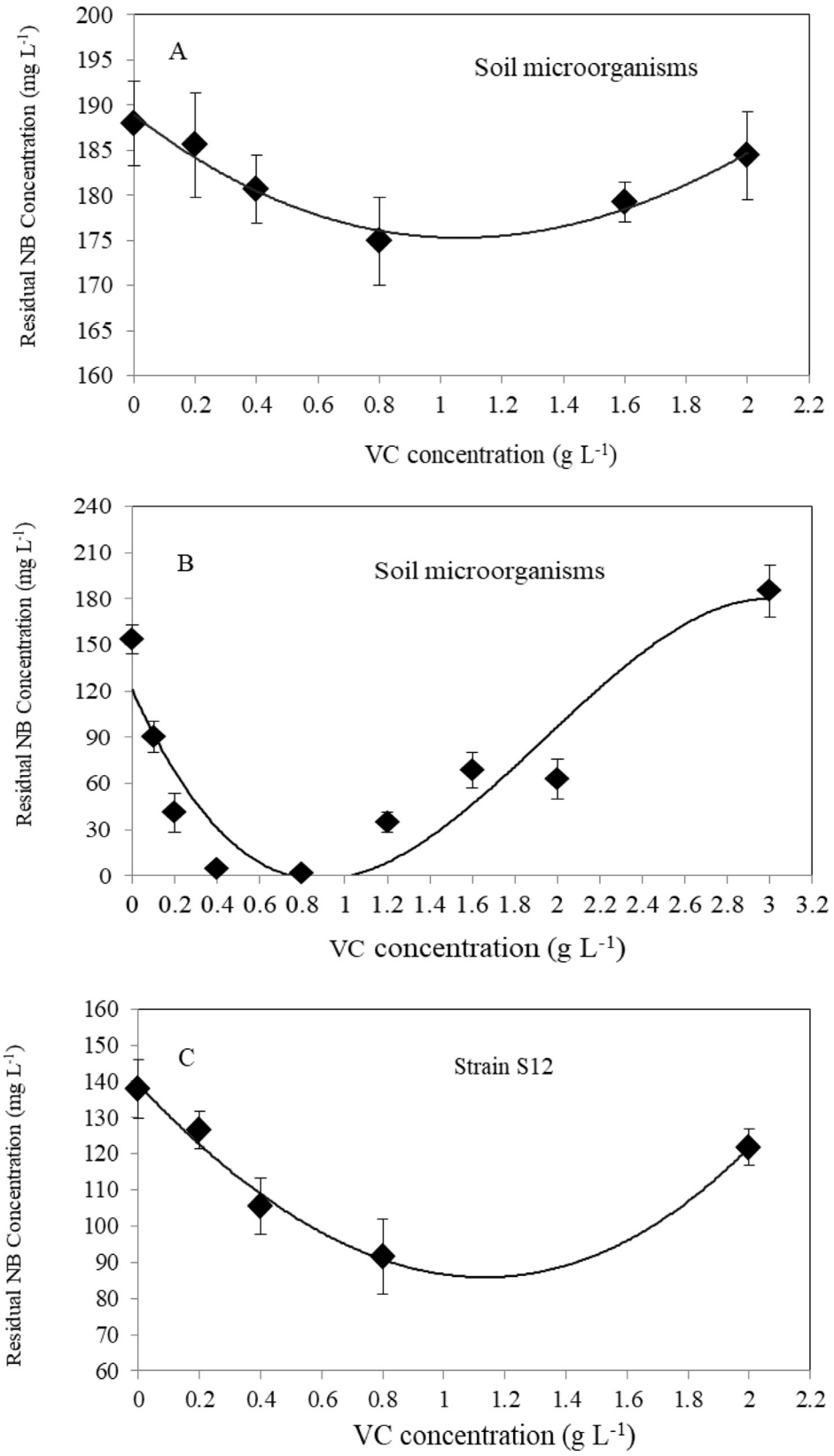


Fig. 2. Effects of reductant (VC) concentration on nitrobenzene biodegradation (200 mgL^{-1}) after 72 h. (A) Inoculated with soil microorganisms in soil 1 but without peptone (sets 4 in Table 2). (B) Inoculated with soil microorganisms in soil 1 and with peptone (set 5). (C) Inoculated with Strain S12 and with peptone (set 5).

1.51 ± 0.27 to 0.48 ± 0.09, and that of strain S12 decreased from 1.14 ± 0.15 to 0.606 ± 0.09. These results demonstrate that increasing reductant (VC) concentration could inhibit microbial growth, which eventually would offset the initial benefit offered by the availability of the excess electron donors at a low VC concentration, and thus, made the decrease in NB biodegradation prevail gradually (e.g., after VC > 1 to 1.2 mgL^{-1}). Therefore, results shown in Figs. 2 and 3 disclose that NB biodegradation was affected by reductant via two mechanisms: the availability of excess electron donors and the effect of microbial growth in the NB biodegradation process.

According to these two new mechanisms, one can explain the results shown in Fig. 2 At a low level of the VC concentration, the increased availability of electron donor with an increasing reductant concentration is the dominant factor that causes the increase in NB biodegradation. This inference is consistent with the previous report that NB biodegradation was improved with an increase in Fe^{2+} concentration (Luan et al., 2015), because Fe^{2+} concentration could enhance or has a limited effect on microbial growth (Wu et al., 2012). At a high level of the VC concentration, the effect of the decreased microbial biomass prevails and as a dominant factor, results in inhibition of NB biodegradation. The antibacterial activity of VC has been fully understood in medical and food field (Hernandezpatlan et al., 2017; Aashima et al., 2018; Lu et al., 2018).

3.3. Degradation kinetics of nitrobenzene

Biodegradation kinetic of NB can be described by the following equation:

$$\frac{dC_{NB}}{d_t} = \frac{C_{NB0} - C_{NBt}}{t} = \mathrm{rNB} \quad (1)$$

where C_{NB0} and C_{NBt} = the initial NB concentration or that at time t, mgL^{-1}; and r_{NB} = the NB biodegradation rate, $mgL^{-1}h^{-1}$. Due to the linear relationship between OD_{600} values and microbial biomass,

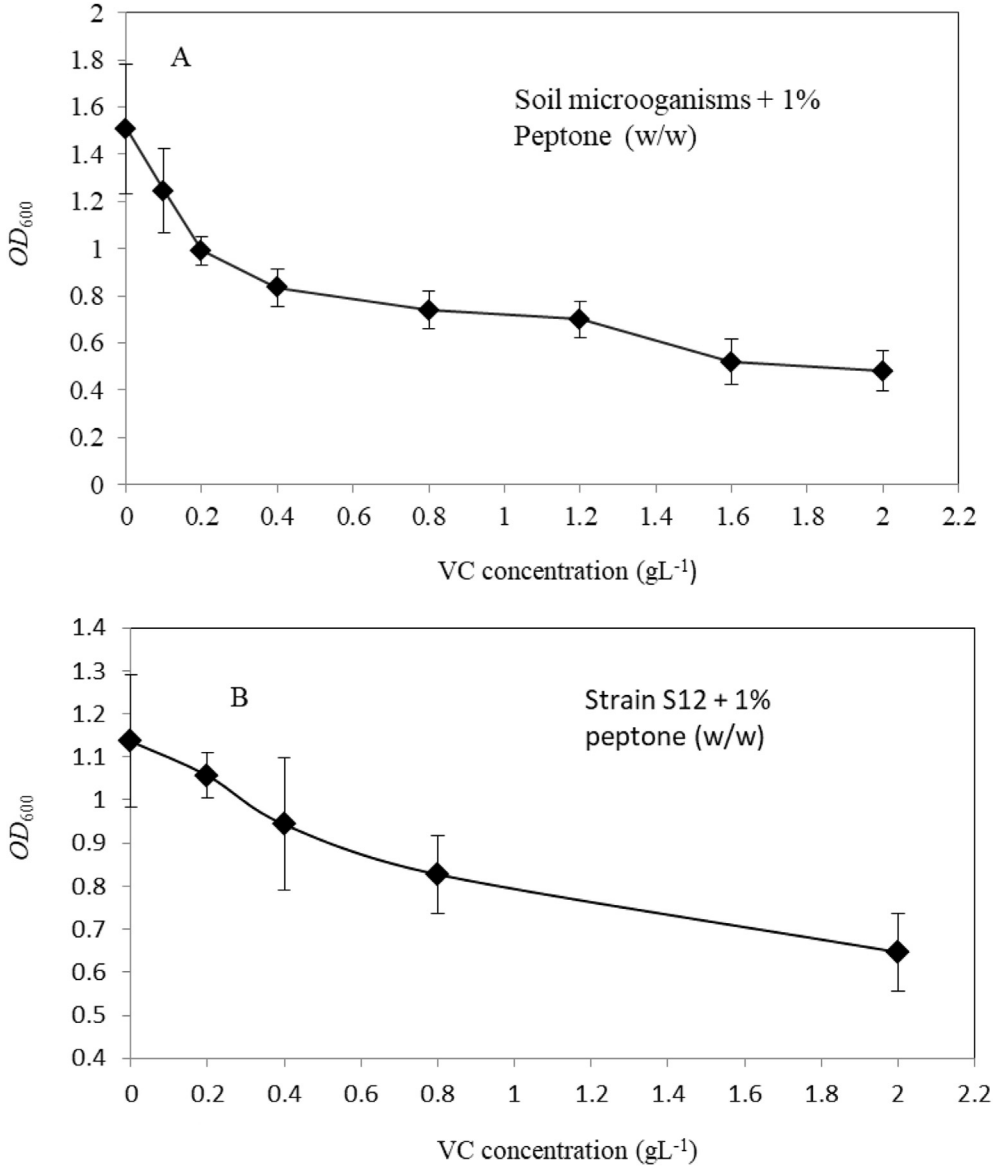


Fig. 3. Effects of reductant (VC) concentration on OD_{600} after 72 h. (A) Inoculated with soil microorganisms and with peptone (set 5). (B) Inoculated with Strain S12 and with peptone (set 5).

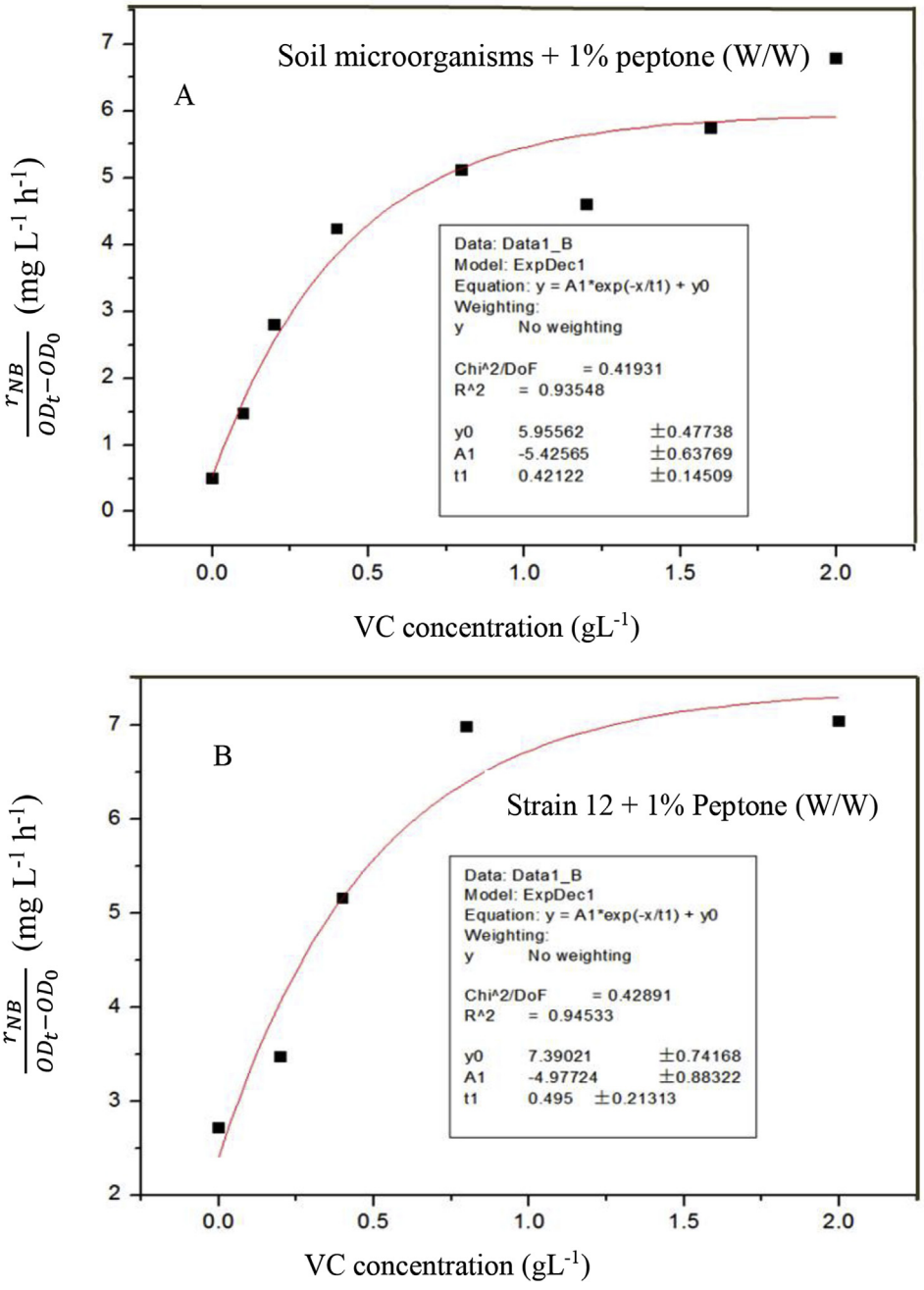


Fig. 4. Pseudo first-order kinetics of nitrobenzene biodegradation as a function of normalized biomass and reductant (VC) concentration. (A) Inoculated with soil microorganisms in soil 1 and with peptone (set 5). (B) Inoculated with Strain S12 and with peptone (set 5).

the data of biomass can be replaced by OD_{600}:

$$OD_t - OD_0 = K(V_t - V_0) \quad (2)$$

where OD_0 and OD_t = initial OD_{600} or that at time t, unitless; V_0 and V_t = the initial quality of microbial biomass or that at time t, mg; K = the coefficient, mg $^{-1}$. The biodegradation rate of NB normalized to the increased biomass during the reaction period is a function of reductant:

$$\frac{dC_{NB}}{(V_t - V_0)dt} = \frac{r_{NB}}{(OD_t - OD_0)} = f(C_{reductant}) \quad (3)$$

Eq. (3) considers the effects of both biomass (normalized by *OD* values) and reductant concentrations on NB concentration in the reactor. By normalizing the biomass change during the reaction period, Eq. (3) eliminates impact of the biomass change due to the reductant's inhibition effect on the biomass and the associated impact on the rate of NB biodegradation. Thus, the curved lines was shown in Fig. 4 reflects the only effects of the reductant availability (as the electron donor) on NB biodegradation.

As shown inFig. 4A andB and , NB biodegradation follows pseudo first-order kinetics as a function of normalized biomass and the VC concentration, and can be represented as:

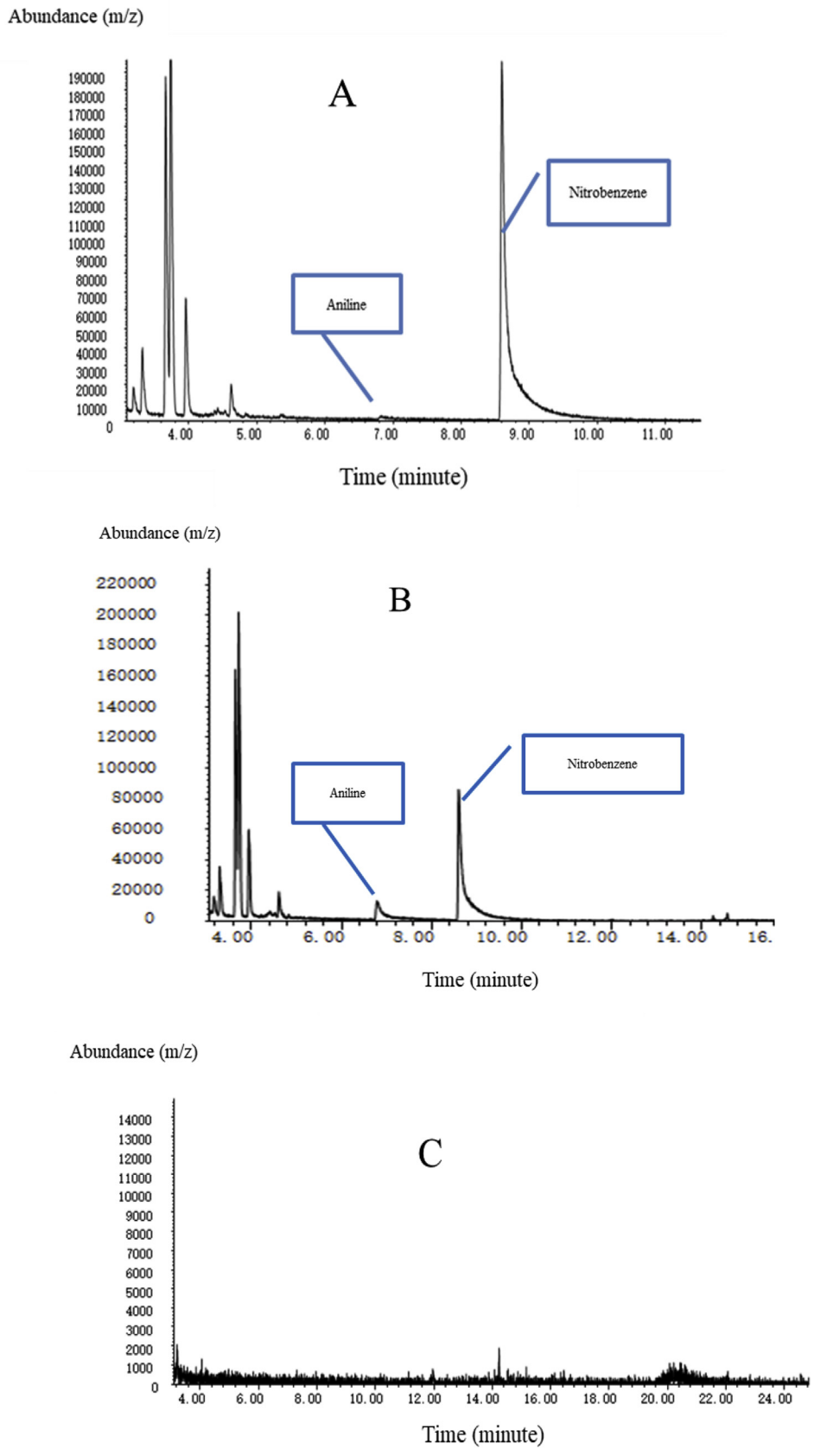


Fig. 5. GC/MS analysis of the metabolites of nitrobenzene.

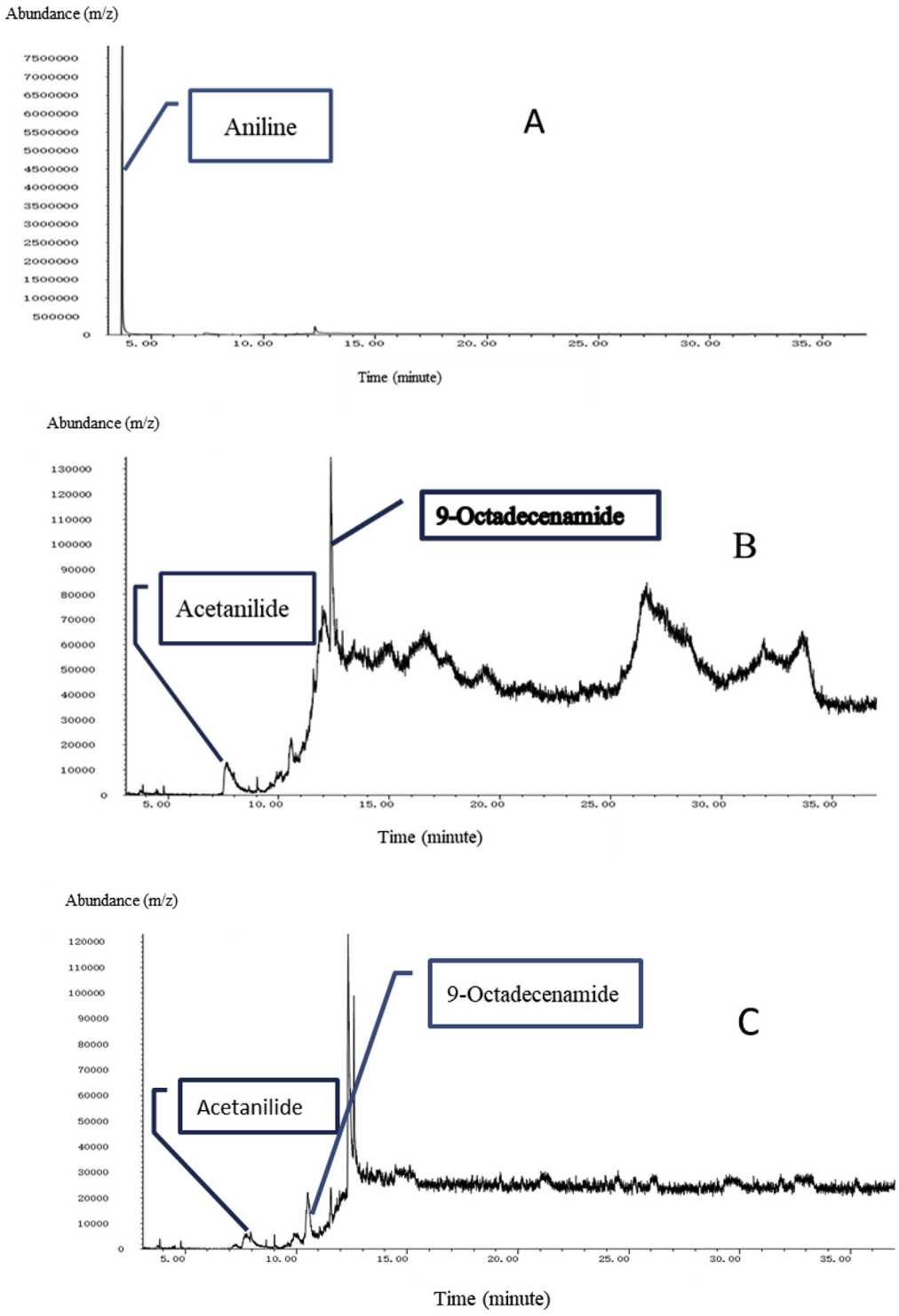
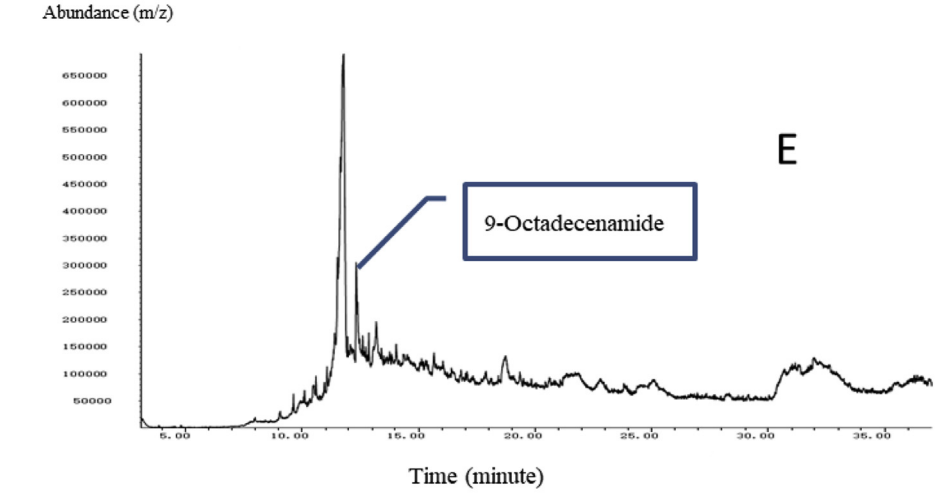
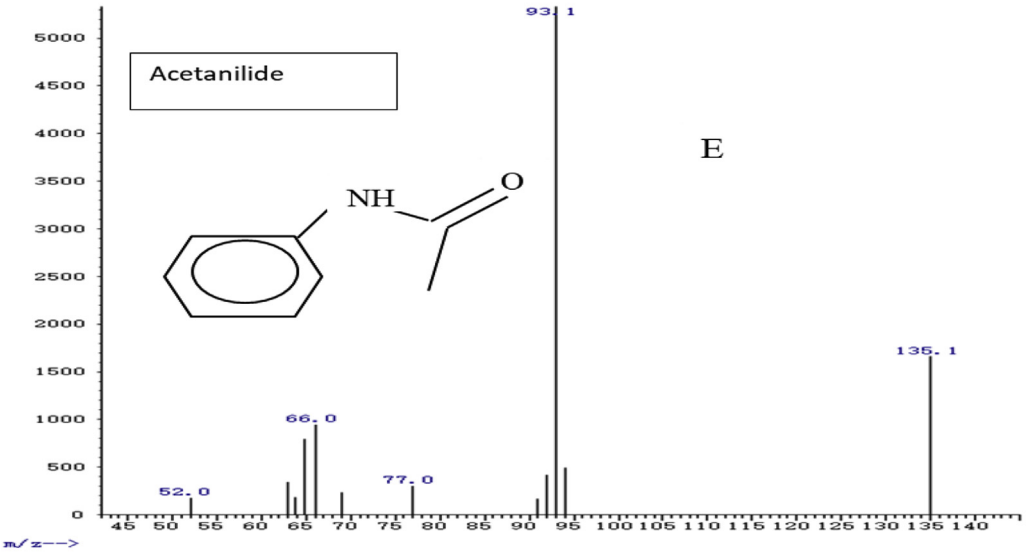


Fig. 6. GC–MS chromatograms and spectra of aniline degradation intermediates at 0, 24, 36, 48 h. A (control 0 h), B (24 h), C (36 h), D (48 h), E (Acetanilide), F (9-Octadecenamide).





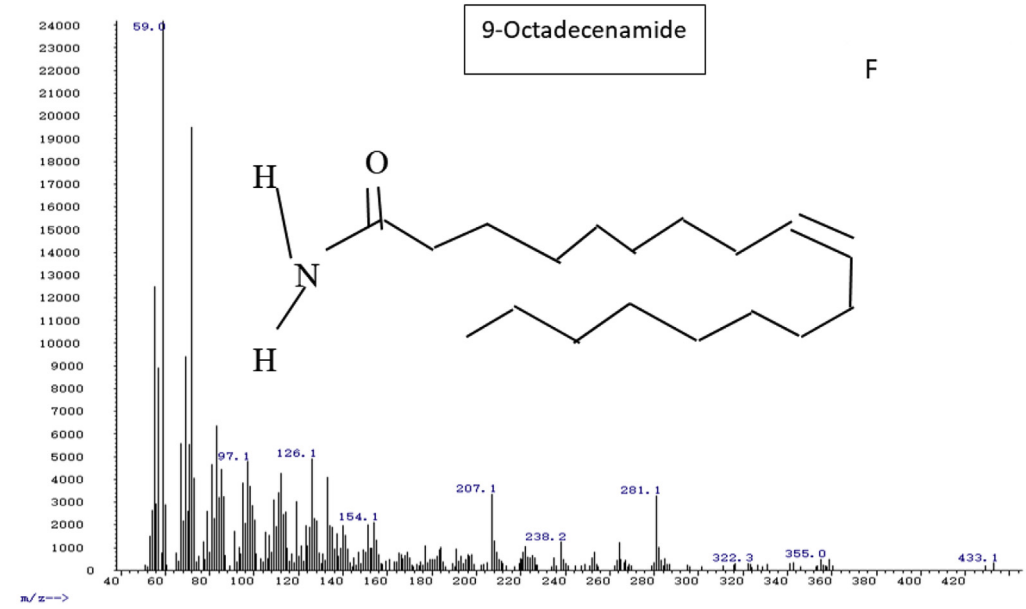


Fig. 6. (*continued*).

$$\frac{r_{NB}}{OD_t - OD_0} = -5.43 \times \exp\left(\frac{-C_{reductant}}{0.42}\right) + 5.97\left(R^2 = 0.94\right) \text{ for soil microorganisms (Fig.4A)} \quad (4)$$

$$\frac{r_{NB}}{OD_t - OD_0} = -4.97 \times \exp\left(\frac{-C_{reductant}}{0.5}\right) + 7.39\left(R^2 = 0.95\right) \text{ for strain S12 (Fig.4B)} \quad (5)$$

For the first time, Eqs. (4) and (5) are established and can be used to disclose the relationship among NB biodegradation under the influence of biomass (pure strain or mixed biomass) and reductant concentration. From Fig. 4 and Eqs. (4) and (5), the curves is rising with the increasing VC concentration. The result demonstrates the positive correlation between the rate of NB biodegradation normalized to the biomass changes and the reductant concentration. The additional peptone enhanced the NB biodegradation through the enhancement of the microbial mass. From Fig. 8, with an increase in peptone concentration (0–10 g/L), the value of OD increased, together with an improvement of the nitrobenzene-biodegrading rate. However, with a further increase in peptone concentration (10–20 g/L), the values of OD and the residue NB concentration remained unchanged. The results show that the optimal peptone concentration was 10 g/L.

Previous studies reported that the reductants-enhanced reduction of nitroaromatic compounds followed the pseudo first-order degradation kinetics (Klupinski et al., 2004; Chun et al., 2006). In the presence of Fe(II), the 4-chloronitrobenzene biodegradation was modeled by the pseudo first-order kinetics, and the removal process would not merely depend on the extent of Fe(II) adsorption but also depend on the dissolved Fe(II) concentration (Klupinski et al., 2004; Chun et al., 2006). While Eq. (3) eliminates the effect of microbial biomass, the NB biodegradation kinetics identified in this study is consistent with previous studies (i.e., first-order or pseudo-first-order kinetics). Hence, Eq. (3) further confirms that the reductant affects the NB biodegradation rate by the available electron donors and stimulating or inhibiting the microbial biomass.

3.4. Biodegradation pathway

There are two biodegradation pathways for NB that could be either reductive (Nishino and Spain, 1995; Takenaka et al., 1997) or oxidative (Ju and Parales, 2010). Between these two pathways, reductive biodegradation is more prevalent due to the nitro group with strong attraction to electrons (Ju and Parales, 2010). He and Spain (1999) demonstrated that, among the 155 isolated NB-degrading strains, 154 strains degraded NB through the partial reductive pathway and one through the oxidative pathway. Previous studies showed that aniline is a normal intermediate of NB biodegradation, and the reduction of NB to aniline represents the rate-limiting step for the overall process of NB degradation (Mu et al., 2014). To our knowledge, co-metabolic pathway of NB has not been reported.

In the present study, samples at 0, 24, 36, 48, and 96 h were analyzed by GC/MS, generating a total ion chromatogram (TIC) in the scan mode (Figs. 5 and 6). The GC/MS peak spectra for the compound showed a positive match probability above 91% when compared to mass spectra in the National Institute of Standards & Technology Mass Spectral Library database. Figs. 5 and 6 indicate that at the beginning, the aniline concentration obviously increased, while the NB concentration was sharply decreased. However, aniline was disappeared after 24 h. The acetanilide was detected after 24 h and 36 h, presumably because the H^+ of the aniline was replaced by acetone due to the complex soil organic compounds. Another intermediate, 9-octadecenamide was found after 24, 36, and 48 h. This result disclosed that aniline could be transformed to acetanilide, and then further to 9-octadecenamide. The *OD* data of soil microorganisms (OD_{600} in MSM = 0.314) demonstrated that indigenous microorganisms from the soil could not utilize NB as the sole carbon and nitrogen source to grow. Hence, NB biodegradation involved in this study should follow the reductive pathway (Peres et al., 1998; Hofstetter et al., 2006) as proposed in Fig. 7.

Previous studies used the following two reasons to explain why NB is transformed from aniline to CO_2 and water (i.e., completely mineralized). First, aniline could be the finial biodegradation product of NB by some kinds of microorganisms, because most microorganisms in previous studies are only capable of reducing NB to the less toxic aniline (Marvinsikkema and de Bont, 1994; Liu

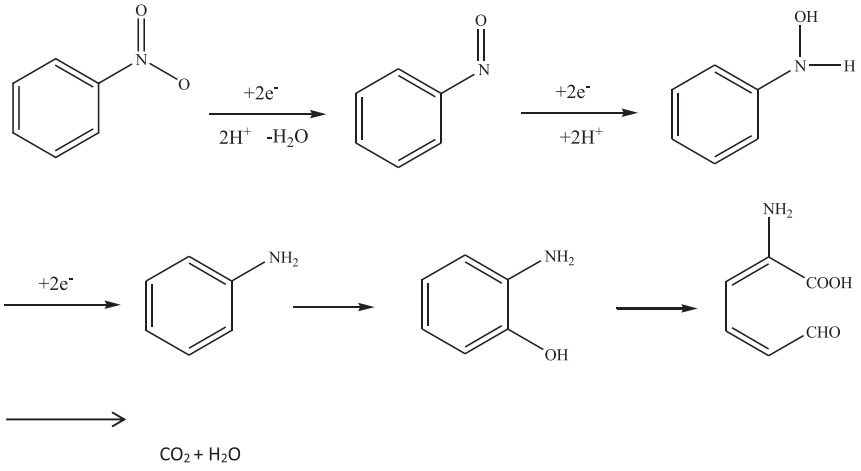


Fig. 7. Proposed possible transformation pathway for NB biodegradation.

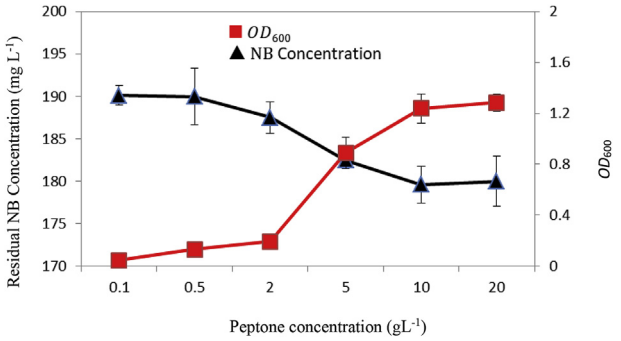


Fig. 8. Effects of peptone concentration on the NB residue concentration (▲) and OD_{600} (■) with inoculated soil microorganisms after 24 h.

et al., 2014). However, other kinds of microorganisms could further biodegrade aniline due to biological diversity in soil (Lyons et al., 1984; Emtiazi et al., 2001). Second, NB could be completely mineralized by some microorganisms that could utilize NB a source of carbon, nitrogen, and energy (Zhao et al., 2011). Up to date, the degradation pathway for aniline has been demonstrated to be the oxidative degradation pathway, by mainly the reduction of aniline to catechol, followed by the ring cleavage and TCA cycle (Liu et al., 2002). In addition, aniline could be transformed into p-aminophenol (Wang et al., 2007) and then be further oxidized to p-nitrosophenol. However, in our study, the two novel intermediate metabolites were found, which demonstrates that complex organic compounds and the soil microbial communities could induce more complex metabolites as the reaction goes on even though the end products are CO_2 and water. Hence, identification of biodegradation pathway of a certain organic compound in the field application could be more difficult because more complicated metabolites could be produced with the existence of more complex organic compounds and more microorganisms in the site.

3.5. Implications

Results of this study demonstrate that Li's approach can be extended and thus, has opened a window for one to inoculate plain soil for effective NB biodegradation in contaminated water, which has notable advantages over inoculation with pure culture, such as cost saving for isolation and enrichment of pure culture, increased metabolic capabilities, complete degradation. For instance, in the presence of peptone and 0.8 gL^{-1} VC, 200 mg L^{-1} NB was completely removed after 72 h in the system inoculated with plain soil. Hence, the approach could serve as a potential treatment process for NB-containing wastewater. Results of this study showed three major contributions (or interesting points), which are discussed as follows:

First, it is interesting to notice that, in general, the biodegradation of NB was at the similar level regardless whether the reductant was inorganic ($FeSO_4$) or organic (e.g., VC, glucose, and mannitol) (Fig. 1). It is also interesting to notice that peptone + glucose and peptone + mannitol were not as good as peptone + $FeSO_4$ (Fig. 1). Fig. A. 1 indicates that organic reductants (e.g., VC, glucose, and mannitol) may not be used efficiently as a carbon source and an electron donor if the system has no appropriate nutrients.

It is interesting to compare results between set 4 (without peptone) and set 5 (with peptone) (as shown in Figs. 2–5). There are two microbial relationships associated with the removal of contaminants, that is, microbial interspecies competition and mutual microbial cooperation [40, 41]. Under substrate sufficient conditions (as in set 5 with both peptone and VC), the mutual microbial cooperation may be a dominating factor on NB biodegradation, which stimulates the biodegradation rate of soil microorganisms to be faster than that of the pure strain. Under substrate insufficient condition (as in set 4 with VC but without peptone), the microbial interspecies competition may be a leading factor in NB biodegradations, which causes the value of biodegradation rate of pure strain to be larger than that of soil microorganisms. Hence, due to the existence of various microorganisms in the natural environment, there isn't an effective strategy to remove NB from the environment by adding a sole organic reductant. However, using the synergistic effects of substrate and organic reductant could effectively remove NB from water or soil. Thus, tests of sets 1 to 5 indicate that Li's approach can be extended to using plain soil to inoculate microorganisms into the aquatic treatment system. Combining results shown in Fig. 1A and B and that obtained from tests of sets 4 and 5, one can understand the importance of adding a good nitrogen source (peptone) into the remediation system; a right combination of the reductant and nitrogen source (e.g., VC + peptone) is critical.

Second, our results demonstrate, for the first time, that the reductant would affect NB biodegradation via two mechanisms: the availability of excess electron donors and inhibition of microbial growth. Comparing Fig. 2B and C, one may conclude that soil microbial communities exhibited a biodegradation performance superior to strain S12 with the addition of peptone and VC. However, if the effect of biomass change is normalized in the kinetics, one would recognize that the results shown in Figs. 3 and 4 indicate that pure culture still had a higher NB biodegradation rate. In the past, no study has focused on whether the reductant's ability to inhibit or improve microbial growth would affect NB biodegradation rate. Results of this study imply that using the availability of excess electron donors as the sole standard to evaluate the optimal reductant and associated conditions could be biased.

Third, Eq. (3) links the two mechanisms together, and Eqs. (4) and (5) can be used to evaluate the NB biodegradation rate as a function of reductant concentration and the biomass change in the system. Therefore, this study provides new insights into how to exploit the synergistic effects of the availability of excess electron donors and biomass growth by controlling the reductant and a substrate in the right concentration range (e.g., VC < 0.8 mgL^{-1} with peptone added) and how to avoid the negative effects of overdosing a reductant and the associated inhibition of biomass growth. Results of this study also warrant the future studies about how peptone (or other substrates) and different reductants would affect stimulation or competitive inhibition of biomass growth and bioremediation of nitroaromatic pollutants (Datta et al., 2014). For example, because the exact composition of peptone depends on the type of protein from which the peptone is derived, detailed studies may be needed to see the effect of peptone types, why peptone is so efficient and how to incorporate peptone's effects into the kinetics (Eq. (3)). Studies can be expended to whether other nitrogen sources would give the similar results as well.

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