Kinetics of nitrobenzene degradation coupled to indigenous microorganism dissimilatory iron reduction stimulated by emulsified vegetable oil

Jun Dong¹, Linjie Ding¹, Zifang Chi^{1,*}, Jiansen Lei², Yan Su³

1. Key Laboratory of Groundwater Resources and Environment, Ministry of Education, Jilin University, Changchun 130021, China. E-mail: dongjun@jlu.edu.cn
2. Exploration Unit Of North China Geological Exploration Bureau, Tianjin 065201, China
3. Shenyang Academy of Environmental Sciences, Shenyang 110000, China

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Abstract: Widespread contamination by nitrobenzene (NB) in sediments and groundwater requires better understanding of the biogeochemical removal process of the pollutant. NB degradation, coupled with dissimilatory iron reduction, is one of the most efficient pollutant removal methods. However, research on NB degradation coupled to indigenous microorganism dissimilatory iron reduction stimulated by electron donors is still experimental. A model for remediation in an actual polluted site does not currently exist. Therefore, in this study, the dynamics was derived from the Michaelis-Menten model (when the mass ratio of emulsified vegetable oil and NB reached the critical value 91:1). The effect of SO_4^{2-} , NO_3^- , $\text{Ca}^{2+}/\text{Mg}^{2+}$, and the grain size of aquifer media on the dynamics were studied, and the NB degradation dynamic model was then modified based on the most significant factors. Utilizing the model, the remediation time could be calculated in a contaminated site.

Key words: nitrobenzene degradation model, iron reduction, groundwater, emulsified vegetable oil, site remediation

* Corresponding author. E-mail: chizifang@jlu.edu.cn (Zifang Chi)

Introduction

Nitrobenzene (NB) is a toxic organic chemical material that is commonly utilized in pharmaceuticals, dyes and other applications. Due to inappropriate disposal during its usage, storage, and transportation processes, NB is already responsible for quite serious pollution in many groundwater systems (Yin et al., 2015). NB does not readily biodegrade and is highly toxic to humans and the environment, so it is listed as a priority pollutant by the US EPA (Dong et al., 2013; 2015). In subsurface environments, the presence of dissimilatory metal-reducing bacteria and organic matter provides a remarkably effective technology for the remediation of contaminated soils and groundwater (Lovley et al., 1989; Lovley and Lonergan, 1990). Geochemical evidence has indicated that organic pollutants, such as nitroaromatic compounds, can be degraded in anaerobic subsurface environments in which iron reduction is an intermediate electron-accepting process (Luan et al., 2009). Fe(III) is one of the most abundant potential electron acceptors in underground environments (Lovley, 1991). Microbial dissimilatory iron reduction coupled to NB degradation has already shown promise in the remediation of NB-polluted aquifers (Heijman et al., 1995).

Under anaerobic subsurface conditions, Fe(III)-reducing microorganisms obtain energy for growth by oxidizing fermentable organic compounds to transform Fe(III) to Fe(II). The microbially generated Fe(II) species can then reabsorb to iron-bearing minerals and catalyze the degradation of various classes of contaminants, including nitroaromatic compounds, which are degraded to aniline (AN) through a series of intermediate products (Tobler et al., 2007). Organic substrate and NB contents, active ions in the groundwater, and the characteristics of aquifer media are the most important factors in the iron reduction-coupled NB degradation process.

The addition of fermentable organic substrates to an aquifer is one of the most common methods of enhancing *in situ* anaerobic bioremediation (Ellis et al., 2000). Emulsified vegetable oil (EVO) is favored due to its long chain molecular structure and low cost (US Air Force, 2007; Lindow, 2004; Tang et al., 2013a). EVO consists of food-grade soybean oil, surfactants, dissolved organic matter, and water blended to form a stable microemulsion with

small and uniformly sized oil droplets (Ma, 2013; Tang et al., 2013b). Once injected into the subsurface, EVO can maintain long-term reducing conditions and provide a long-lasting carbon source and electron donor for bioremediation (Sheu et al., 2015). Recent laboratory and field studies have shown that injecting EVO into a subsurface can effectively enhance anaerobic bioremediation of chlorate, perchlorate (Borden et al., 2007), nitrate, chromate, AMD (Lindow, 2004), chlorinated solvents (Sheu et al., 2015), and U(VI) (Tang et al., 2013b) in contaminated groundwater and sediment. Addition of edible oil can rapidly reduce contaminant concentrations in the aqueous phase by partitioning off a portion of the solvent mass into the edible oil (US Air Force, 2007).

Many studies have been conducted on NB degradation using zero valent iron (ZVI) or nano-scale zero-valent iron (NZVI) because of their fast and efficient action (Fu et al., 2014; Mueller et al., 2012); however, the poor mobility of NZVI, and especially ZVI, can cause the clogging of aquifers, and also might be toxic to indigenous bacteria and hinder their participation in the cleanup process (Chen et al., 2011). Overall, the addition of ZVI improves the remediation cost and increases the probability of secondary contamination. By comparison, the addition of EVO is economical, safe and can provide a long-lasting carbon source and electron donor for bioremediation (Dong et al., 2014). Biodegradation of the oil then stimulates anaerobic conditions and multiplies the indigenous microorganisms, including dissimilatory iron-reducing bacteria, which are ubiquitous in aquifers, and couple the oxidation of EVO with the reduction of Fe(III) in natural minerals in the aquifer media to generate Fe(II) species.

Relatively few studies as of now have investigated the dynamics of NB degradation coupled to microbial iron reduction in underground environments, especially regarding adding the electron donor EVO to stimulate the process. Many studies have, though, investigated iron reduction dynamics, which can be described by the Michaelis–Menten model, Monod model, or first-order kinetics equation. The kinetic parameters have proven to differ significantly between different experimental environments (Bonneville et al., 2004; 2006; Jaisi et al., 2007; Liu et al., 2001; McCormick et al., 2002; Ross et al., 2009), in which

most kinetic models have used either pure culture microbes or synthetic iron mineral. Prior to now, no research project has estimated the EVO electron donor dose or remediation period in any actual contaminated site. An NB degradation kinetic model that closely reflects real-world conditions is urgently required in order to guide engineers at work remediating actual contaminated sites.

The present study investigated the kinetics of NB degradation using EVO as an electron donor to stimulate indigenous microorganism dissimilatory iron reduction by simulating an *in situ* underground environment. The research consisted of two parts: (1) developing the NB degradation dynamics model in the presence of excess electron donor (EVO) in the simulated underground system; and (2) investigating the major factors that affect NB degradation in order to refine the NB degradation model accordingly.

1. Materials and methods

1.1 Materials

The preparation of EVO: A homogenizer (HJ-6A, Jiangsu Jinyi Instruments Technology Co., China) was used to make an emulsion with commercial soybean oil, Tween-80 (analysis grade), yeast extract (Sinopharm Chemical Reagent Co., Ltd., China) and deionized water. The mass concentration of oil used was 10%. EVO was prepared by homogenizing for 24 hr at 3000 r/min.

NB solution was prepared by dissolving analytically pure nitrobenzene (Sinopharm Chemical Reagent Co., Ltd., China) in deionized water and mixing until uniform. The final concentration of NB was 40 mg/L.

Five types of sand with D_{50} of 0.04 mm, 0.23 mm, 0.42 mm, 0.61 mm, and 0.8 mm were used as aquifer media and were prepared by sieving and grading from commercial river sand.

The inorganic salts used were sodium sulfate (Na_2SO_4), sodium nitrate (NaNO_3), magnesium chloride (MgCl_2) and calcium chloride (CaCl_2). All the inorganic salts were obtained from Sinopharm Chemical Reagent Co., Ltd., China.

1.2 Experimental design

1.2.1 Verification of NB biodegradation coupled with dissimilatory iron reduction

Four gas-tight flasks (with 250 mL capacity) fitted with rubber stoppers were used as microcosms for the NB degradation simulations. 200 g sand ($D_{50}=0.23$ mm) was placed in each of four flasks as aquifer media, and the four flasks were filled with deionized water, NB (41.7 mg/L), EVO (18.18 g/L), EVO (18.18 g/L) and NB (40 mg/L), respectively. All of the flasks were incubated anaerobically in a light-tight constant temperature incubator (Shanghai Boxun Medical Biological Instrument Corp., China) at 30°C. Samples were taken once a day, and concentrations of NB, AN, total Fe, and Fe(III) were analyzed. Each experiment was replicated three times.

1.2.2 EVO addition stimulating NB biodegradation coupled with dissimilatory iron reduction

Four flasks with 200 g sand ($D_{50}=0.23$ mm) as aquifer media, 220 mL liquid phase composed of NB 40.2 mg/L, Na_2SO_4 150 mg/L, NaNO_3 5 mg/L, $\text{MgCl}_2 + \text{CaCl}_2$ 100 mg/L (molar ratio of $\text{MgCl}_2:\text{CaCl}_2=1:1$) and variable concentrations of EVO (0.18, 0.36, 3.63, 18.18 g/L) were incubated anaerobically in a light-tight constant temperature incubator at 30°C. Samples were taken once a day, and concentrations of NB, AN, total Fe, and Fe(III) were analyzed. Each experiment was replicated three times.

1.2.3 Major factors affecting NB degradation in groundwater

The experiments were designed using the Design Expert 8.0.6.1 Trial (Stat-Ease Inc., USA). SO_4^{2-} , NO_3^- and total hardness ($\text{Ca}^{2+}/\text{Mg}^{2+}$) were chosen as important factors in the groundwater because NO_3^- and SO_4^{2-} are competitive electron acceptors for the process of dissimilatory iron reduction (Acht nich et al., 1995), while Ca^{2+} , Mg^{2+} may precipitate on the surface of the sand and decrease the reaction rate of dissimilatory iron reduction (Li, 2012). The grain size of aquifer media was also selected as a factor for study because it could affect the content of total iron in the aquifers (Sheng, 2015). So, SO_4^{2-} , NO_3^- , total hardness ($\text{Ca}^{2+}/\text{Mg}^{2+}$), and the grain size of aquifer media were used as the four variables, with each at five coded levels ($-\alpha$, -1, 0, +1, $+\alpha$) as shown in **Table 1**. A four-factor, five-level central composite design (CCD) was developed to explore the effects of the four variables on the NB degradation and AN generation. The selection of variable levels was based on the

classification in The Quality Standard of Underground Water (GB/T 14848-9). The CCD contained a total of 30 experimental trials that included 16 trials for factorial design, eight trials for axial points and six trials for replication of the central points (**Table 2**). The response values (Ys) in each trial were the NB degradation rates and AN concentrations. The concentrations of NB and EVO in the 30 experimental trials were 40 mg/L and 18.18 g/L, respectively. The experimental processes followed were the same as described above.

1.3 Analytical methods

The samples were extracted with a syringe and filtered using a 0.22 μm filter (Tianjin Jinteng Experiment Equipment Co., Ltd., China), and filtrates were analyzed for the concentrations of Fe(III), Fe(II), NB and AN. Fe(III) and total iron concentrations were determined using the thiocyanate colorimetric assay method at a wavelength of 480 nm, and the concentration of Fe(II) was obtained by subtracting the concentration of Fe(III) from the total iron (Goswami and Kalita, 2013). NB and AN were identified by High Performance Liquid Chromatography (HPLC) (Agilent 1260, Wilmington, DE). HPLC analyses were performed on an Agilent C18 column (150 mm $\times$ 4.6 mm, 5 μm). The column temperature was 25°C. The mobile phase was a mixture of acetonitrile and acetic acid-ammonium acetate buffer solution (by volume ratio 65%:35%) delivered by a pump at a flow rate of 1.0 mL/min. The absorbance wavelength for NB was 262 nm and for AN was $\lambda_{\text{ex}}/\lambda_{\text{em}}=280/340$ nm. Injection volume was 5 μL (Peng et al., 2009).

2. Results and discussion

2.1 Verification of NB biodegradation coupled with dissimilatory iron reduction

The following experiment was conducted to demonstrate the feasibility of NB biodegradation coupled with dissimilatory iron reduction. As shown in **Fig. 1a**, the concentration of Fe(II) in the EVO + sand system began to increase on the second day, while Fe(II) in the NB + EVO + sand system remained low until the 8th day. Fe(II) was not detected at all in the system without EVO. The final concentrations in the two systems with EVO were very close: 53 mg/L in the NB system, and 60 mg/L in the EVO + sand system. The difference in the two systems was caused by Fe(II) in the NB system reacting with NB

just after being generated, that is to say, the process of NB degradation coupled with dissimilatory iron reduction took place in the NB + EVO + sand system. The addition of EVO was clearly shown to stimulate the process of dissimilatory iron reduction effectively.

As shown in **Fig. 1b**, NB began to rapidly degrade on the 6th day when Fe(II) began to accumulate substantially in the NB + EVO + sand system, as shown in **Fig. 1a**, confirming that Fe(II) in the system would not accumulate when there was NB present at the early stage. As the reaction progressed, the production of Fe(II) exceeded the demand for NB degradation, so the concentration of Fe(II) began to accumulate on the 6th day. The consistency of the time when Fe(II) started to accumulate and NB rapid degradation began confirmed that the NB degradation was the result of reduction by microbially generated Fe(II) species. As shown in **Fig. 1b**, NB was degraded completely by the 9th day in the NB + EVO + sand system, while the concentration of NB in the NB + sand system showed a slight reduction during the initial phase, which illustrated that sand has a definite adsorption capacity for NB (Ma, 2013), and indigenous microorganisms in the sand did not degrade NB without EVO addition. NB degradation in the iron reduction system can be roughly divided into three phases: (1) sharp decline on the first day due to high intermiscibility between EVO and NB; (2) a slow decline phase from the second day to the 6th day, during which time indigenous microorganisms in the system went through an adaptation period and underwent rapid growth along with the generation of Fe(II) (US Air Force, 2007); and (3) a rapid drop below the detection limit from the 7th day to the 9th day as the redox reaction between NB and Fe(II) proceeded at a rapid rate, NB concentration dropped below 20 mg/L, and Fe(II) began to accumulate. The product of NB degradation coupled with dissimilatory iron reduction is AN; the trend of AN generation corresponded to that of NB degradation.

2.2 EVO addition stimulating NB biodegradation coupled with dissimilatory iron reduction

The addition of EVO to an aquifer can effectively enhance the anaerobic bioremediation of organic pollutants in groundwater and sediment. The main functional component is linear long-chain aliphatic compounds in soybean oil, including palmitic acid and octadecanoic acid.

The theoretically required quantity of soybean oil can be predicted based on the average molecular formula of the liquid soybean oil. This study assumed that the substrate was completely mineralized to carbon dioxide without any partially oxidized intermediates produced (Lindow, 2004). Electrons were then eventually delivered to NB through a series of electron acceptors such as iron, as shown in Eqs. (1) and (2). According to reaction stoichiometry, 16.94 g soybean oil is sufficient to reduce one mole of NB, and the required concentration of EVO in each microcosm is 55.3 mg/L.

Fig. 2a shows the NB degradation process in the four study microcosms. NB in all systems declined significantly on the first day after the addition of EVO, consistent with previous research (US Air Force, 2007). Although EVO addition was in excess in all four systems, 0.18 and 0.36 g/L EVO concentrations were proven incapable of supporting enough electrons for the rapid degradation of NB. EVO was also the limiting factor in both systems, because EVO, as a hydrogen-releasing compound, can only provide limited electrons within a certain period of time – this limits the reaction rate of indigenous microbial dissimilatory iron reduction (Ma, 2013). EVO was no longer a limiting factor, however, when EVO concentrations met or exceeded 3.63 g/L. There were no significant differences in the NB degradation rate/time in systems with EVO concentrations of 3.63 and 18.18 g/L; NB concentrations in both systems began to rapidly decline on the 6th day and were completely degraded on the 9th day. It is therefore valid to conclude that NB is the sole limiting factor when EVO concentration is greater than or equal to 3.63 g/L, in this case when the mass ratio of EVO and NB reached 91:1.

The concentrations of the product AN in the four systems are shown in **Fig. 2b**. AN generation corresponds to NB degradation. AN continually increased in systems with EVO concentration of 0.18 or 0.36 g/L and showed no peak during the test period, while AN concentrations in systems with EVO concentration of 3.63 or 18.18 g/L peaked at 20.63 mg/L and 21.95 mg/L at the 9th day and 8th day, respectively. The concentrations remained

relatively stable. The final mass balance was 67% for NB reduction. Mass loss was attributed to AN degradation by indigenous microorganisms (Wen et al., 2015) and intermediates that were not counted (Dong et al., 2015).

Changes in Fe(III) and Fe(II) can also indirectly demonstrate that the NB degradation is coupled to indigenous microbial dissimilatory iron reduction. As shown in **Fig. 2c** and **2d**, the concentrations of Fe(III) and Fe(II) were both very low before NB degradation was complete. The chemical reaction between microbially generated Fe(II) and NB occurs very rapidly, as demonstrated by Eq. (2). That is to say, the Fe(II) generation process described by Eq. (1) was the limiting step throughout the entire process (Tang et al., 2013a). As a result, Fe(II) cannot accumulate in the presence of NB.

2.3 NB degradation model

Biochemical reaction rates are typically expressed using the Michaelis–Menten equation:

$$V = V_{\max} C_s / (C_s + K_m) \quad (3)$$

where, V (mg/L/day) is the substrate degradation rate, $V_{\max}$ (mg/L/day) is the maximum substrate degradation rate, C_s (mg/L) is the substrate concentration, and K_m (mg/L) is the apparent half-saturation constant.

In the system with EVO concentration of 18.18 g/L, which provided the carbon and energy source for indigenous microorganisms, was not the limiting factor due to the large excess added. The NB degradation rate did not continue to increase with increase in EVO addition at concentrations over 3.63 g/L. As mentioned above, microbially generated Fe(II) species reacted with NB as soon as they were generated. Thus, NB degradation in the two systems can also be expressed by the Michaelis–Menten equation, and the concentration of NB is the sole limiting factor.

NB concentration reduction within 24 hr occurred mainly due to physical absorption between EVO and NB. At the 7th, 8th and 9th days, Fe(II) began to accumulate and the NB concentration rapidly dropped due to the redox reaction between NB and Fe(II) (**Fig. 2**). These data do not reflect changes in NB caused by indigenous microorganism metabolism,

therefore, data was selected from the 1st day to the 6th day to build the NB degradation model.

An NB degradation equation deduced from the Michaelis–Menten equation is proposed as follows:

$$V_{NB} = V_{\max,NB} C_{NB} / (C_{NB} - K_{m,NB}) \quad (4)$$

Where, V_{NB} (mg/L/day) is the NB degradation rate, $V_{\max,NB}$ (mg/L/day) is the maximum NB degradation rate, C_{NB} (mg/L) is the NB concentration, and $K_{m,NB}$ (mg/L) is the apparent half-saturation constant of NB.

Eq. (4) can then be converted into the following:

$$1/V_{NB} = -K_{m,NB} / V_{\max,NB} \times 1/C_{NB} + 1/V_{\max,NB} \quad (5)$$

The relationship between $1/V_{NB}$ and $1/C_{NB}$ can be simplified as:

$$y = ax + b \quad (6)$$

where $y = 1/V_{NB}$ denotes the inverse of the NB degradation rate, $x = 1/C_{NB}$ denotes the inverse of the NB concentration at reaction time t , $a = -K_{m,NB}/V_{\max,NB}$ is the slope of the y - x line graph, and $b = 1/V_{\max,NB}$ is the intercept of the graph.

According to the slope and intercept of the $1/V_{NB}$ - $1/C_{NB}$ plot, based on the experimental data gathered at the EVO concentration of 18.18 g/L through three parallel experiments, $K_{m,NB}$ and $V_{\max,NB}$ were calculated as shown in **Fig. 3**.

The experimental data showed good agreement with Eq. (6); all correlation coefficients $R^2 > 0.9$. **Table 3** displays all the relevant parameters. Results showed that $V_{\max,NB}$ and $K_{m,NB}$ in the three parallel experiments were very close for NB degradation, so a kinetic model for NB degradation under excess EVO addition was established. The NB degradation model expression is shown in Eq. (7). The equation further indicates that the NB degradation process followed Michaelis–Menten kinetics when the EVO concentration was in excess, and that NB concentration was the sole limiting factor. The rate of NB degradation would increase with decreasing NB concentration.

$$V_{NB} = (0.412 \pm 0.009) C_{NB} / \{C_{NB} - (17.83 \pm 0.47)\} \quad (7)$$

2.4 NB degradation model verification

The above NB degradation model (with excess EVO) was verified through experimentation. **Fig. 4** shows a comparison of measured and simulated NB concentration in the system with EVO concentration of 3.63 g/L. The NB degradation model was found to agree well with the experimental data, proving that the NB degradation model proposed above is applicable when the mass ratio of EVO and NB reaches the critical value of 91:1, and that the NB concentration is the sole limiting factor.

2.5 Major factors affecting NB degradation in groundwater

30 experimental trials were conducted in Design Expert 8.0.6.1 to explore the effects of the four factors on the rate of NB degradation (Y1) and AN generation (Y2). These four variables included total hardness (50-250 mg/L), SO_4^{2-} (50-450 mg/L), NO_3^- (1-17 mg/L), and grain size (0.04-0.8 mm) (**Table 2**). The results obtained were then analyzed by ANOVA to assess significance levels. Both of the responses, Y1 (NB degradation) and Y2 (AN generation), were well-fit to the quadratic model with R^2 0.93 for Y1 and 0.84 for Y2.

Table 4 summarizes the effects of the model terms and associated p values. Both of the quadratic models were significant (where p value < 0.05 indicates the significance of a model at 95% confidence level). The sign and value of the effect values represent the tendency and magnitude of the variables' influence on the responses – the negative sign for the effect values of A, B, and D indicated that the variables had an inhibitive effect on NB degradation and AN accumulation. The positive sign for the C for Y1 and Y2 indicated that the rate of NB degradation and AN generation increased with increased NO_3^- . The effect values of all four variables in the fitted model for Y2 were greater than for Y1, which suggests that AN generation is more sensitive than NB degradation (Hao et al., 2012; Verma et al., 2009).

Response surface analyses were also plotted in three-dimensional model graphs. **Fig. 5a-c** shows the response surface plots for the effects of two independent variables on the NB degradation rate, while the other two variables were kept at the central level. The response surfaces in **Fig. 5a-c** would almost be planar, and the quadratic effects for AB, AC, AD, BC, BD and CD in **Table 4** close to zero indicate that the interactive effects between the variables

are very slight (Sabio et al., 2015). Only the linear effect and quadratic effect of grain size are significant for the NB degradation rate. **Fig. 5a** shows the response surface for the influence of grain size and nitrate on NB degradation, through which it was observed that the NB degradation rate can be improved significantly by decreasing the grain size of aquifer media. NB degradation rate can also be improved by increasing the nitrate concentration, because nitrate in groundwater can function as an intermediate electron acceptor similar to iron, and in fact, is easier to utilize (Dong, 2006). The influence of sulfate and nitrate on NB degradation in **Fig. 5b** confirmed that the NB degradation rate decreases as sulfate increases, although sulfate can also serve as an electron intermediary and product-sulfide can react with Fe(II) in the system (Fullerton et al., 2013). As shown in **Fig. 5c**, total hardness can slow the NB degradation rate. Ca-Mg ions in the liquid phase readily precipitate with hydroxyl ions, and the precipitate clings to the surface of the aquifer media and decreases the degradation rate of NB (Arnold and Roberts, 2000).

The degree of importance of the four factors was altogether dissimilar, as discussed above. This study sorted the influence of the four variables on the NB degradation rate according to the absolute value of the effects as follows: grain size of aquifer media > nitrate > sulfate > total hardness.

The **Fig. 5d-f** shows the response surface plots for the effects of two independent variables on the AN generation, while the other two variables were kept at the central level. The linear effect and quadratic effect of grain size, and the quadratic effect for AB have a significant influence on the AN generation. **Fig. 5d** demonstrates the effects of grain size and nitrate on AN generation. It was observed that the maximum accumulation of AN could be improved by decreasing the grain size of aquifer media and increasing the nitrate concentration. **Fig. 5e** shows that a high rate of AN generation was observed at sulfate concentration of 264.89 mg/L and nitrate concentration of 11.34 mg/L. **Fig. 5f** shows that the predicted maximum accumulation was 13.30 mg/L at a high value of sulfate concentration and low value of total hardness. AN accumulation decreased with the increase or decrease of both independent variables.

Altogether, the above analyses confirm that the grain size of aquifer media is the most significant factor among the four. The effects of the other three factors are negligible when the grain size of the aquifer media varies. What is more, the quadratic effects of AB, AC, AD, BC, BD, CD (representing interactions between the variables) for NB degradation rate are close to zero, while the quadratic effects are very different from zero for AN generation as shown in **Table 4**; this illuminates the clear interactive effects on AN generation and the scant interactions on NB degradation (Sabio et al., 2015). Therefore, this study concluded that NB degradation was primarily influenced by a single factor among the four, while AN generation was affected most by the interaction effects between the four independent variables.

2.6 Model modification

According to the above analysis of the effects of the four independent variables (SO_4^{2-} , NO_3^- , total hardness, and the grain size of aquifer media) on NB degradation, it was concluded that the grain size of aquifer media plays a key role in the process of NB degradation, while the other three factors play smaller roles in the process. Research therefore continued while focusing on the effects of the grain size of aquifer media on the NB degradation rate, and the NB degradation model was modified according to the experimental results.

As shown in **Fig. 6**, changes in the grain size of aquifer media had rather different effects on NB degradation. In systems with grain sizes 0.04, 0.23, and 0.42 mm, NB was thoroughly degraded at varying degradation rates. The smaller the grain size, the faster the NB degradation. In systems with grain sizes 0.61 and 0.8 mm, the degradation effect of NB was not obvious – most of the NB reduction was attributable to its physical adsorption by EVO (US Air Force, 2007), and there was no generation of AN. Based on the Michaelis–Menten model, NB degradation models were established for grain sizes 0.04, 0.23, and 0.42 mm (**Fig. 7**). $V_{\text{max-D}}$ and $K_{\text{m-D}}$ were obtained for each, as shown in **Table 5**.

The relationships between $1/V_{\text{max-D}}$ and $\ln D$, $K_{\text{m-D}}$ and D were then obtained as shown in **Fig. 8**. **Table 6** details the expression of the equations.

$$V_{\text{NB}} = \left\{ -1 / (0.65 \ln D + 0.53) \pm 0.01 \right\} C_{\text{NB}} / (C_{\text{NB}} - 34.92D - 10.22 \pm 0.47) \quad (8)$$

From the above results, a modified NB degradation dynamics model coupled to dissimilatory iron reduction was derived (Eq. (8)). Based on the modified model, the remediation period in an actual polluted site can be calculated.

3. Conclusion

The most notable conclusions of this study can be summarized as follows:

- (1) This study verified that NB reduction is effectively coupled with Fe(III) reduction conducted by indigenous microorganisms.
- (2) EVO addition was proven to readily stimulate microbial dissimilatory iron reduction, where NB can be reduced completely into AN.
- (3) A NB reduction Michaelis–Menten model was successfully established for excess EVO addition (where the mass ratio of EVO and NB reached the critical value 91:1).
- (4) The effects of four factors on NB reduction were thoroughly analyzed, and results indicated that the grain size of aquifer media plays the most significant role in NB reduction. The NB reduction dynamics model was modified accordingly.
- (5) Using the NB reduction dynamics model derived in this research, the remediation period can be estimated based on the aquifer medium size and the concentration of NB. The proposed NB reduction dynamics model has significance for guiding real-world remediation engineering of contaminated sites.

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JES-D-15-02440- table

Table 1 The variables and their levels for the central composite design (CCD)

Variables	symbols	Code levels				
		-α	-1	0	+1	+α
Total hardness (mg/L)	A	50	100	150	200	250
Sulfate(mg/L)	B	50	150	250	350	450
Nitrate (mg/L)	C	1	5	9	13	17
Grain size (mm)	D	0.04	0.23	0.42	0.61	0.80

Table 2 Experimental design and results of the CCD

std	Factor 1 A:	Factor 2	Factor 3	Factor 4	response	
	total	B:	C:	D:	NB	Max-AN
	hardness	SO ₄ ²⁻	NO ₃ ⁻	grain size	(mg/L/day)	(mg/L)
	(mg/L)	(mg/L)	(mg/L)	(mm)		
1	100	150	5	0.23	1.78	12.51
2	200	150	5	0.23	1.55	12.55
3	100	350	5	0.23	1.545	13.28
4	200	350	5	0.23	1.30	12.20
5	100	150	13	0.23	1.59	12.48
6	200	150	13	0.23	1.39	12.68
7	100	350	13	0.23	1.67	12.41
8	200	350	13	0.23	1.66	11.78
9	100	150	5	0.61	0.19	0.00
10	200	150	5	0.61	0.38	0.43
11	100	350	5	0.61	0.66	5.54
12	200	350	5	0.61	0.33	0.00
13	100	150	13	0.61	0.34	0.00

14	200	150	13	0.61	0.73	7.71
15	100	350	13	0.61	0.80	10.12
16	200	350	13	0.61	0.30	0.00
17	50	250	9	0.42	0.71	12.51
18	250	250	9	0.42	0.94	11.86
19	150	50	9	0.42	0.72	11.89
20	150	450	9	0.42	0.31	11.71
21	150	250	1	0.42	0.83	12.01
22	150	250	17	0.42	0.89	12.14
23	150	250	9	0.04	3.60	10.31
24	150	250	9	0.8	0.32	0.00
25	150	250	9	0.42	0.75	11.25
26	150	250	9	0.42	0.88	11.84
27	150	250	9	0.42	0.50	11.57
28	150	250	9	0.42	0.71	12.16
29	150	250	9	0.42	0.61	11.88
30	150	250	9	0.42	0.30	12.19

Table 3 Maximum reaction rate and half-saturation constant of NB degradation

	$1/V_{NB}-1/C_{NB}$	R^2	$V_{max,NB}$ (mg/L/day)	$K_{m,NB}$ (mg/L)
	$y=-42.68x+2.42$	0.94	0.412	17.61
NB degradation	$y=-42.93x+2.41$	0.95	0.415	17.83
	$y=-45.43x+2.48$	0.91	0.403	18.3

Table 4 The quantitative factor effects and associated p value for the responses

Source	Y1		Y2	
	Effect	p -Value	Effect	p -Value
Model		<0.0001		<0.0001
A	-0.021	0.7089	-0.43	0.4566

B	-0.022	0.6869	+0.28	0.6306
C	0.037	0.5081	+0.46	0.4296
D	-0.64	< 0.0001	-4.03	< 0.0001
A ²	0.046	0.3788	-0.34	0.5312
B ²	-0.032	0.5423	-0.43	0.4226
C ²	0.055	0.2944	-0.36	0.4987
D ²	0.33	< 0.0001	-2.09	0.0012
AB	-0.077	0.2642	-1.61	0.0334
AC	0.020	0.7719	+0.21	0.7675
AD	0.027	0.6916	-0.38	0.5902
BC	0.028	0.6771	-0.26	0.7151
BD	0.038	0.5776	+0.50	0.4742
CD	0.030	0.6558	+0.82	0.2537
Lack of fit		1.97		87.5

Table 5 $V_{\max-D}$ and K_{m-D} in different media size

Grain size	$1/V_{NB}-1/C_{NB}$	R^2	$V_{\max-D}$ (mg/L/day)	K_{m-D} (mg/L)
0.04 mm	$y=-8.43x+0.67$	0.96	1.55	12.59
0.23 mm	$y=-42.68x+2.42$	0.94	0.415	17.83
0.42 mm	$y=-590.84x+23.53$	0.99	0.042	25.11

Table 6 Equation expression between $V_{\max-D}$, K_{m-D} and D

The dependent variable	Equation expression	R^2
$V_{\max-D}$	$V_{\max-D} = -1/(0.6468*\ln D+0.53)$	0.99
K_{m-D}	$K_{m-D} = 34.921D + 10.221$	0.99