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Monitoring nitrate natural attenuation and analysis of indigenous micro-organism community in groundwater

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ABSTRACT

In the result of monitoring nitrate in about 6,000 groundwater wells around agricultural area, an average detection is about 9.0 mg/L and the excess rate of quality standard for drinking water was serious level as 45%. A shallow aquifer in some agricultural sites was highly contaminated showing 105–311 NO_3-mg/L. In this work, we evaluated monitored-natural attenuation (MNA) of nitrate by biological denitrification in highly nitrate-contaminated aquifer, known as effective technology to clean-up high concentration of nitrate, for confirming whether the groundwater is naturally remediated. We monitored nitrate degradation rate, microbial reaction (sulfate reduction, etc.) for 16 months according to oxidant and reduction rate. We also monitored microbial community change and amount of functional genes (*noeZ* and *nirK*) that encode for enzyme involved in denitrification pathway. In the result, since the groundwater was quiet oxidant condition, nitrate reduction was not monitored. However, there are functional genes and various microbial families related to denitrification process were detected such as methylomonas and methylomirabilis. These results indicate that there are possibilities of natural denitrification if the redox condition and other factors (carbon source, etc.) are supported. We also calculated the Hazard Index using RBCA Tool Kits to obtain remediation need of nitrate-contaminated groundwater.

Keywords: Nitrate; Biological denitrification; Functional genes (*nirK*, *nosZ*); Microbial family

1. Introduction

In the result of monitoring nitrate concentration in more than 6,000 shallow groundwater wells near livestock complexes across the nation, the excess rate of quality standard for drinking water was 45% and the average detection of nitrate concentration was about 9.0 mg/L [1]. Previous studies found that the shallow and aquifer water in the northern area of Nonsan had the maximum nitrate concentration of 49 mg/L and exceeded the drinking water standards by 22% [2].

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In addition, around 54% of 90% groundwater wells in Sunchang-gun were exposed to artificial contamination, and exceeded the quality standards for drinking water by 16% [3]. Nitrate concentration in groundwater is affected by multiple factors including dishwater, soil nitrogen and fertilizer, and excreta [4] and exacerbates the pollution of groundwater if not removed by physicochemical reactions such as biological decomposition and adsorption, and oxidation–reduction [5]. The groundwater with a high level of nitrate concentration can negatively affect animal health or produce quality, and when consumed as drinking water, it can cause severe diseases in humans such as cyanoderma and cancers [6].

The European Union (EU) established the EU Nitrates Directive in 1991 based on which the European Commission (EC) sets up strategies for the effective management of nitrate in groundwater, while the US designates priority management zones for the same purpose. However, their efforts cannot bear fruit without the application of technology that can reduce the level of nitrate concentration. The most representative nitrate reduction technologies include ion exchange, reverse osmosis, and biological denitrification [7,8]. Biological denitrification is a process that uses the anaerobic reduction process that transforms nitrate into the most existing and stable nitrogen gas and send back to the atmosphere, taking a critical role in biological nitrogen removal [9] and requiring relatively less cost [10]. It was reported that denitrification process uses NO_3 as terminal electron acceptor and takes place in the soil and groundwater environment with no oxygen or a low level of oxygen partial pressure (below DO 0.2 mg/L) [11]. In particular, the most well-known bacteria involved with denitrification process are Pseudomonas, Bacillus, Thiobacillus, and Propionibacillus.

Denitrification is a process of nitrate reduction to nitrogen gas (Eqs. (1) and (2)). The enzymes involved in each step include nitrate reductase (Nir), nitric oxide reductase, and nitrous oxide reductase. Especially, two classes of nitrite reductase (NiR) enzymes: cytochrome cd1 enzyme consisted of *nirS* and Cu-containing enzyme consisted of *nirK* have been identified for denitrification from nitrate to nitrite. The last step, N_2 reduction, is catalyzed by nitrous oxide reductase, and the enzyme involved in this process is *nosZ* [12].

$$NO_3^- + H_2 \rightarrow NO_2^- + H_2O \quad (1)$$

$$NO_2^- + 1.5H_2 + H^+ \rightarrow 0.5N_2 + 2H_2O \quad (2)$$

A research conducted in Nebraska, the Unite States, used acetate as carbon source to reduce the concentration of nitrate by 45% [13], and one in Korea carried out an on-site injection/extraction test in an alluvium region where the maximum nitrate concentration of 30 mg/L was reduced to below 3 mg/L [14].

This study established a monitoring well in a contaminated area by nitrate with the concentration of 105–311 NO_3 mg/L per year, and evaluated the possibility of nitrate natural attenuation by indigenous micro-organism and biochemical characteristics by monitoring nitrate natural attenuation, SO_4 changes, and other positive and negative ions, and analyzing denitrification-related genes and micro-organism communities based on the real-time RT-PCR for approximately 16 months. In addition, this study emphasized the necessity for reduction of nitrate concentration in contaminated groundwater based on the risks calculated using the RBCA Tool Kit.

2. Methods and materials

2.1. Field site characteristics

The field site where a high concentration of nitrate (105–311 NO_3 mg/L) was detected is basically used for ranch and takes the form of even ground nearby pigsties. The site is located on the eastern incline of the ridge developed from northeast to southwest direction. The field site is surrounded by agricultural areas, livestock manure, and manure storages (Fig. 1), and takes the form of even ground but has slopes on the periphery. Based on these characteristics, it is assumed that the construction site might have been prepared by organizing the slopes. The annual average concentrations of NH_4-N, NO_3, and Cl were 4.5, 173.9, and 140.7 mg/L, respectively (Table 1).

2.2. Slug test

A slug test is designed to cause a momentary change of the water level in a single well, observe the recovery patterns, and assume the hydraulic constants (hydraulic conductivity and storage coefficient) around the well. This test causes a change in the water level by extracting groundwater or injecting water in a moment (Fig. 2) [15]. The hydraulic constants derived from this test indicate the characteristics of media surrounding the test well, and are frequently used as supplementary data for pumping tests in the regions where a massive amount of pumping is not available. A slug test, unlike other site tests, is relatively simple

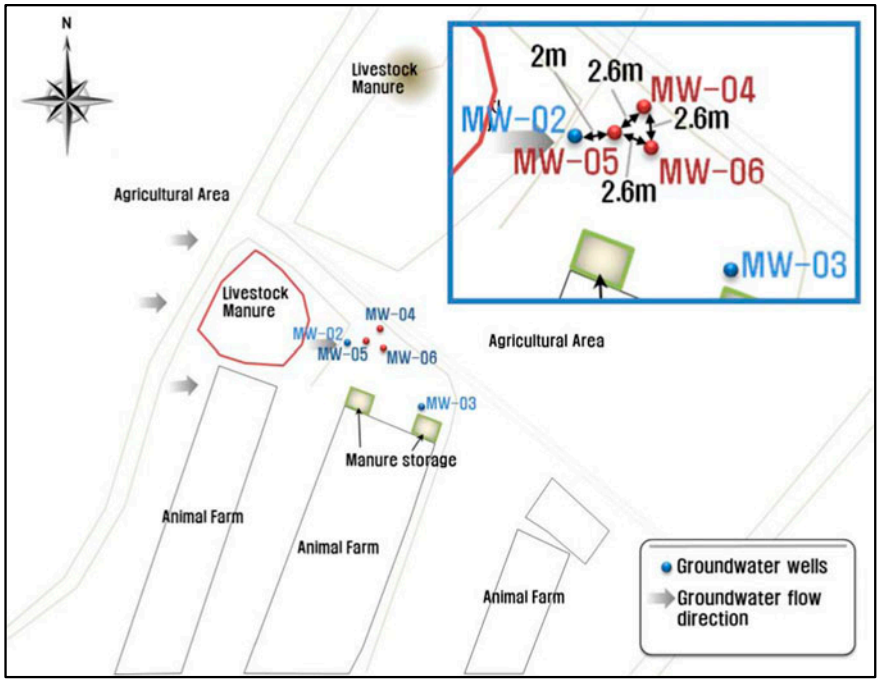


Fig. 1. Monitoring well-establishment position.

Table 1
NH_4-N, NO_3-N, EC, Cl Total coliforms in the site

Term	EC	NH_3-N	NO_3	Cl	Total coliforms
1	335	8.8	228.5	188.8	3,700
2	296	5.7	204.0	131.6	50
3	301	2.7	126.8	83.5	5,400
4	327	0.7	136.1	158.8	4,200
A.V.	314.8	4.5	173.9	140.7	15,487

and economical since it can be conducted in a single well. Moreover, it can be easily applied to the areas that consist of media with low permeability where the changes of water level in aquifer happen slowly. Also, when the test well is spatially widely distributed, the spatial distribution of hydraulic constants can be estimated.

For the interpretation of slug test results, a group of methods such as the Hvorslev method [16], Cooper et al. method [17], and Bouwer and Rice method [15] can be used according to the field characteristics. This study adopted the Bouwer and Rice method for interpretation since the depth of the test well was about 10 m, and most of the excavation area were the alluvial layers consisting of sand and silty sand, which justifies the assumption that the test was conducted in an unconfined aquifer. The formula of the Bouwer and Rice method is described below (Eq. (3)).

$$K = \frac{r_0^2 \ln(R_e/R)}{2L_e} \frac{1}{t} \ln\left(\frac{H_t}{H_0}\right) \quad (3)$$

where K is the hydraulic conductivity, r_c—well casing radius; R_e—well investigation radius, R—well excavation radius, L_e—well screen or open hole area, H_0—hydrograph when $t = 0$, H_t—hydrograph when t = elapsed time after H_0.

2.3. Measurement of field parameters and sampling of groundwater

For sampling, groundwater was purged until the measurement values of on-site water quality parameters (EC, pH, etc.) were stabilized. The stabilization of on-site water quality measurement values was evaluated based on the data of the United States Geological Survey (USGS) (Table 2) [18]. The amount of pumping was controlled to prevent the occurrence of disruption or air bubbles during sampling, and samples were carefully collected by not causing headspace in the top of the water sample bottles (aseptic sampling bottle or sterile bottle) so that changes in samples by air contact could be prevented. The samples were transported and stored while refrigerated at 0–4℃ to prevent any changes, and those expected to be highly contaminated were separately stored and transported.

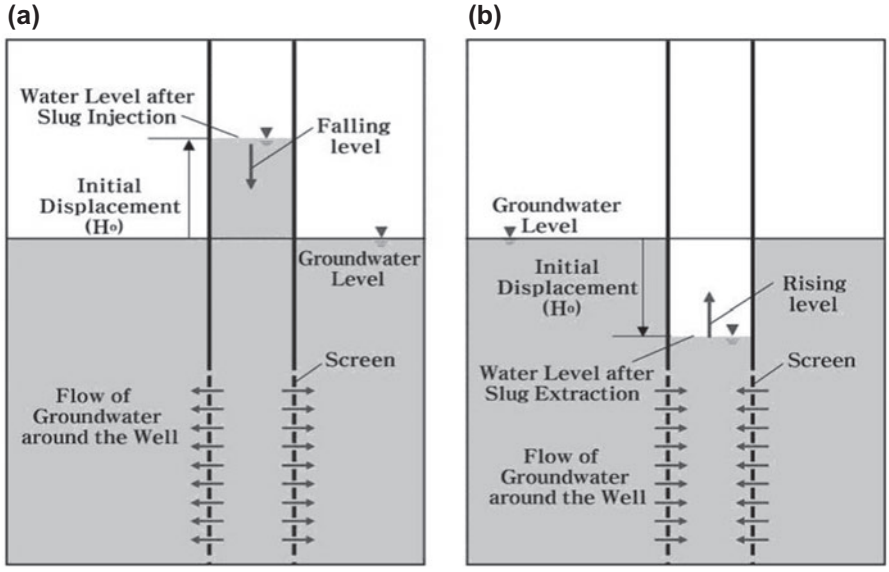


Fig. 2. Schematic diagram of slug test: (a) injection method and (b) extraction method.

Table 2
Stabilization criteria for recording direct field measurements (USGS 2008)

Content	Stabilization criteria
Electric conductivity	Conductivity ≤100 μS/cm, within 5% of full scale Conductivity >100 μS/cm, within 3% of full scale
Temperature	Within ±0.2°C of the thermistor thermometer
Hydrogen-ion concentration (pH)	Within ±0.1–0.2 pH unit
Dissolved oxygen	Within ±0.2 mg/L (±0.3 mg/L for continuous monitor)

2.4. Positive and negative ion analysis using ion chromatography

A total of 10 ions: 5 positive (Na^+, K^+, NH_4^+, Ca^{2+}, and Mg^{2+}) and 5 negative (NO_3^-, NO_2^-, Cl^-, SO_4^{2-}, and PO_4^{2-}) were analyzed using ion chromatography. Samples of 10 mL for each were collected for ion chromatography analysis, filtered using 0.45 μm PVDF membrane, and analyzed using CH/850 Professional IC (Metrohm, Herisau, Switzerland) and Metrosep C4, 150/4.0 (Metrohm, Herisau, Switzerland) and Metrosep A supp 5, 150/4.0 (Metrohm) were used as column, and 0.1 mM H_2SO_4 as mobile phase.

2.5. Multiple-parameter analysis

A total of seven parameters including T-N, Mn, MnO_4^{2-}, $KMnO_4$, Fe^{2+}, T-Fe, and S^{2-} were analyzed using multiple-item analyzer. Samples of 10–25 mL for each were collected for multiple-item analysis, filtered using 0.45 μm PVDF membrane, and analyzed using DR/890 Colorimeter (Loverland, CO, USA).

2.6. DNA extraction

The frozen samples were thawed and transferred to a sterilized centrifuge tube, and then went through the process of centrifugation for 10 min at 4°C and 3,000 rpm. The DNA of the cell pellet, of which supernatant was carefully removed after centrifugation, was extracted and separated using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). According to the instruction manual, germs were floated in buffer solution (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme), and then left for reaction at 37°C for 30 min and at 70°C for 30 min with Proteinase *K* added. After adding ethanol, the DNA was purified using the column. The concentration and purity (260/280 ratio) of the purified DNA were verified using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, USA).

2.6.1. PCR and sequence analysis

After extracting genomic DNA, RT-PCR analysis was conducted using a gene amplification device (MyGenieTM 32 Thermal Block, Daejeon, Korea). The Table 3 describes the sequence of primers used to amplify the functional genes (*nirK*, *nirS*, *nosZ*) that constitute the enzymes involving in denitrification process; amplification degree of PCR product; and RT-PCR conditions. After performing a PCR reaction, an agarose gel electrophoresis was carried out. For the verification of the final sequence, the PCR product collected from agarose gel was ligated to pGEM-T vector and transformed into *E. coli* JM109. After selecting transformed clones, DNA sequencing was performed using T7 promoter primer or SP6 promoter primer, and ABI 3100 automatic sequencer. The specific process of analysis followed the Maniatis method [19]. The sequences found were analyzed using the Lasergene program (DNA STAR, Inc., Madison, WI, USA) and the database of a BLAST search of GenBank.

2.6.2. Micro-organism community analysis

Micro-organism communities were monitored using the pyrosequencing based on 16S rRNA gene. The variable regions (V1–V3) of 16S rRNA were amplified from genomic DNA using fusion primers. The analysis of amplification conditions, sequence library construction, and sequences was conducted using 454 GS FLX Junior Sequencing System (Roche, Brandford, CT, USA).

2.7. Risk assessment

The risk assessment was conducted using the RBCA Tool Kit. The RBCA Tool Kit for Chemical Releases is a software developed by the American Society for Testing and Materials (ASTM) that contains all calculation process and function for the evaluation of Tier 1 and Tier 2 suggested by the ASTM. The assessment process using the RBCA Tool Kit selects Tier 1 and Tier 2 according to its purpose, and is conducted in the following order: selection of exposure pathways and input of exposure factors, selection of contaminants and input of concentration, input of field characteristic data, and confirmation of results [20–22].

The concentration and factors used for the risk assessment using the RBCA are described in Table 4. The risk assessment used the concentration of 95 percentile and calculated non-carcinogen risks only due to the absence of toxicity value for evaluating the carcinogenic risks of nitrate. Only intake pathway was considered among the exposure pathways to groundwater (Fig. 3), and the most conservative Tier 1 was selected for calculation using the RBCA Took Kit.

3. Results and discussion

3.1. Slug-test results

Most of the media in the aquifer surrounding the test wells designed for the slug test were composed of sand and silty sand, and the initial water levels (depth to water) of each test well are as follows: 3.91 m at MW-04, 2.30 m at MW-05, and 2.40 m at MW-06. The specific data on the test wells are described in Table 5.

Table 3
Base sequence of denitrification enzyme and PCR condition

Primer	Priver sequence	Product size (bn)	PCR condition	
nirK876	ATYGGCGGBAYGGCGA	165	95℃, 10 min	
			95℃, 15 s	5 cycle touchdown (−1℃)
			63–58℃	
			30 s	
			72℃, 30 s	
NirK5R	GCTGCATCAGRTTRTGG		95℃, 15 s	40 cycle
			58℃, 30 s	
			72℃, 30 s	
NosZ2F	CGCRACGGCAASAAGGTSMSSGT	259	95℃, 10 min	
			95℃, 30 s	5 cycle touchdown (−1℃)
			65–60℃	
			30 s	
			72℃, 30 s	
NosZ2R	CAKRTGCAKSGCRTGGCAGAA		95℃, 15 s	40 cycle
			60℃, 15 s	
			72℃, 30 s	

Since different accuracies of variation values of initial water level can result in different interpretations, the slug test was performed using an automatic water level meter to observe the changes in water level every 0.5 s. The data on the changes in water level according to the elapsed time were entered into the hydraulic analysis program AQTESOLV (Ver. 4.5, HydroSOLVE, Inc.) for interpretation. Based on the assumption that the test wells were established in an unconfined aquifer, the Bouwer and Rice method was adopted to estimate hydraulic conductivity. The values of hydraulic conductivity according to the slug test are as follows: 1.29×10^{-4} cm/s at MW-04, 1.63×10^{-4} cm/s at MW-05, and 2.95×10^{-4} cm/s at MW-06 (Table 6). The average hydraulic conductivity of test wells is in the range of $1.29–2.95 \times 10^{-4}$ cm/s, indicating the values of soil consisting of silt or loess to coarse sand [23]. This implies that the intuitive classification of particle sizes which were recorded in the process of soil sampling had the similar results. Based on the data on groundwater level collected from the slug test, the groundwater flow direction and level distribution are described in Fig. 4. Groundwater is distributed from MW-05 and MW-06 to the direction of MW-04 and MW-02, forming a comparatively steep slope, and to the direction of MW-03 monitoring well, a relatively an easy slope. However, the data used here cannot be considered as an absolute groundwater level since they were collected from one-time slug test and seasonal changes are expected.

3.2. Groundwater field parameters and nitrate changes

3.2.1. On-site water quality measurement

The results (Table 7) of measuring the water quality in the monitoring wells (MW-03, 04, 05, 06) show that the average pH level was 5.7–6.4, which is slightly acid and lower than that of the national groundwater monitoring network [24]. The electrical conductivity was 999.6–1161.6 μS/cm, which is higher than the average pH level of the national groundwater

Table 4
Parameter and concentration for Risk assessment in RBCA Tool Kits

Well	Cons. (mg/L)	Parameter	
MW-03	253	Contact rate (CRw)	2 L/d
MW-04	325	Exposure frequency (EF)	350 d/y
MW-05	270	Exposure duration (ED)	30 y
MW-06	295	Body weight (BW)	70 kg
		Averaging time (AT)	10,950 d (30 × 365)
		Reference dose (RfD)	1.6 mg/kg/d

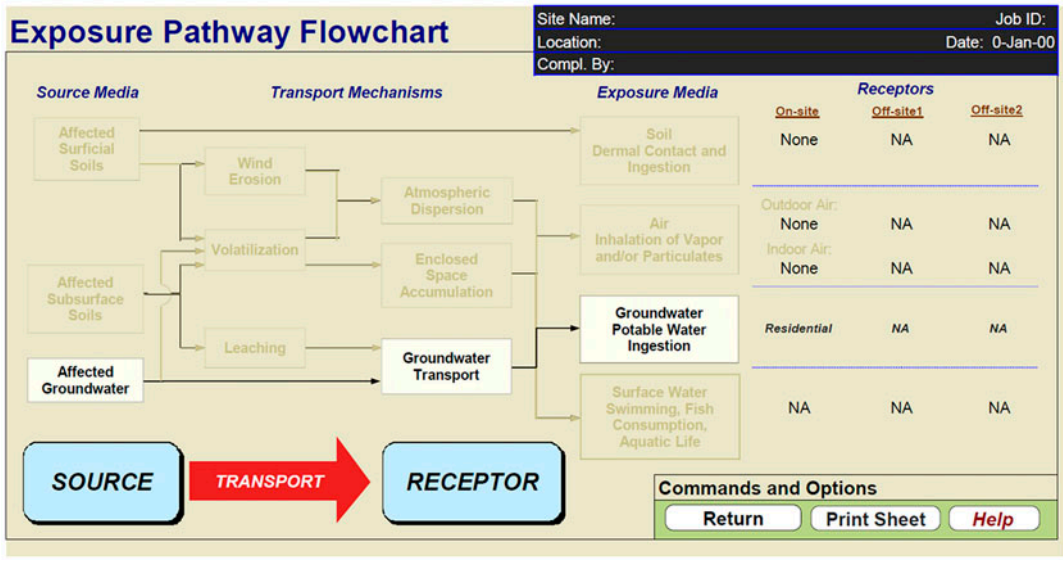
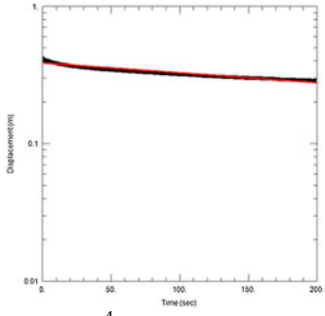
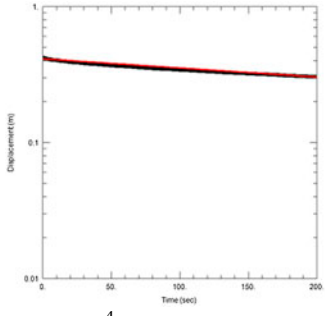
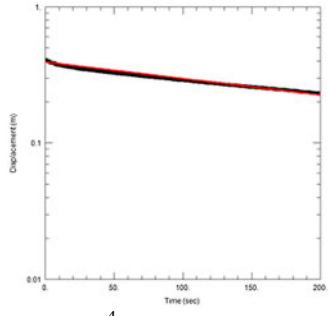


Fig. 3. Exposure pathway screen of RBCA Tool Kit.

Table 5
Designs of test wells

Well	Initial water lever (DTW, m)	Well depth (m)	Inner diameter (mm)	Well screen length (m)	Well casing length (m)
MW-04	3.91				
MW-05	2.30	10	50.8	7	7
MW-06	2.40				

Table 6
Slug test and hydraulic conductivity interpretation

Well	MW-04	MW-05	MW-06
Slug test interpretation			
Hydraulic conductivity (cm/s)	1.29×10^{-4}	1.63×10^{-4}	2.95×10^{-4}

monitoring network 874.3 μS/cm. This indicates that an increase in nitrate and ammonia nitrogen concentration has led to a dramatic increase of electrical conductivity in groundwater. The average oxidation reduction potential was 231.6 mV, and the average concentration of dissolved oxygen in groundwater was 3.8 mg/L. When groundwater has dissolved oxygen concentration of less than 2 mg/L and oxidation reduction potential of less than 200 mV, it indicates a reducing environment [25] where denitrification cannot take place due to a high level of oxygen concentration.

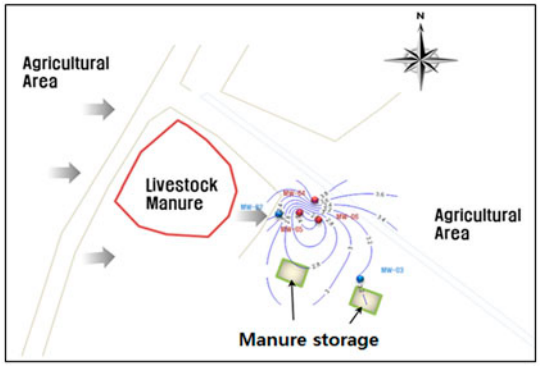


Fig. 4. Groundwater flow direction and groundwater level distribution.

3.2.2. NO_3 changes in shallow groundwater

The preliminary research shows the result of monitoring NO_3 in five test wells in Fig. 5. The average concentration of NO_3 in monitoring wells is as follows: MW-03 (227.6 mg/L), MW-04 (279.1 mg/L), MW-05 (147.8 mg/L), and MW-06 (242.4 mg/L). MW-04 and MW-06 had a higher level of NO_3 concentration than the other two as 73.0 mg/L on average, which implies that sources of contamination from nearby livestock manure and agricultural activities had continuously moved to MW-04 and MW-06 along with groundwater flow as seen in Fig. 4. The comparison of NO_3 monitoring results with precipitation allows the confirmation of their correlation (Fig. 5). The value of K was 1.29–2.95×10^{-4}, and the level of NO_3 concentration in aquifer where groundwater moves relatively slowly was detected in a consistent manner during the monitoring period. In July 2013, however, when the amount of precipitation drastically increased, the level of NO_3 concentration significantly decreased. This implies that the increased amount of precipitation lowered the level of NO_3 concentration. Nevertheless, the level of NO_3

Table 7
Field parameter of temperature, pH, EC, ORP, DO in monitoring wells

	Temp. (℃)	pH	EC (μs/cm)	ORP (mV)	DO (mg/L)
MW-03	13.5	5.9	1109.2	268.0	3.7
MW-04	14.3	5.7	1076.2	232.1	4.1
MW-05	14.7	6.4	1161.6	186.7	4.6
MW-06	14.5	5.9	999.6	239.7	3.0

concentration increased back to the original level after 9 months, which indicates that NO_3 coming from nearby sources of contamination had continuously flowed into groundwater.

Although previous researches [26,27] reported that precipitation can lead to an increase or a decrease in the level of NO_3 concentration, the results of this study are expected to contribute to the evaluation of

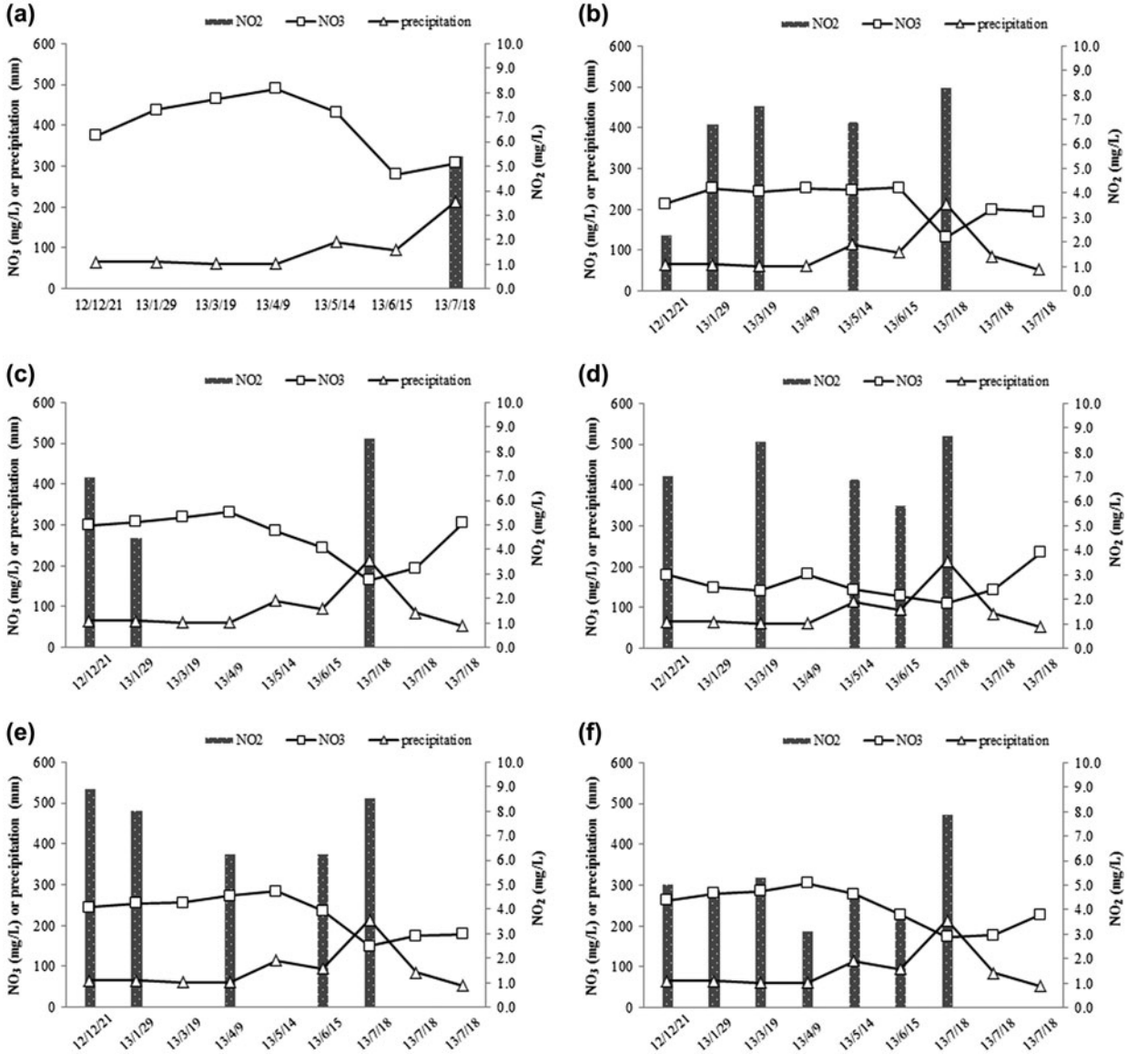


Fig. 5. Monitoring NO_3 concentration with precipitation and NO_2 in 5 different wells (a–e) and average concentration (f).

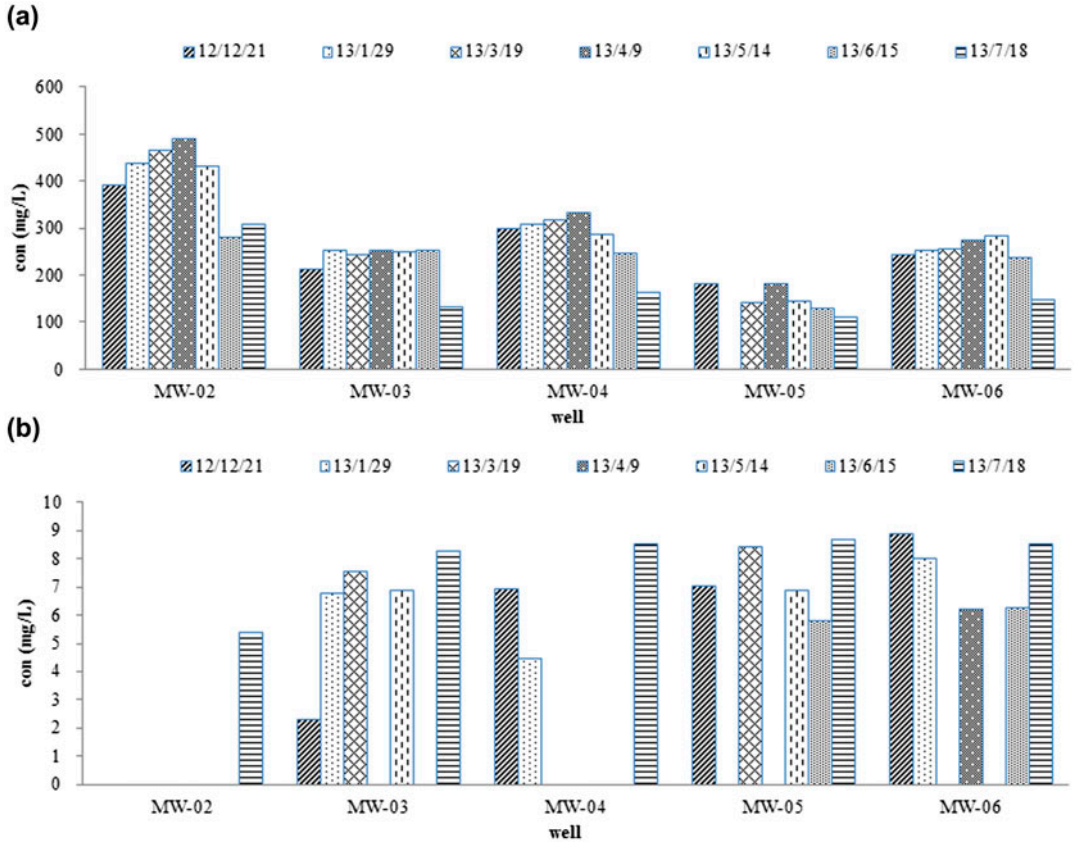


Fig. 6. Monitoring results of NO_3 (a) and NO_2 (b) in groundwater well 2–5 for 16 months.

changes in non-point pollutant sources. As seen in Fig. 6, the results of monitoring nitrate and nitrite in five groundwater wells for 16 months show that the level of concentration was the highest at MW-02, which is the closest to the livestock manure and the lowest at MW-05. When it comes to seasonal changes, the observed values from December to May were higher than those from June to July but with no

Table 8
Monitoring results of chemical elements (Fe, Mn, S) according to oxidate state and HI in well 3–6

Well	Sampling date	ORP (mV)	Fe^{2+} (mg/L)	T-Fe (mg/L)	Mn (mg/L)	Mn of MnO_4 and $KMnO_4$ (mgMn/L)	MnO_4^- (mg/L)	$KMnO_4$ (mg/L)	S^{2-} (mg/L)	SO_4^{2-} (mg/L)	HI
MW-03	13 June 2015	269.0	0.04	0.05	4.0	4.9	10.6	14.1	0.0	169.1	4.3
	14 April 2014	283.0	0.04	0.06	13.9	13.9	30.1	40.2	0.0	76.0	
	14 May 2014	211.2	0.03	0.06	11.7	11.7	25.3	33.7	0.0	169.7	
MW-04	13 June 2015	238.5	0.02	0.02	4.3	4.3	9.4	12.5	0.0	185.5	5.6
	2 April 2014	244.0	0.05	0.06	2.0	2.0	4.3	5.8	0.0	68.8	
	14 May 2014	173.8	0.02	0.09	2.4	2.4	5.3	7.0	0.0	190.8	
MW-05	15 June 2013	273.3	0.02	0.03	10.8	10.8	23.3	31.0	0.0	185.3	4.6
	2 April 2014	230.8	0.02	0.10	9.7	9.7	20.9	27.9	0.0	87.7	
	14 May 2014	183.7	0.00	0.01	7.2	7.2	15.5	20.7	0.0	187.4	
MW-06	13 June 2015	269.9	0.03	0.03	23.0	22.9	49.6	66.2	0.0	158.1	5.1
	2 April 2014	255.6	0.03	0.20	16.1	16.1	34.9	46.5	0.0	86.4	
	14 May 2014	198.7	0.03	0.03	11.5	11.4	24.7	33.0	0.0	183.7	

Table 9
Standard reduction potential (E^0) of selected redox couples

Redox scale			
N_2O/N_2	+1,355 mV	NO_2^-/NH_4^+	+440 mV
ClO_2^-/Cl^-	+1,199 mV	NO_3^-/NO_2^-	+430 mV
$2NO/N_2O$	+1,175 mV	NO_2^-/NO	+350 mV
O_2/H_2O	+820 mV	HSO_3^-/HS^-	−110 mV
ClO_4^-/ClO_3^-	+788 mV	CO_2/CH_4	−240 mV
Fe^{3+}/Fe^{2+}	+772 mV	SO_4^{2-}/HSO_3^-	−516 mV
ClO_3^-/ClO_4^-	+709 mV		
MnO_2/Mn^{2+}	+380 mV		

Note: Standard reduction potentials (pH 7; 25°C) were derived from [28].

significant differences. The nitrate concentration in MW-02 (Fig. 6) was the highest among five wells during the monitoring period, while the value of nitrite was very low or not even detected except in July.

These results indicate that nitrate was reduced to nitrite by indigenous micro-organisms in some wells but at a very slow rate. In addition, what is interesting is that nitrite was not detected in MW02 that showed the highest level of nitrate concentration except in July when the temperature was high. This implies that the degree of nitrate reduction can be different depending on the well.

3.3. Multiple-parameter analysis results

The Table 8 shows the result of monitoring iron in 12 samples of groundwater that iron exists in the form of Fe^{2+} regardless of season or well location. This means that iron in groundwater exists in the form of Fe^{2+}, a more reduced form than Fe^{3+}. The ORP of on-site groundwater was 173–283 mV, which is considered to provide an appropriate condition where Fe^{3+} can be reduced to Fe^{2+} by iron reducer (Table 8). However, sulfur in groundwater existed in the oxidized form (SO_4^{2-}) during the monitoring period. As seen from Table 9, the redox scale of the field allows iron reduction by iron reducer, but fails to provide a condition where sulfur can be reduced by sulfate reducer (−516 mV). Moreover, the field is considered as an appropriate condition for denitrification

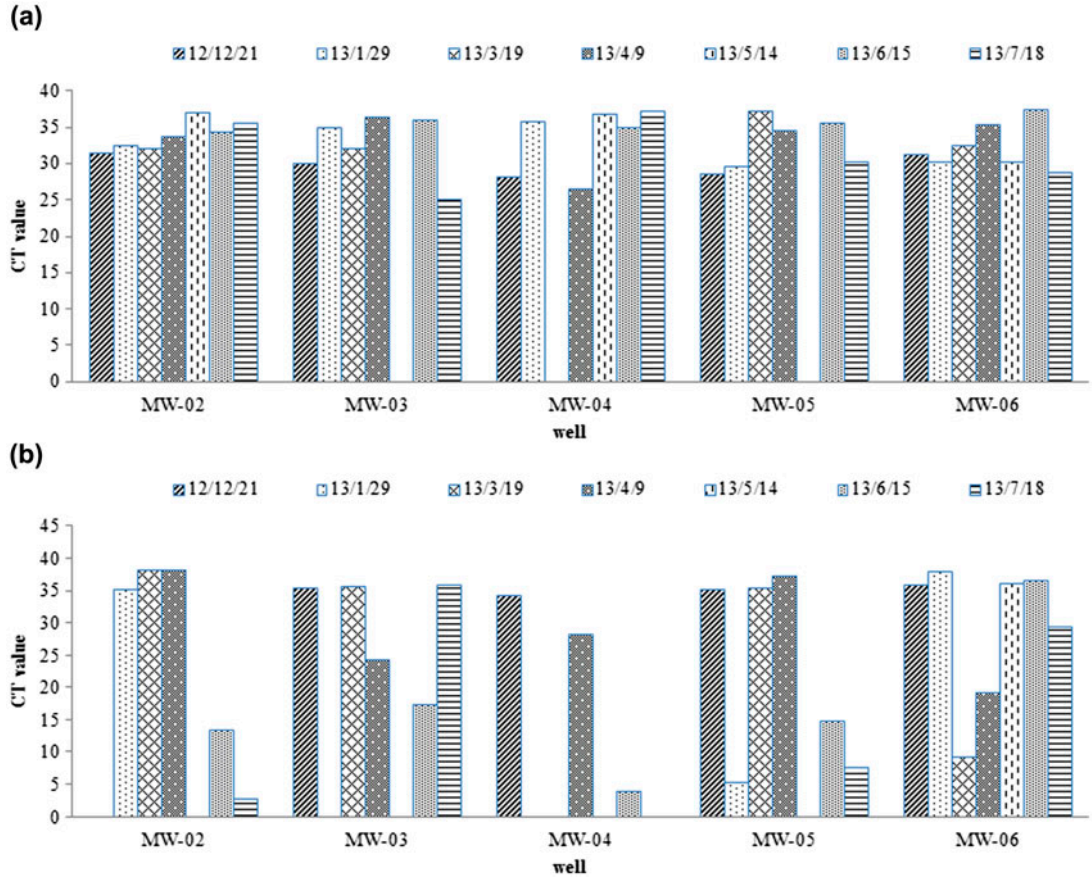


Fig. 7. Monitoring results of *nosZ* (a) and *nirK* (b) in groundwater well 2–5 for 16 months.

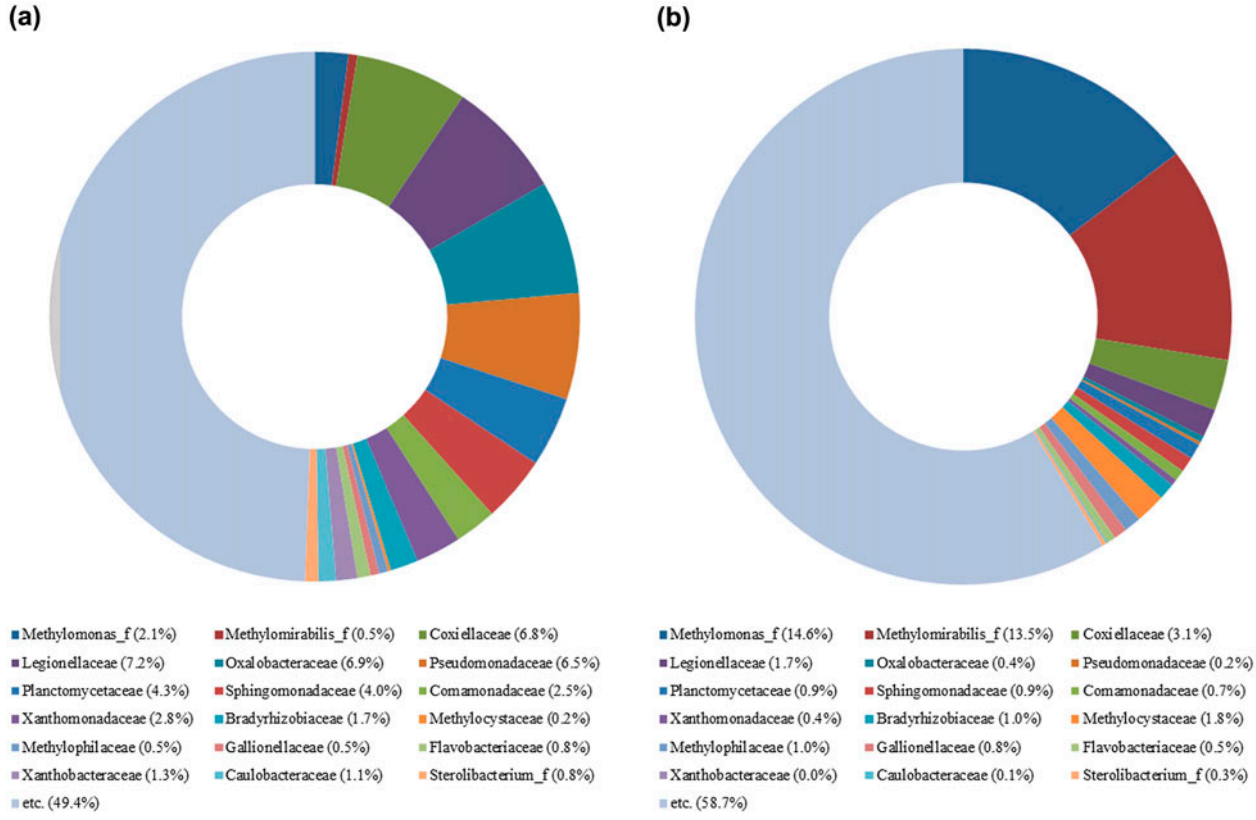


Fig. 8. Composition of micro-organism family in MW-05 (a) and MW-06 (b).

(430 mV). In this regard, when electrons are supplied by proper carbon sources, it seems that denitrification will be possible by indigenous micro-organisms in the field.

3.4. Micro-organism analysis results (including denitrification)

In the result of analyzing functional genes (*nosZ*, *nirK*) that constitute enzymes related to denitrification in monitoring wells(MW-02, 03, 04, 05, 06) (Fig. 7), *nosZ* was detected at a more relatively constant concentration than *nirK* in all monitoring wells regardless of the period. Since *nosZ* plays a role in reducing nitrite to nitrogen gas, the detection of the more *nosZ* leads to the expectation of the more active reduction to nitrogen gas. The result of analyzing *nosZ* in the groundwater of the observed field shows that the CT values were mostly more than 30, confirming the existence of *nosZ* in almost every sample from all monitoring wells despite the low level of enzyme concentration in groundwater. This allows a prediction that the reduction of nitrite to nitrogen gas is continuously proceeded in the concerned field. Moreover, the observation that the *nosZ* CT value was the highest in MW-02 where nitrite was rarely detected leads to the confirmation of the relation between nitrite and the concerned enzyme in groundwater. The reduction of nitrate to nitrite is affected by enzymes such as *nirK* and *nirS*. Fig. 7 shows the result of real-time PCR of *nirK* where the CT values were measured in the diverse range of ND–37.97. And the level of nitrate concentration in groundwater was very high as 110.8–490.4 mg/L. However, the correlation between these two graphs was not clearly shown as known before. In order to identify what kind of micro-organisms exists in the groundwater for test except the two enzymes with denitrification function, the micro-organism communities in MW-05 and MW-06 were analyzed at the level of family as seen in Fig. 8. In MW05, 304 kinds of families were found including Legionellaceae, Oxalobacteraceae, Pseudomonadaceae, Coxiellaceae, and Planctomycetaceae, and 289 families, of which each took up less than 1%, were combined to account for 49.4%. A total of 370 kinds of families were found in MW-06, and 354 families, of which each

took up less than 1%, were combined to account for 58.7%. Major families found include Methylomonas, Methylomirabilis, and Coxiellaceae. A total of 474 kinds of families were found in MW-05 and MW-06, and 200 kinds of families were detected in both wells, taking up approximately 42.2% of the total families. Families detected in both wells include Methylomonas, Methylomirabilis, Methylocystaceae, Methylophilaceae, Oxalobacteraceae, and Sterolibacterium that engage in denitrification; Legionellaceae, Sphingomonadaceae, Gallionellaceae, Flavobacteriaceae, and Methylophilaceae that are involved with organic fermentation; and Pseudomonadaceae and Comamonadaceae that participate in the reduction of sulfate (Fig. 8).

3.5. Risk assessment results

The results of risk assessment using the RBCA are described in Table 4. To assess non-carcinogen risks, the Hazard Quotient (HQs) are first calculated, and then the HQs of all routes are added and integrated into the Hazard Index (HI). The HQ and HI values of over 1 indicate the possibility of risks due to the exposure to pollutants. When calculating the non-carcinogen risks of nitrate, the HQs of each well were MW-03 (4.3), MW-04(5.6), MW-05(4.6), and MW-06(5.1). Since all HQ values exceeded 1, it is concluded that risks of nitrate exist in this region.

4. Conclusion

In the results of monitoring the groundwater contaminated by high concentration nitrate in agricultural and livestock areas for approximately 16 months, the concentration of nitrate was observed at a constant level during the monitoring period except the rainy season that witnessed a temporary reduction in the concentration level. In particular, the concentration of nitrite was monitored at a constant level (average –3.0 mg/L) in monitoring wells.

(1) The detection of denitrification-related functional genes (*noeZ* and *nirK*) and of denitrification-related micro-organism communities (Methylomonas, Methylomirabilis, Methylocystaceae, Methylophilaceae, Oxalobacteraceae, Sterolibacterium, etc.) indicates the existence of denitrification-related micro-organisms in the field groundwater.
(2) In addition, the results of monitoring the redox-active compounds of iron, manganese, and sulfur ion and the field ORP show that the conditions are suitable for active iron reducers and denitrifers but not for active sulfate reducers.
(3) Since the field has appropriate conditions for the existence of denitrification-related micro-organisms and oxidation–reduction potential enough for denitrification, it is considered that some nitrite was detected during the monitoring period because of denitrification. However, the amount of electron donors is considered not enough to remove high concentration nitrate.
(4) The risk assessment using the RBCA Tool Kits shows the value of HI was 4.9 on average, meaning that active reduction of nitrate in the field groundwater is required. This phenomenon is commonly observed in most agricultural groundwater, and more active measures need to be taken to remove pollutant sources and reduce the concentration of nitrate in the groundwater contaminated by high concentration nitrate in the future.

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References

[1] Research on the Actual Pollution of Livestock Area Background Concentration, Nier, 2012.
[2] E.Y. Kim, D.C. Koh, K.S. Ko, I.W. Yeo, Prediction of nitrate contamination of groundwater in the northern Nonsan area using multiple regression analysis, J. Soil Groundw. Environ. 13(5) (2008) 57–73.
[3] S.R. Jeon, S.J. Park, H.S. Kim, S.K. Jung, Y.U. Lee, J.I. Chung, Hydrogeochemical characteristics and estimation of nitrate contamination sources of groundwater in the Sunchang area, Korean J. Geol. Soc. Korean 47 (2) (2011) 185–197.
[4] K.H. Kim, S.T. Yun, G.T. Kim, B.Y. Chae, S.O. Choi, K.J. Kim, H.S. Kim, C.W. Lee, Nitrate contamination of alluvial groundwaters in the Keum river watershed area: Source and behaviors of nitrate, and suggestion to secure water supply, J. Eng. Geol. 12(4) (2002) 471–484.
[5] H.J. Suk, C.M. Chon, Model development for analysis of nitrate leaching and its field application in a rural area, Econ. Environ. Geol. 42(6) (2009) 561–574.
[6] J. Ginocchio, Nitrate levels in drinking water are becoming too high, Water Serv. 88(10058) (1984) 143–147.
[7] Emerging Technologies for Enhanced In Situ Biodenitrification (EISBD) of Nitrate-Contaminated Ground Water. ITRC, 2000.
[8] Nitrate and Nitrite in Drinking Water, Health Canada, 2013.
[9] M.H. Gerardi, Nitrification and Denitrification in the Activated Sludge Process, Wiley-Interscience, John Wiley and Sons Inc. , New York, NY, 2002.

[10] B.E. Rittmann, L.M. Perry, Environmental Biotechnology: Principles and Applications, McGraw-Hill, New York, NY, 2001.
[11] P.A. Matson, R.C. Harriss, Biogenic Trace Gases: Measuring Emissions From Soil and Water, Blackwell, Oxford, 1995.
[12] N. Throback, K. Enwall, A. Jarvis, S. Hallin, Reassessing PCR primers targeting nirS, nirK, and nosZ genes for community surveys of denitrifying bacteria with DGGE, FEMS Microbiol. Ecol. 49 (2004) 401–417.
[13] I.A. Khan, R.F. Spalding, Enhanced *in situ* denitrification for a municipal well, Water Res. 38 (2004) 3382–3388.
[14] In-situ Bioremediation Using Single Well Push-Pull Test and Well to Well Test for Nitrate Contaminated Ground Water, Ministry of Environment, 2005.
[15] H. Bouwer, R.C. Rice, A slug test for determining hydraulic conductivity of unconfined aquifers with completely or partially penetrating wells, Water Res. Res. 12(3) (1976) 423–428.
[16] M.J.Hvorslev, Time Lag and Soil Permeability in Ground-Water Observations. Bulletin 36, US Army Crops of Engineers. Waterways Experiment Station, Vicksburg, 1951.
[17] H.H. Cooper, J.D. Bredehoeft, S. Papadopulos, Response of a finite-diameter well to an instantaneous charge of water, Water Res. Res. 3(1) (1967) 263–269.
[18] Documentation for the 2008 update of the United States National Seismic hazard maps. USGS, 2008.
[19] S. Kahng, B.A. Iwata, A.B. Lewin, Behavioral treatment of self-injury, 1964–2000, Am. J. Mental Retardation 107 (2002) 212–221.
[20] ASTM, Standard Guide for Risk-Based Corrective Action Applied at Petroleum Release Sites (E 1739–95), ASTM International, West Conshohocken, PA, USA, 2010.
[21] ASTM, Standard Guide for Risk-Based Corrective Action (E2081–00), ASTM International, West Conshohocken, PA, USA, 2010.
[22] T.W. Nam, H.R. Ryu, Y.J. Kim, S.O. Ko, K.T. Baek, K.P. Nam, Development of Korean risk-based corrective action (K-RBCA), J. Soil Groundwater Environ. 16 (1) (2011) 1–11.
[23] P.A. Domenico, F.W. Schwartz, Physical and Chemical Hydrogeology. John Wiley & Sons, Inc., New York, NY, 1990, pp. 26, 65, 118.
[24] Ministry of Environment, National Groundwater Monitoring Network, Sejong, South Korea, 2013.
[25] E.E. Cey, D.L. Rudolph, R. Aravena, G. Parkin, Role of the riparian zone in controlling the distribution and fate of agricultural nitrogen near a small stream in southern Ontario, J. Contam. Hydrol. 37 (1999) 45–67.
[26] S.G. Yun, Behavior of nitrate and accompanying cations derived from urea under upland condition, J. Korean Soc. Soil Sci. Fert. 27(1) (1994) 15–20.
[27] T.G. Oh, K.S. Kim, Y.J. Kim, S.R. Kim, Nitrate and chloride characteristics in the groundwater in Jeju area, J. Environ. Res. 3 (2006) 1–14.
[28] R.K. Thauer, K. Jungermann, K. Decker, Energy-conservation in chemotropic anaerobic bacteria, Bacteriol. Rev. 41 (1977) 100–180.