1 **Combined use of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ tracers to study anaerobic**
2 **bacterial processes in groundwater flow systems**

3
4 **Takahiro Hosono^{1*}, Takahiro Tokunaga², Akane Tsushima^{1#}, Jun Shimada²**

5
6 ¹Priority Organization for Innovation and Excellence, Kumamoto University, 2-39-1 Kurokami,
7 Kumamoto 860-8555, Japan

8 ²Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto
9 860-8555, Japan

10 -----
11 *Corresponding author: Tel & Fax: +81-96-342-3935. Email addresses: hosono@kumamoto-u.ac.jp

12 #Present address: Institute of Low Temperature Science, Hokkaido University, Kita-19, Nishi-8,
13 Kita-ku, Sapporo 060-0819, Japan

14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29 Resubmitted to:

30 **Water Research**

31
32 23 December, 2013

Abstract

We present an approach for determining the major anaerobic bacterial processes in aquifers, using the combined stable isotope ratios of major elements (C, N, and S) as net recorders of the biogeochemical reactions. The Kumamoto groundwater is constituted of two major flow systems, A-A' and B-B', within 10³ km-scale area. Previous study [Hosono, T., Tokunaga, T., Kagabu, M., Nakata, H., Orishikida, T., Lin, I-T., Shimada, J., 2013. The use of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ tracers with an understanding of groundwater flow dynamics for evaluating the origins and attenuation mechanisms of nitrate pollution. *Water Res.* 47, 2661-2675.] investigated the nitrate sources and extent of denitrification using $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ tracers. In the present study, we studied a type of denitrification (heterogenic vs. autotrophic) and occurrence of sequential anaerobic processes along the flow systems with newly measured $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$. In A-A' flow system, C, N, and S isotopic compositions did not change along the flow direction. However, in B-B' flow system significant sulfate reduction (with a maximum $\delta^{34}\text{S}_{\text{SO}_4}$ enrichment of +55‰) occurred along with denitrification (with a maximum $\delta^{15}\text{N}_{\text{NO}_3}$ enrichment of +38‰) as the groundwater flowed down-gradient. Depletions in $\delta^{34}\text{S}_{\text{SO}_4}$ (−8‰ maximum) were found only sporadically. These observations imply that autotrophic denitrification could occur in very limited parts of the study area. Moreover, the occurrence of methanogenic reactions was suggested by the enriched $\delta^{13}\text{C}_{\text{DIC}}$ signature (+8‰ maximum) at a denitrification hotspot. By characterizing C, N, and S isotope compositional changes in wide redox range (from aerobic oxidation of organic carbon, through denitrification, to sulfate reduction, until methanogenesis), we could develop the model of C, N, and S isotopic evolutionary patterns under different redox scenarios. Proposed model is useful in obtaining a comprehensive understanding of the major anaerobic bacterial processes in aquifer systems, including distinguishing between heterotrophic and autotrophic denitrification.

Keywords; stable isotopic compositions, denitrification, anaerobic biogeochemical process, groundwater, Kumamoto

1. Introduction

Nitrate pollution is one of the most profound ongoing groundwater problems around the world. Besides studying the pollutant provenance, much emphasis has recently been placed on understanding the biogeochemical processes that control nitrate attenuation in aquifers (Rivett et al., 2008), because of the primary importance of nitrate in natural water resource governance. Denitrification is a microbially facilitated nitrate reduction process that may ultimately produce molecular nitrogen through a series of intermediate gaseous nitrogen oxide compounds (Knowles et al., 1982), and can be expressed as the following half-equation:

74

75 In general, two different types of denitrification, heterotrophic and autotrophic denitrification,
 76 occur in natural aquifers (Korom, 1992; Rivett et al., 2008), aside from the anaerobic ammonium
 77 oxidation (anammox) reaction (Strous and Jetten, 2004). Both the electrons and carbon needed for
 78 the heterotrophic reaction come from the microbial oxidation of organic carbon, and this reaction
 79 can be expressed using the following example stoichiometric equation:

80

82

83 The electrons required for autotrophic denitrification come from the oxidation of reduced-state
 84 elements (Korom, 1992), the most typical electron donors in natural aquifer conditions being
 85 reduced iron (Fe^{2+}) and reduced sulfur, usually represented as pyrite (FeS_2). The stoichiometric
 86 equations for these reactions are shown below (Korom, 1992; Ottley et al., 1997):

87

89

91

92 The nitrogen stable isotope ratio of nitrate in water ($\delta^{15}\text{N}_{\text{NO}_3}$) increases exponentially as
 93 microbial denitrification (equations 2–4) progresses because of kinetic isotope fractionation effects
 94 (Wellman et al., 1968; Delwiche and Steyn, 1970; Bryan et al., 1983; Sutka et al., 2006; Torrentó et
 95 al., 2011). This has been used in regional groundwater studies since the 1970s as a useful parameter
 96 for revealing, and estimating the extent of, denitrification (i.e. Kreitler, 1979; Kaplan and Magaritz,
 97 1986; Mariotti et al., 1988; Smith et al., 1991). These studies were summarized as being
 98 conventional approaches to understand the distribution and efficiency of denitrification processes in
 99 contaminated aquifer (Kendall, 1998). However, it is impossible to judge whether heterotrophic or
 100 autotrophic denitrification dominates in aquifer environments using only $\delta^{15}\text{N}_{\text{NO}_3}$ evidence. An
 101 approach, using the combined stable isotopic compositions of dissolved inorganic carbon (DIC; most
 102 being HCO_3^-), sulfur in dissolved sulfate (SO_4^{2-}), and $\delta^{15}\text{N}_{\text{NO}_3}$, has been used to determine which
 103 denitrification process dominates, assuming that these ions are the major products of the
 104 denitrification reactions (equations 2 and 4) and that the isotopic compositions differ for the different
 105 denitrification processes (Aravena and Robertson, 1998; Otero et al., 2009).

106 Aravena and Robertson (1998) observed a clear depleted tendency in groundwater $\delta^{13}\text{C}_{\text{DIC}}$
 107 values (−1.9 to −8.6‰) as denitrification progressed, as shown by a drastic enrichment in $\delta^{15}\text{N}_{\text{NO}_3}$
 108 (6.4 to 58.3‰) along the vertical groundwater flow in a contaminated shallow aquifer (≈6 m from

the surface) in southern Ontario, Canada. They attributed the $\delta^{13}\text{C}_{\text{DIC}}$ pattern to the increasing DIC contribution from an organic C source with a lower $\delta^{13}\text{C}$ signature (-29 to -26‰), and suggested that it was enhanced by oxidation of organic carbon via heterotrophic denitrification. A decline in $\delta^{13}\text{C}_{\text{DIC}}$ can be regarded as an indicator of heterotrophic denitrification if other electron acceptors for oxidation can be excluded. In addition, Otero et al. (2009) reported a clear declining tendency in $\delta^{34}\text{S}_{\text{SO}_4}$ (10 to -20‰) accompanying denitrification, with a $\delta^{15}\text{N}_{\text{NO}_3}$ enrichment of 10 to 35‰, in a heavily polluted deep aquifer (>100 m deep, with a maximum nitrate concentration of up to 400 mg L^{-1}) in eastern Spain. No clear patterns were observed in the $\delta^{13}\text{C}_{\text{DIC}}$ changes, which were between -16 and -7‰ . The authors suggested that the declining $\delta^{34}\text{S}_{\text{SO}_4}$ trend was caused by the dissolution of sulfate originating from the oxidation of sulfide with a low $\delta^{34}\text{S}$ in the aquifer, and concluded that the denitrification here proceeded autotrophically. Changes in $\delta^{13}\text{C}_{\text{DIC}}$, $\delta^{15}\text{N}_{\text{NO}_3}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ accompanying the two different denitrification types (equations 2 and 4), taken from the key studies described above, are summarized in Fig. 1. Unfortunately, reaction 3 does not produce ions with C-N-S isotope tracers, so we will now focus on reaction 4, because it is a major example of autotrophic denitrification.

The concept illustrated in Fig. 1 is very helpful in determining which type of denitrification dominates in a particular study area. However, it is also important to consider the possibility that groundwater $\delta^{34}\text{S}_{\text{SO}_4}$ and $\delta^{13}\text{C}_{\text{DIC}}$ values may be altered by other anaerobic microbial reactions, such as bacterial sulfate reduction (Rye et al., 1981; Strebel et al., 1990; Dogramaci et al., 2001; Tuttle et al., 2009) and methanogenesis (Chapelle and Knobel, 1985; Lansdown et al., 1992; Martini et al., 1998; Cheung et al., 2010). An example of a stoichiometric equation for sulfate reduction is:

This reaction can proceed heterotrophically in anaerobic aquifers in the presence of dissolved organic compounds. In a similar way to the $\delta^{15}\text{N}_{\text{NO}_3}$ described earlier, the $\delta^{34}\text{S}_{\text{SO}_4}$ value increases as this reaction progresses (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Nakai and Jensen, 1964; Rees, 1973) and this effect can be observed in aquifer systems worldwide (i.e. Rye et al., 1981; Strebel et al., 1990; Dogramaci et al., 2001; Yamanaka and Kumagai, 2006; Tuttle et al., 2009). Bacterial methanogenesis is the other major reaction that leads to the fractionation of stable isotope ratios in anaerobic aquifer environments (Chapelle and Knobel, 1985; Lansdown et al., 1992; Martini et al., 1998; Cheung et al., 2010), and a stoichiometric equation for this can be expressed as:

The lighter ^{12}C isotope becomes enriched relative to the ^{13}C isotope in the CH_4 that is produced but

145 depleted in the CO_2 as the reaction progresses, resulting in an increase in the $\delta^{13}\text{C}_{\text{DIC}}$ in the
146 groundwater (Chapelle and Knobel, 1985; Aravena et al., 2003; Conrad, 2005; Sharma and Frost,
147 2008).

148 These reactions typically occur after denitrification (Korom, 1992), and are very likely to be
149 found in areas where denitrification has been confirmed. After pioneer study by Aravena and
150 Robertson (1998), multi-isotope concept for tracing the correlation of carbon, nitrogen, and sulfur
151 cycles has been applied in nitrate attenuation study for both laboratory-scale (Carrey et al., 2013;
152 Vidal-Gavilan et al., 2013) and field-scale (Böhlke et al., 2002; Vitòria et al., 2008; Otero et al.,
153 2009; Puig et al., 2013). However, this concept has been applied under specific aquifer condition
154 where denitrification is the only major anaerobic process (Fig. 1) and studies involving all major
155 bacterial reactions (heterotrophic and autotrophic denitrification, sulfate reduction, and
156 methanogenesis) within certain aquifer system are still rare. As mentioned previously, the effects of
157 C and S isotopic changes through denitrification reaction could be masked by the influence of
158 isotopic changes through further anaerobic processes; however, this important problem has not yet
159 been clarified. Aquifers with reducing conditions are very common nature (i.e. North America;
160 McMahon and Chapelle, 2008, Asia; Hosono et al., 2011a). Therefore, combination use of
161 multi-isotope compositions, followed by understanding of C, N, and S isotope changing patterns
162 under variety redox conditions, should be developed as it to be a general application for worldwide
163 groundwater systems. In addition, multi-isotope approach in combination with an understanding of
164 groundwater flow dynamics has not been adequately tested in large-scale aquifer systems (10^3 km^3
165 scale in area, ~200 m in depth), which is prime important concept to understand anaerobic processes
166 in circulated groundwater systems (Hosono et al., 2013).

167 The Kumamoto area, western Japan (Fig. 2), is the largest user of groundwater in Japan, in
168 which about one million peoples depend entirely on groundwater for their domestic water, including
169 for drinking (Oshima, 2010; Shimada, 2012). Internationally, this area has been received
170 considerable attention as the advanced rejoin in groundwater management based on understanding of
171 natural hydrologic system (<http://www.un.org/waterforlifedecade/winners2013.shtml>). Previous
172 study (Hosono et al., 2013) has deduced the origins of nitrate pollution and distributions and degrees
173 of denitrification using $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ tracers with a comprehensive understanding of
174 groundwater flow dynamics and land-use. In the present study, the C, N, and S isotope changing
175 patterns are characterized along groundwater flow systems in Kumamoto, in combination of
176 previously determined $\delta^{15}\text{N}_{\text{NO}_3}$ with newly measured $\delta^{34}\text{S}_{\text{SO}_4}$ and $\delta^{13}\text{C}_{\text{DIC}}$, and a type of
177 denitrification and occurrence of further anaerobic processes are discussed. Our main goal was to
178 develop the understanding of C, N, and S isotopic tracing method (called the “CNS-IT” method)
179 studying the occurrence of sequential anaerobic processes in contaminated aquifers, including
180 judgment between heterotrophic and autotrophic denitrification.

181

182 **2. Materials and methods**

183

184 **2.1. Study site**

185 The “Kumamoto groundwater area” is defined as an area (945 km²) surrounded by the divide of the
186 Midori River watershed to the north, the Shira River to the south, the Aso Mountain (1,592 m) to the
187 east, and the Ariake Sea and Kinpo Mountain (665 m) to the west (Fig. 2). Because of the aquifer’s
188 importance, it has been monitored for a long time, and its potential and flow dynamics are well
189 understood. Detailed information on topography, climate, geology, hydrogeology, groundwater flow
190 and age, and land-use and groundwater use has been described previously (Hosono et al., 2013).
191 Only the essence of necessary information is described in the followings.

192 Hydrogeologically, Paleozoic metasedimentary rocks and Tertiary–Quaternary Pre-Aso
193 volcanic rocks form the low-permeability basement. The major aquifers are the overlying
194 Quaternary Aso volcanic rocks (sub-divided into Aso-1–4, from the lower to the upper parts; Ono
195 and Watanabe, 1985) and alluvium deposits (Kumamoto Prefecture and Kumamoto City, 1995)
196 (Appendix 1). The aquifers are of two types, unconfined and confined (Appendix 1). The unconfined
197 aquifer is composed of unwelded Aso-4 pyroclastic and alluvial sedimentary deposits, which overlie
198 an aquiclude of impermeable lacustrine sediments, and the confined aquifer is composed of Aso-1–3.
199 The groundwater is recharged from the Ueki, Kikuchi, and Takayubaru highlands (Fig. 2 and
200 Appendix 1), where permeable Aso-4 pyroclastic deposits are found (Kumamoto Prefecture and
201 Kumamoto City, 1995; Taniguchi et al., 2003), and from the mid-stream Shira River (Fig. 2 and
202 Appendix 1) because of the absence of aquiclude over the deep aquifer (Tanaka et al., 2010). Lake
203 Ezu (Fig. 2) is the largest Kumamoto groundwater discharge point ($1.5\text{--}1.8 \times 10^8 \text{ m}^3 \text{ y}^{-1}$) (Ono et al.,
204 2013). Except Lake Ezu, there are about 30 natural spring sites in and around the Kumamoto area
205 (Fig. 2) with its size ranging $1.0 \times 10^3\text{--}1.6 \times 10^7 \text{ m}^3 \text{ y}^{-1}$. Most of these are flowing-out at the foot of
206 Mt. Aso slope and highlands and hills (Fig. 2) in the end of local groundwater flow systems.

207 The most important confined groundwater flow system is the A–A’ flow shown in Fig. 2. This
208 flow can be divided into four zones (Fig. 2 and Appendix 1): A1, flow between the recharge area in
209 the Kikuchi highlands to the mid-stream area of the Shira River; A2, the mixing zone for
210 groundwater from zone A1, recharge water from the Takayubaru highlands, and recharge water from
211 the Shira River; A3, the most dynamic lateral flow through the Aso-2 volcanic lava aquifer toward
212 the Ezu Lake discharge area; and A4, flow toward the stagnant coastal area. Due to the high
213 discharging flux in Lake Ezu, lateral groundwater flows are not important in the extension of zone
214 A4 (Appendix 1). The other major confined groundwater flow system within the study area is the B–
215 B’ flow, which can be divided into three zones: B1, southward flow from the Ueki highlands
216 recharge area to the basement lifting point (shown in Appendix 1); B2, flow across the basement

217 uplift toward the plain area entry; and B3, southwestward flow to the stagnant coastal area. In
218 general the unconfined and confined groundwater flow characteristics are similar, and are basically
219 controlled by regional geology and topography. In addition, two major groundwater flow systems
220 (A-A' and B-B') are not hydrogeologically connecting each other.

221 The main land uses in the Ueki, Kukuchi, and Takayubaru highlands are vegetable and
222 livestock farming (Fig. 2). A recent isotope tracer study (Hosono et al., 2013), using $\delta^{15}\text{N}_{\text{NO}_3}$ and
223 $\delta^{18}\text{O}_{\text{NO}_3}$, revealed that long-term chemical fertilizer application in these highland areas is a primary
224 cause of the current groundwater nitrate pollution in Kumamoto (Tomiie et al., 2011). Pollution
225 attenuation mechanisms along the A-A' and B-B' flow systems have been investigated (Hosono et
226 al., 2013), and dilution was found to be the main attenuation process in the A-A' groundwater,
227 except for in zone A4, whereas attenuation by denitrification was found to be important in the B-B'
228 flow. The denitrification process was found to be more active in the stagnant area than in the
229 recharge area. Furthermore, the most significant denitrification area was located on the B-B' flow
230 line, under a point where the topography changed, where the hills meet the plain (marked
231 "denitrification hotspot" in Fig. 2). From a clear enriched trend of $\delta^{15}\text{N}_{\text{NO}_3}$ (10 to 48‰) with
232 simultaneous enrichment of $\delta^{18}\text{O}_{\text{NO}_3}$ and decline of NO_3^- in groundwater under anaerobic ($\text{DO} < 2$
233 mg L^{-1}) condition, and because this isotopic change cannot be explained by mixing of any possible
234 source materials (Hosono et al., 2013), $\delta^{15}\text{N}_{\text{NO}_3}$ value more than 10‰ can be regarded as a parameter
235 of denitrification. Denitrification has been well characterized, as mentioned above, so this area was
236 chosen as an ideal setting for us to further investigate the anaerobic biogeochemical processes
237 involved by analyzing $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ in the groundwater.

238

239 2.2. Samples

240 A total of 258 (83 unconfined and 175 confined) groundwater samples were collected between
241 November 2009 and November 2011 (Fig. 2). Of these, 199 samples were obtained from monitoring
242 wells governed by Japanese government, prefecture, or city, while rests of 59 samples were from
243 private production wells. With the geological descriptions of the borehole cores and information of
244 screens (usually 5-20 m in width) and well depths (4.4–210 m) we could define the aquifer type
245 (confined or unconfined) of which collected groundwater belongs. Samples were collected from the
246 same wells at 68 sites in January–March 2011 and October–November 2011 to compare the
247 hydrochemistry and isotopic compositions in the high potential groundwater season (August–
248 November) and the low potential groundwater season (January–April).

249 Groundwater samples were collected using a portable pump (MP1, Grundfos, Denmark). Water
250 already present in the borehole ($\approx 5 \times 10^3 \text{ L}$) was pumped out before taking a sample, so that, as
251 much as possible, freshly replenished water was collected. Temperature, electrical conductivity, pH,
252 redox potential (ORP), and dissolved oxygen (DO) were continuously monitored using field meters

(HORIBA D-54, Japan) during sampling, and sample collection began once these values became constant. Ion concentrations (HCO_3^- , NO_3^- , SO_4^{2-} , and Cl^-) and $\delta^{15}\text{N}_{\text{NO}_3}$ were measured in all collected samples (Hosono et al., 2013), whereas representative samples were selected for $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ analyses.

River (at 15 sites) and spring water (at 30 sites) samples were also collected in and around the study area (Fig. 2) in April 2011 (low water season) and July 2011 (high water season). Since all of these waters showed $\text{DO} > 6 \text{ mg L}^{-1}$ and $\text{ORP} > 150 \text{ mV}$ (Appendix 2) and no microbiologically-induced $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ enrichment signatures (Hosono et al., 2013), effects of further anaerobic biogeochemical reactions (sulfate reduction and methanogenesis) on $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ isotopic variations are unexpected. We used concentrations and C, N, and S isotopic compositions of river and spring waters as the references of juvenile compositions which have not been altered through anaerobic bacterial processes.

2.3. Analysis

Water samples were filtered through $0.2 \mu\text{m}$ cellulose acetate filters, at the sampling site for the analysis of concentrations (100 mL samples) and in the laboratory after transportation (within 1 day) for $\delta^{13}\text{C}_{\text{DIC}}$ (500 mL) and $\delta^{34}\text{S}_{\text{SO}_4}$ (2000 mL) analyses. The filtered water samples were adjusted to pH 12–12.5 with NaOH for $\delta^{13}\text{C}_{\text{DIC}}$ analysis and acidified to pH 2.5–3 with distilled 6 M HCl for $\delta^{34}\text{S}_{\text{SO}_4}$ analysis, then the DIC and SO_4^{2-} were collected as BaCO_3 and BaSO_4 , respectively, for isotope analysis by adding 10% BaCl_2 (aq). HCO_3^- contents and NO_3^- , SO_4^{2-} , and Cl^- concentrations were measured by titrating with sulfuric acid and by ion chromatography (Compact IC 761, Metrohm, Switzerland), respectively.

The $\delta^{13}\text{C}_{\text{DIC}}$ of BaCO_3 and $\delta^{34}\text{S}_{\text{SO}_4}$ of BaSO_4 were measured on CO_2 and SO_2 gasses, respectively, in a continuous-flow gas-ratio mass spectrometer (Thermo Fisher Delta V Advantage, Germany) interfaced with an elemental analyzer (Thermo Fisher Flash 2000). The standards used were based on international standards (NBS123 and OGS-1), as well as several carbonate and sulfate materials that have been compared between laboratories. The precision of the $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ measurements were estimated to be better than $\pm 0.2\text{‰}$ from repeated internal standard measurements. The $\delta^{15}\text{N}_{\text{NO}_3}$ compositions were determined following the “denitrifier method” of Sigman et al. (2001) and Casciotti et al. (2002). Analytical methods and results for $\delta^{15}\text{N}_{\text{NO}_3}$ are after Hosono et al. (2013).

3. Results and discussion

3.1. Seasonal variations

The $\delta^{13}\text{C}_{\text{DIC}}$, $\delta^{15}\text{N}_{\text{NO}_3}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ values in groundwater can vary because of a number of abiotic

and biotic processes (separately or in combination), such as the mixing of different source materials, aerobic bacterial decomposition of organic material, anaerobic bacterial denitrification, sulfate reduction, and methanogenic reactions (Chapelle and Knobel, 1985; Böhlke and Denver, 1995; Aravena and Robertson, 1998; Böhlke et al., 2002; Vengosh et al., 2002; Widory et al., 2004; Vitòria et al., 2008; Otero et al., 2009; Hosono et al., 2009, 2010, 2011b; Cheung et al., 2010; Puig et al., 2013). The $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ variations in groundwater in Kumamoto do not clearly correlate with $\delta^{15}\text{N}_{\text{NO}_3}$ (Fig. 3), for which a value of more than 10‰ can be regarded as indicating denitrification (as explained previously). This should be primarily caused by the combined effects of the processes described above in the saturated groundwater systems. Analyzing the $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ values, while having a good understanding of the denitrification patterns and groundwater flow systems, should be an efficient way of reducing the difficulty of interpreting the complex isotopic compositions that can be seen (Böhlke and Denver, 1995; Böhlke et al., 2002; Hosono et al., 2013) (Fig. 4).

Before discussing the concentration and isotope compositional changes along the groundwater flows studied, it is important to describe their seasonal changes. Systematic differences in groundwater NO_3^- concentrations and $\delta^{15}\text{N}_{\text{NO}_3}$ values between the high and low potential seasons have been shown not to exist in most areas (Hosono et al., 2013). We found good correlations between the HCO_3^- and SO_4^{2-} concentrations and the $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ values in the two seasons (Appendix 3), suggesting that these values are not seasonal, but reflect spatial diversity (Appendix 4). For example, the HCO_3^- and SO_4^{2-} concentrations are generally higher in coastal plain area than in the recharge area, and a similar tendency was seen for $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$. The causes of the variations in the NO_3^- , HCO_3^- , and SO_4^{2-} concentrations and the $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ compositions along the groundwater flows need to be assessed with regard to spatial variation but not a seasonal variation.

3.2. Source characteristics of A-A' groundwater flow systems

For the A-A' groundwater, the $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ values were isotopically homogeneous throughout the flow paths, being -20 to -17‰ and 8–10‰, respectively, except in the mixing zone (zone A2) and the stagnant zone (zone A4) (Fig. 4). The HCO_3^- and SO_4^{2-} concentrations were also stable throughout the flow paths. These results match the $\delta^{15}\text{N}_{\text{NO}_3}$ and NO_3^- results (Fig. 4), which suggested that denitrification does not occur in zones A1–A3 and that zone A2 groundwater is heterogeneous, in terms of its chemistry, because of the mixing of different source waters (Hosono et al., 2013). These observations, along with the previous results, suggest that other anaerobic bacterial reactions, such as sulfate reduction and methanogenesis, are not active for most of the A-A' flow. It can, therefore, be assumed that there are homogeneous sources of HCO_3^- and SO_4^{2-} , giving $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ values with narrow ranges, -20 to -17‰ and 8–10‰, respectively.

Looking only at the samples with no denitrification signature and $\text{DO} > 6 \text{ mg L}^{-1}$ and $\text{ORP} > 150 \text{ mV}$ (Appendix 2), the average $\delta^{13}\text{C}_{\text{DIC}}$ decreased systematically with increasing HCO_3^- concentration, from river water (-15.2‰ and 56 mg L^{-1} , $n = 23$ and 29 , respectively), through spring water (-17.2‰ and 59 mg L^{-1} , $n = 35$ and 57), to unconfined groundwater (-18.3‰ and 71 mg L^{-1} , $n = 20$ and 35), and confined groundwater (-18.5‰ and 80 mg L^{-1} , $n = 50$ and 97) (gray arrows in Figs. 3a and 5a). In general, freshwater $\delta^{13}\text{C}_{\text{DIC}}$ can be altered by four major processes: (1) the oxidation of organic matter; (2) the dissolution of carbonate minerals; (3) exchange with atmospheric carbon dioxide; and (4) photosynthetic activity (at the surface) (Hélie et al., 2002). Processes (2) and (3) are major factors in increasing $\delta^{13}\text{C}_{\text{DIC}}$ to near 0‰ , whereas process (1) increases HCO_3^- and decreases $\delta^{13}\text{C}_{\text{DIC}}$ to around -27‰ (Clark and Fritz, 1997; Schulte et al., 2011). It is, therefore, reasonable to interpret our results as indicating that the river water had the lowest organic C source contributions, and that this changed, with organic C sources becoming more significant with time, as the water entered the deeper zones. The lowest $\delta^{13}\text{C}_{\text{DIC}}$ (-21 to -17‰ , that is, the water most affected by organic C sources) was found in groundwater from the Ueki and Kikuchi recharging areas (Fig. 4), and the $\delta^{13}\text{C}_{\text{DIC}}$ (and HCO_3^- concentrations) did not change within the A–A' groundwater flow paths between the recharge and the stagnant zones, suggesting that carbonate dissolution has not been occurred which can increase $\delta^{13}\text{C}_{\text{DIC}}$ values and HCO_3^- concentrations. Aquifer geology (Quaternary volcanic materials) is composed of silicate minerals. Furthermore, inverse geochemical modeling (PHREEQCI) demonstrated that the downstream changes in groundwater cation ions could be largely explained by the weathering of plagioclase to kaolinite, with possible contributions from weathering of biotite and pyroxene, and dissolution of secondary phase calcite is not important (Hossain et al., 2013). Our findings are corresponding well with these facts and results.

The $\delta^{34}\text{S}_{\text{SO}_4}$ values in most of the river and spring water samples were within a narrow range, 3 – 12‰ , whereas the groundwater $\delta^{34}\text{S}_{\text{SO}_4}$ values had a wide range, 0 – 60‰ (Fig. 5b), suggesting that there were processes in the subsurface environment that led to a high degree of isotope fractionation. Three SO_4^{2-} concentration peaks can be seen for groundwater samples in Fig. 5b: (1) 35 mg L^{-1} for $\delta^{34}\text{S}_{\text{SO}_4} = 0$ – 4‰ ; (2) 70 mg L^{-1} for $\delta^{34}\text{S}_{\text{SO}_4} = 8$ – 12‰ ; and (3) 230 mg L^{-1} for $\delta^{34}\text{S}_{\text{SO}_4} = 40$ – 60‰ . Similar SO_4^{2-} concentration peaks were found for river and spring waters with $\delta^{34}\text{S}_{\text{SO}_4}$ around 5‰ (spring water from the Aso volcano as a reference of volcanic origin sulfur in the study area) and $\delta^{34}\text{S}_{\text{SO}_4} = 7$ – 12‰ (spring water affected by agricultural activities and some Shira River water samples; Hosono et al., 2013). The origin of the first peak was assumed to be the addition of sulfate formed by the oxidation of sulfide minerals in the aquifer, with $\delta^{34}\text{S}$ values generally $< 5\text{‰}$, as it has been reported by many researchers (i.e. Hendry et al., 1986; Massmann et al., 2003; André et al., 2005; Schwientek et al., 2008; Vitòria et al., 2008; Cheung et al., 2010; Pauwels et al., 2010). The third peak groundwater samples were collected from the coastal aquifers (Appendix 4b and d).

Increasing trend of Cl^- concentrations with increasing $\delta^{34}\text{S}_{\text{SO}_4}$ values (40 to 60‰) of this water (Appendix 5) clearly suggests the contribution of saline (remnant marine source) water, which has already been affected by bacterial sulfate reduction prior to mixing (Feast et al., 1997; Barbecot et al., 2000; Hosono et al., 2011b). The second peak $\delta^{34}\text{S}_{\text{SO}_4}$ range (8–12‰) corresponds with the surface and spring waters second peak (7–12‰). Some of these water samples are most concentrated in NO_3^- (~80 mg L⁻¹; Appendix 6), suggesting the partial contribution of sulfate from agrochemicals applied. These observations suggest that the groundwater sulfate in zones A1 and A3 ($\delta^{34}\text{S}_{\text{SO}_4}$ = 8–10‰) has both natural and anthropogenic origins that are mixed to give a homogeneous composition.

370

3.3. Anaerobic biogeochemical processes along the B-B' groundwater flows

For the B-B' flow, the groundwater in the recharge area under the Ueki highlands had similar $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ signatures to the A-A' groundwater (Fig. 4). This suggests that the origins of the HCO_3^- and SO_4^{2-} in the B-B' water are similar to the origins of the A-A' water, although the $\delta^{13}\text{C}_{\text{DIC}}$ range is slightly lower in the B-B' (-21 to -20‰) than the A-A' water (-20 to -17‰) probably because the B-B' water is more enriched in DIC with organic C source. Importantly, it is clear that the B-B' groundwater is fundamentally different from the A-A' groundwater in that it has a high degree of isotopic variations along its flow path (Fig. 4). In fact, it has already been shown that significant denitrification occurs at several points along the B-B' groundwater flow (Hosono et al., 2013). The main reactions were seen in the down-gradient areas of zones B1 and B2, where nitrate reaching up to 50 mg L⁻¹ was transported along the groundwater flow and leached into the anaerobic environment ($\text{DO} < 2 \text{ mg L}^{-1}$), and in the stagnant area in zone B3 (Fig. 4). It is not, therefore, surprising that other anaerobic reactions may occur in these anaerobic conditions, causing changes in the groundwater $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$.

Most interestingly, significant increases in $\delta^{13}\text{C}_{\text{DIC}}$ (-21 to -13‰) and $\delta^{34}\text{S}_{\text{SO}_4}$ (8 to 20‰) were found in zones B1 and B2 with increasing HCO_3^- (70 to 200 mg L⁻¹) and decreasing SO_4^{2-} concentrations (20 to 0.1 mg L⁻¹) (Fig. 4). These isotopic changes at the end of the flow paths occurred much later than the beginning of the denitrification reactions, which started a lot further up-gradient in each zone (Fig. 4). Geological materials constituting B1 and B2 zone aquifers are the same silicate minerals as can be seen in A-A' aquifer. From a hydrogeological and hydrogeochemical point of view, there is no reasonable reason to explain these isotopic changes by the contributions of different carbon source materials (Hossain et al., 2013). Instead, it seems highly likely that they are caused by anaerobic bacterial sulfate reduction (equation 5) and methanogenesis (equation 6) reactions. Indeed, ORP range of the groundwater samples from these zones ($\text{DO} < 2 \text{ mg L}^{-1}$) was generally negative with minimum value of -200 mV (Appendix 2), under which condition sulfate reduction and methanogenesis could occur after denitrification (Morris et al., 2003). The

occurrence of the methanogenic reaction is supported by the fact that high CH_4 concentrations (up to 1 mg L^{-1}) were detected at each site in preliminary analyses (on selected samples). Considering the location of each reaction in the groundwater flow pathway (Fig. 4), the order we observed is consistent with the reaction sequence generally observed in natural aquifer systems (denitrification $\rightarrow$ sulfate reduction $\rightarrow$ methanogenesis) (Edmunds et al., 1982; Korom, 1992). This finding may be extrapolated to most of the Kumamoto area for the groundwater with elevated $\delta^{13}\text{C}_{\text{DIC}}$ ($> 17\text{‰}$), $\delta^{15}\text{N}_{\text{NO}_3}$ ($> 10\text{‰}$), and $\delta^{34}\text{S}_{\text{SO}_4}$ ($> 10\text{‰}$) in the reducing environment ($\text{DO} < 2 \text{ mg L}^{-1}$, $\text{ORP} < 0 \text{ mV}$) (Fig. 3).

The most significant increase in groundwater $\delta^{34}\text{S}_{\text{SO}_4}$ along the flow path was found in the stagnant coastal aquifer in zone B3 (Fig. 4 and Appendix 4d), where denitrification typically occurs (Hosono et al., 2013). As mentioned previously, because this isotopic change accompanies increases in SO_4^{2-} (Figs. 4 and 5) and Cl^- concentrations (Appendix 5), it is suggested that the fresh groundwater system receives marine sulfate that has undertaken the sulfate reduction (Feast et al., 1997; Barbecot et al., 2000; Hosono et al., 2010). In addition, the groundwater $\delta^{13}\text{C}_{\text{DIC}}$ in zone B3 showed somewhat increasing tendency along the flow path (Fig. 4 and Appendix 4c), although there was evidence of significant denitrification and sulfate reduction. The ORP values of these groundwater samples were generally negative with minimum value of -322 mV (Appendix 2). Slight, and similar, increases in $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{34}\text{S}_{\text{SO}_4}$, and $\delta^{13}\text{C}_{\text{DIC}}$ were found at the end of the zone A4 stagnant groundwater (Fig. 4). These results indicate that the increase in HCO_3^- concentration in the coastal groundwater can be attributed to the contribution of HCO_3^- from the methanogenic reaction (Figs. 3a and 5a). Weaker $\delta^{13}\text{C}_{\text{DIC}}$ increasing tendency in zone B3 (Fig. 4) is likely due to significant contribution of HCO_3^- (with lower $\delta^{13}\text{C}_{\text{DIC}}$ value) through sulfate reduction (equation 5) of high concentrate marine source sulfate, which effect can to some extent compensate the effect of methanogenesis reaction. A detailed analysis of the $\delta^2\text{H}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ values and CH_4 concentrations is required to elucidate the HCO_3^- enrichment mechanism further (Martini et al., 1998; Cheung et al., 2010; Warner et al., 2013).

Since aquifer types (Appendix 1), constituting materials, and land-use (Fig. 2) do not differ between A–A' and B–B', these are not likely the fundamental factors creating the gap of biogeochemical systems observed. The gap of the groundwater flow velocity ($\text{A–A}' > \text{B–B}'$) is one of the known hydrologic differences (Appendix 1), which might cause difference in biogeochemical processes among them. This is the important problem to be answered in future work with additional information on organic carbon contents and bacterial condensation data.

429

430 3.4. Assessment of the type of denitrification

Sulfate reduction and methanogenesis are both heterotrophic microbial reactions. The significant occurrence of these reactions suggests that aquifers contain sufficient organic compounds,

433 which are essential for this type of bacteria. As mentioned above, the groundwater $\delta^{13}\text{C}_{\text{DIC}}$ in the
 434 recharge area (the up-gradient area of zone A1 and B1) was affected by an organic C source and had
 435 the lowest $\delta^{13}\text{C}_{\text{DIC}}$ values (-21 to -19‰). This isotopic alteration makes it impossible to use $\delta^{13}\text{C}_{\text{DIC}}$
 436 as a parameter for directly assessing the types of denitrifiers, heterotrophs or autotrophs, present (Fig.
 437 1). However, by confirming that there is no decreasing tendency in $\delta^{34}\text{S}_{\text{SO}_4}$ and the presence of
 438 sulfate reduction (no additional SO_4^{2-} was observed along the flow) and methanogenesis, we can
 439 assess with better confidence whether the denitrification seen in zones B1 and B2 was heterotrophic.
 440 Similarly, SO_4^{2-} concentrations did not change or rather decrease in zone A4 along the flow with
 441 increasing in $\delta^{34}\text{S}_{\text{SO}_4}$. Exceptionally, high SO_4^{2-} concentrations ($60\text{--}75 \text{ mg L}^{-1}$) were found in the
 442 groundwater at the end of zone B2 (Fig. 4), but the $\delta^{34}\text{S}_{\text{SO}_4}$ compositions were still around 10‰ . In
 443 consequence, there were no isotopic and geochemical evidences of the occurrence of autotrophic
 444 denitrification (equation 4) within B1, B2, and A4 groundwater flows. Interestingly, we found very
 445 high concentrations of Mg^{2+} in this water, and we speculate that materials with high Mg and SO_4
 446 enter the groundwater in this zone. However, we have not yet obtained a clear answer to this
 447 question. It appears that using $\delta^{34}\text{S}_{\text{SO}_4}$ to trace autotrophic denitrification becomes difficult in coastal
 448 groundwater that might be affected by marine sulfate with $\delta^{34}\text{S}_{\text{SO}_4}$ up to 20‰ and variety of
 449 concentrations. Confirming the occurrence of sulfate reduction ($\delta^{34}\text{S}_{\text{SO}_4}$ increases more than 20‰)
 450 could be an indirect evidence of denitrification progressing heterotrophically as well, that is the case
 451 of the most part of zone B3.

452 The combined use of $\delta^{13}\text{C}_{\text{DIC}}$, $\delta^{15}\text{N}_{\text{NO}_3}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ has successfully been used to show that
 453 denitrification in confined groundwater in carbonate–sandstone aquifers in eastern Spain progressed
 454 autotrophically, from the drastic decline in $\delta^{34}\text{S}_{\text{SO}_4}$ (10 to -20‰) with increasing $\delta^{15}\text{N}_{\text{NO}_3}$ (10 to
 455 35‰) (Otero et al., 2009). In contrast, the groundwater $\delta^{34}\text{S}_{\text{SO}_4}$ in Kumamoto was typically higher
 456 than 5‰ , and autotrophic denitrification does not appear to be the major denitrification process. The
 457 contrast between the denitrification processes in these two areas must be caused by differences in the
 458 amount of organic compounds and other electron donors used by the bacteria in each aquifer. At the
 459 local scale (Fig. 4), we found one site outside of the coastal zone (the up-gradient area of zone B3)
 460 that had groundwater with an increase in $\delta^{15}\text{N}_{\text{NO}_3}$ (up to 12‰), a decrease in $\delta^{34}\text{S}_{\text{SO}_4}$ (to close to 0‰),
 461 and a $\delta^{13}\text{C}_{\text{DIC}}$ (-19‰) within the background range (-21 to -17‰). A few groundwater samples
 462 showed similar isotopic trends (increasing $\delta^{15}\text{N}_{\text{NO}_3}$ with decreasing $\delta^{34}\text{S}_{\text{SO}_4}$) in the Kumamoto area
 463 (Fig. 3b), suggesting that autotrophic denitrification occurs here. A similar very weak tendency has
 464 also been reported in other Quaternary volcanic aquifers in Indonesia (Hosono et al., 2011b). These
 465 results suggest that autotrophic denitrification can occur locally in volcanic aquifers, in contrast to
 466 the other aquifers (Schwientek et al., 2008; Vitòria et al., 2008; Otero et al., 2009; Pauwels et al.,
 467 2010) in different climate and geological conditions.

468

3.5. Development of the CNS-IT method

As discussed in the previous section, the efficiency of the combination use of C, N, and S isotope compositions for evaluating the type of denitrification, as illustrated in Fig. 1, depends on the aquifer conditions in the target area. There are two major concerns when applying this approach. One concern is that the $\delta^{13}\text{C}_{\text{DIC}}$ cannot be used as a direct parameter to track heterotrophic denitrification if the groundwater has already been saturated by an organic C source. In this case, $\delta^{34}\text{S}_{\text{SO}_4}$ should be used as a main parameter to assess if autotrophic denitrification has been taken place. The other is that the sulfur isotopic evidence for the occurrence of autotrophic denitrification might disappear if high marine sulfate forms a contribution in coastal areas. In these cases, the $\delta^{34}\text{S}_{\text{SO}_4}$ and $\delta^{13}\text{C}_{\text{DIC}}$ values should rather be used as parameters for evaluating the progress of heterotrophic sulfate reduction and methanogenesis, respectively. In this way, the evaluation enables us to assess the most likely denitrification type (heterotrophic or autotrophic) in the study area.

The $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ evolution patterns through the major anaerobic bacterial reactions, individually or in combination, in groundwater in the cases that carbonate dissolution and mixing of different sources have not been taken place along the flow systems (as in Kumamoto), are shown in Fig. 6. If no major anaerobic bacterial reactions except denitrification (case 1) occur in an aquifer, the combined C-N-S isotopic tracers can be used to determine the type of denitrification, based on the concept illustrated in Fig. 1. If the groundwater has already been saturated with an organic C source, a decline in $\delta^{13}\text{C}_{\text{DIC}}$ through heterotrophic denitrification might not be seen (case 1b). If sulfate reduction occurs in addition to denitrification, its effects on the changes in $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ can be counterbalanced (case 2). If methanogenic reactions occur in addition to denitrification, the groundwater $\delta^{13}\text{C}_{\text{DIC}}$ may increase despite the denitrification process (case 3). Mixture effects should be considered when interpreting the data from case 4. In this framework, the Kumamoto groundwater case corresponds to cases 4b and 4c. In summary, $\delta^{34}\text{S}_{\text{SO}_4}$ declining with increasing $\delta^{15}\text{N}_{\text{NO}_3}$ (Fig. 6) can be regarded as evidence for autotrophic denitrification. The occurrence of other anaerobic reactions (sulfate reduction and methanogenesis) could be indirect evidence for heterotrophic denitrification. Along these understandings, the possible compositional areas through each type of denitrification reaction can be shown as in Fig. 6. Comparison of the sample data with this developed model (CNS-IT method) helps distinguishing between the denitrification types in aquifer systems. The $\delta^{13}\text{C}_{\text{DIC}}$ parameter should be treated more carefully when carbonate dissolution can occur in the aquifers along groundwater flow. This would enhance the increase of groundwater $\delta^{13}\text{C}_{\text{DIC}}$ (Clark and Fritz, 1997; Schulte et al., 2011), and this effect could disturb isotopic enrichment effect through oxidation of organic carbon and methanogenic reactions.

The developed CNS-IT method seems to work well to explain the occurrence of major anaerobic bacterial reactions and to determine the denitrification type in the Kumamoto groundwater

505 systems (Figs. 3 and 6). However, further verification is needed to fully discuss biogeochemical
506 cycle in quantitative point of view with additional important parameters, such as DOC, H₂S, and
507 CH₄ concentrations, $\delta^{13}\text{C}_{\text{CH}_4}$, and bacterial identification. Nevertheless, the $\delta^{13}\text{C}_{\text{DIC}}$, $\delta^{15}\text{N}_{\text{NO}_3}$, and
508 $\delta^{34}\text{S}_{\text{SO}_4}$ values and their respective concentrations are the core parameters for the major
509 biogeochemical reactions, and their combined use is a major step forward. Proposed model (Fig. 6)
510 summarized here could be useful in anaerobic biogeochemical studies of contaminated aquifers
511 around the world.

512

513 **7. Conclusions**

514

515 Occurrences of anaerobic biogeochemical reactions (denitrification, sulfate reduction, and
516 methanogenesis), including distinguishment between heterotrophic and autotrophic denitrification,
517 were investigated in detail (analyzing 348 water samples) along two major groundwater flow
518 systems in Kumamoto area, southern Japan, using C-N-S isotopic tracing (called as CNS-IT) method.
519 Contrasting biogeochemical systems was observed between two groundwater flow systems, A–A’
520 and B–B’. None of above anaerobic bacterial process has been taken place in A–A’ groundwater
521 system, whereas, sequential anaerobic bacterial activities (heterotrophic denitrification → sulfate
522 reduction → methanogenesis) dominantly controlled nitrogen, carbon, and sulfur cycles in
523 groundwater of B–B’ flow system. This study developed the CNS-IT method to study denitrification
524 mechanism with understanding of wide-range anaerobic bacterial processes in groundwater flow
525 systems. Methodology summarized in this work could be applied for studies in the other
526 contaminated aquifers of the world.

527

528 **Acknowledgments**

529 The groundwater flow models used in this study were constructed by Geosphere Environmental
530 Technology Corporation, Tokyo, Japan. This study was funded by a Grant-in-Aid for Young
531 Scientists (A) (no. 24681007) and by the CREST Project (JST: Japan Science and Technology
532 Agency).

533

534 **References**

- 535 André, L., Franceschi, M., Pouchan, P., Atteia, O., 2005. Using geochemical data and modelling to
536 enhance the understanding of groundwater flow in a regional deep aquifer, Aquitaine Basin,
537 south-west of France. *J. Hydrol.* 305, 40–62.
- 538 Aravena, R., Harrison, S.M., Barker, J.F., Abercrombie, H., Rudolph, D., 2003. Origin of methane in
539 the Elk Valley coalfield, southeastern British Columbia, Canada. *Chem. Geol.* 95, 219–227.
- 540 Aravena, R., Robertson, W.D., 1998. Use of multiple isotope tracers to evaluate denitrification in

ground water: study of nitrate from a large-flux septic system plume. *Ground Water* 36, 975–982.

Barbecot, F., Marlin, C., Gibert, E., Dever, L., 2000. Hydrochemical and isotopic characterisation of the Barhonian and Bajocian coastal aquifer of the Caen area (northern France). *Appl. Geochem.* 15, 791–805.

Bryan, B.A., Shearer, G., Skeeters, J.L., Kohl, D.H., 1983. Variable expression of the nitrogen isotope effect associated with denitrification of nitrite. *J. Biol. Chem.* 258, 8613–8617.

Böhlke, J.K., Denver, J.M., 1995. Combined use of groundwater dating, chemical, and isotopic analysis to resolve the history and fate of nitrate contamination into two agricultural watersheds, Atlantic coastal plain, Maryland. *Water Resour. Res.* 31, 2319–2339.

Böhlke, J.K., Wanty, R., Tuttle, M., Delin, G., Landon, M., 2002. Denitrification in the recharge area and discharge area of a transient agricultural nitrate plume in a glacial outwash sand aquifer, Minnesota. *Water Resour. Res.* 38. 10.1029/2001WR000663.

Carrey, R., Otero, N., Soler, A., Gómez-Alday, J.J., Ayora, C., 2013. The role of Lower Cretaceous sediments in groundwater nitrate attenuation in central Spain: Column experiments. *Appl. Geochem.* 32, 142–152.

Casciotti, K.L., Sigman, D.M., Galanter Hastings, M., Böhlke, J.K., Hilkert, A., 2002. Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal. Chem.* 74, 4905–4912.

Chapelle, F.H., Knobel, L.L., 1985. Stable carbon isotopes of HCO_3^- in the Aquia Aquifer, Maryland: Evidence for an isotopically heavy source of CO_2 . *Ground Water* 23, 592–599.

Cheung, K., Klassen, P., Mayer, B., Goodarzi, F., Aravena, R., 2010. Major ion and isotope geochemistry of fluids and gases from coalbed methane and shallow groundwater wells in Alberta, Canada. *Appl. Geochem.* 25, 1307–1329.

Clark, I.D., Fritz, P., 1997. *Environmental Isotopes in Hydrology*. Lewis Publishers, New York, 328 pp.

Conrad, R., 2005. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. *Organic Geochem.* 36, 739–752.

Delwiche, C.C., Steyn, P.L., 1970. Nitrogen isotope fractionation in soils and microbial reactions. *Environ. Sci. Technol.* 4, 929–935.

Dogramaci, S.S., Herczeg, A.L., Schiff, S.L., Bone, Y., 2001. Controls on $\delta^{34}\text{S}$ and $\delta^{18}\text{O}$ of dissolved sulfate in aquifers of the Murray Basin, Australia and their use as indicators of flow processes. *Appl. Geochem.* 16, 475–488.

Edmunds, W.M., Bath, A.H., Miles, D.L., 1982. Hydrochemical evolution of the East Midlands Triassic sandstone aquifer, England. *Geochim. Cosmochim. Acta* 46, 2069–2081.

Feast, N.A., Hiscock, K.M., Dennis, P.F., Bottrell, S.H., 1997. Vertical chemical and isotopic

577 gradients in Chalk groundwaters. *Appl. Geochem.* 12, 803–812.

578 Harrison, A.G., Thode, H.G., 1958. Mechanism of the bacterial reduction of sulphate from isotope
579 fractionation studies. *Trans. Faraday Soc.* 53, 84–92.

580 Hendry, M.J., Cherry, J.A., Wallick, E.I., 1986. Origin and distribution of sulfate in a Fractured Till
581 in Southern Alberta, Canada. *Water Resour. Res.* 22, 45–61.

582 Hosono, T., Delinon, R., Nakano, T., Kagabu, M., Shimada, J., 2011b. Evolution model of $\delta^{34}\text{S}$ and
583 $\delta^{18}\text{O}$ in dissolved sulfate in volcanic fan aquifers from recharge to coastal zone and through the
584 Jakarta urban area, Indonesia. *Sci. Total Env.* 409, 2541–2554.

585 Hosono, T., Ikawa, R., Shimada, J., Nakano, T., Saito, M., Onodera, S., Lee, K.-K., Taniguchi, M.,
586 2009. Human impacts on groundwater flow and contamination deduced by multiple isotopes in
587 Seoul City, South Korea. *Sci. Total Env.* 407, 3189–3197.

588 Hosono, T., Nakano, T., Shimizu, Y., Onodera, S., Taniguchi, M., 2010a. Hydrogeological constraint
589 on nitrate and arsenic contamination in Asian metropolitan groundwater. *Hydrol. Process.* 379,
590 23–36.

591 Hosono, T., Siringan, F., Yamanaka, T., Umezawa, Y., Onodera, S., Nakano, T., Taniguchi, M.,
592 2010. Application of multi-isotope ratios to study the source and quality of urban groundwater
593 in Metro Manila, Philippines. *Appl. Geochem.* 25, 900–909.

594 Hosono, T., Tokunaga, T., Kagabu, M., Nakata, H., Orishikida, T., Lin, I.-T., Shimada, J., 2013. The
595 use of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ tracers with an understanding of groundwater flow dynamics for
596 evaluating the origins and attenuation mechanisms of nitrate pollution. *Water Res.* 47, 2661–
597 2675.

598 Hossain, S., Hosono, T., Tokunaga, T., Ide, K., Shimada, J., 2013. Geochemical modeling of
599 groundwater evolution in a volcanic aquifer system of Kumamoto area, Japan. American
600 Geophysical Union (AGU) 2013 Fall meeting, San Francisco, 9–13 December 2013, abstract,
601 H11H-1247.

602 Hélie, J.-F., Hillaire-Marcel, C., Rondeau, B., 2002. Seasonal changes in the sources and fluxes of
603 dissolved inorganic carbon through the St. Lawrence River— isotopic and chemical constraint.
604 *Chem. Geol.* 186, 117–138.

605 Kaplan, I.R., Rittenberg, S.C., 1964. Microbiological fractionation of sulphur isotopes. *J. Gen.*
606 *Microbiol.* 34, 195–212.

607 Kaplan, N., Magaritz, M.A., 1986. Nitrogen-isotope study of the sources of nitrate contamination in
608 groundwater of the pleistocene coastal plain aquifer, Israel. *Water Res.* 20, 131–135.

609 Kendall, C., 1998. Tracing nitrogen sources and cycling in catchments. In *Isotope Tracers in*
610 *Catchment Hydrology*; Kendall, C.; McDonnell, J.J. Eds.; Elsevier Science B.V., pp 519–576.

611 Knowles, R., 1982. Denitrification. *Microbiol. Rev.* 46, 43–70.

612 Korom, S.F., 1992. Natural denitrification in the saturated zone: a review. *Water Resour. Res.* 28,

613 1657–1668.

614 Kreitler, C.W., 1979. Nitrogen-isotope ratio studies of soils and groundwater nitrate from alluvial
615 fan aquifers in Texas. *J. Hydrol.* 42, 147–170.

616 Kumamoto Prefecture and Kumamoto City., 1995. Integrated Groundwater Survey Report of the
617 Kumamoto area. Kumamoto Prefecture and Kumamoto City, 122 pp (in Japanese).

618 Lansdown, J.M., Quay, P.D., King, S.L., 1992. CH₄ production via CO₂ reduction in a temperate
619 bog: A source of ¹³C-depleted CH₄. *Geochim. Cosmochim. Acta* 56, 3493–3503.

620 Mariotti, A., Landreau, A., Simon, B., 1988. ¹⁵N isotope biogeochemistry and natural denitrification
621 process in groundwater: Application to the chalk aquifer of northern France. *Geochim.*
622 *Cosmochim. Acta* 52, 1869–1878.

623 Martini, A.M., Walter, L.M., Budai, J.M., Ku, T.C.W., Kaiser, C.J., Schoell, M., 1998. Genetic and
624 temporal relations between formation waters and biogenic methane: Upper Devonian Antrim
625 Shale, Michigan Basin, USA. *Geochim. Cosmochim. Acta* 62, 1699–1720.

626 Massmann, G., Tichomirowa, M., Merz, C., Pekdeger, A., 2003. Sulfide oxidation and sulfate
627 reduction in a shallow groundwater system (Oderbruch Aquifer, Germany). *J. Hydrol.* 278,
628 231–243.

629 McMahon, P.B., Chapelle, F.H., 2008. Redox processes and water quality of selected principal
630 aquifer systems. *Ground Water* 46, 259–271.

631 Morris. B.L., Lawrence, A.R.L., Chilton, P.J.C., Adams, B., Calow, R.C., Klinck, B.A., 2003.
632 Groundwater and its susceptibility to degradation: a global assessment of the problem and
633 options for management. Early Warning and Assessment Report Series, RS. 03-3. United
634 Nations Environmental Programme, Nairobi, Kenya.

635 Nakai, N., Jensen, M.L., 1964. The kinetic isotope effect in bacterial reduction and oxidation of
636 sulfur. *Geochim. Cosmochim. Acta* 28, 1893–1912.

637 Ono, K., Watanabe, K., 1985. Geological map of Aso volcano (1:50,000). Geological Map of
638 Volcanoes 4, Geological Survey of Japan (in Japanese with English abstract).

639 Ono, M., Tokunaga, T., Shimada, J., Ichiyanagi, K., 2013. Application of continuous ²²²Rn monitor
640 with dual loop system in a small lake. *Ground Water* 51, 706–713.

641 Oshima, I., 2010. Administration for groundwater management in the Kumamoto area. *J.*
642 *Groundwater Hydrol.* 52, 49–64 (in Japanese with English Abst.).

643 Otero, N., Torrentó, C., Soler, A., Menció, A., Mas-Pla, J., 2009. Monitoring groundwater nitrate
644 attenuation in a regional system coupling hydrogeology with multi-isotopic methods: the case
645 of Plana de Vic (Osona, Spain). *Agric. Ecosyst. Env.* 133, 103–113.

646 Ottley, C.J., Davison, W., Edmunds, W.M., 1997. Chemical catalysis of nitrate reduction by iron (II).
647 *Geochim. Cosmochim. Acta* 61, 1819–1828.

648 Pauwels, H., Pettenati, M., Greffié, C. 2010. The combined effect of abandoned mines and

649 agriculture on groundwater chemistry. *J. Contam. Hydrol.* 115, 64–78.

650 Puig, R., Folch, A., Menció, A., Soler, A., Mas-Pla, J., 2013. Multi-isotopic study (^{15}N , ^{34}S , ^{18}O , ^{13}C)
651 to identify processes affecting nitrate and sulfate in response to local and regional groundwater
652 mixing in a large-scale flow system. *Appl. Geochem.* 32, 129–141.

653 Rees, C.E., 1973. A steady-state model for sulphur isotope fractionation in bacterial reduction
654 processes. *Geochim. Cosmochim. Acta* 37, 1141–1162.

655 Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N., 2008. Bemment, C.D. Nitrate attenuation in
656 groundwater: A review of biogeochemical controlling processes. *Water Res.* 42, 4215–4232.

657 Rye, R.O., Back, W., Hanshaw, B.B., Rightmire, C.T., Pearson, F.J., 1981. The origin and isotopic
658 composition of dissolved sulphide in groundwater from carbonate aquifers in Florida and Texas.
659 *Geochim. Cosmochim. Acta* 45, 1941–1950.

660 Schulte, P., van Geldern, R., Freitag, H., Karim, A., Négrel, P., Petelet-Giraud, E., Probst, A., Probst,
661 J.L., Telmer, K., Veizer, J., Barth, J.A.C., 2011. Applications of stable water and carbon
662 isotopes in watershed research: Weathering, carbon cycling, and water balances. *Earth Sci. Rev.*
663 109, 20–31.

664 Schwientek, M., Einsiedl, F., Stichler, W., Stögbauer, A., Strauss, H., Maloszewski, P., 2008.
665 Evidence for denitrification regulated by pyrite oxidation in a heterogeneous porous
666 groundwater system. *Chem. Geol.* 255, 60–67.

667 Sharma, S., Frost, C.D., 2008. Tracing coalbed natural gas-coproduced water using stable isotopes of
668 carbon. *Ground Water* 46, 329–334.

669 Shimada, J., 2012. Sustainable management of groundwater resources for 700,000-plus residents: a
670 practical example of the transboundary management of groundwater resources in the
671 Kumamoto area, Japan. In *The Dilemma of Boundaries: Toward a New Concept of Catchment*.
672 Taniguchi, M.; Shiraiwa, T., Eds.; Springer Japan, pp 235–246.

673 Sigman, D.M., Casciotti, K.L., Andreani, M., Barford, C., Galanter, M., Böhlke, J.K., 2001. A
674 bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal.*
675 *Chem.* 73, 4145–4153.

676 Smith, R.L., Howes, B.L., Duff, J.H., 1991. Denitrification in nitrate-contaminated groundwater:
677 Occurrence in steep vertical geochemical gradients. *Geochim. Cosmochim. Acta* 55, 1815–
678 1825.

679 Strebel, O., Böttcher, J., Fritz, P., 1990. Use of isotope fractionation of sulfate–sulfur and sulfate–
680 oxygen to assess bacterial desulfurication in a sandy aquifer. *J. Hydrol.* 12, 155–172.

681 Strous, M., Jetten, M.S.M., 2004. Anaerobic oxidation of methane and ammonium. *Annu. Rev.*
682 *Microbiol.* 58, 99–117.

683 Sutka, R.L., Ostrom, N.E., Ostrom, P.H., Breznak, J.A., Gandhi, H., Pitt, A.J., Li, F., 2006.
684 Distinguishing nitrous oxide production from nitrification and denitrification on the basis of

isotopomer abundances. Appl. Environ. Microbiol., 72, 638–644.

Tanaka, K., Funakoshi, Y., Hokamura, T., Yamada, F., 2010. The role of paddy rice in recharging urban groundwater in the Shira River Basin. Paddy Water Env. 8, 217–226.

Taniguchi, M., Shimada, J., Uemura, T., 2003. Transient effects of surface temperature and groundwater flow on subsurface temperature in Kumamoto Plain, Japan. Phys. Chem. Earth 28, 477–486.

Tomiie, K., Itomitsu, N., Matsuyama, K., Kakimoto, R., Kawagoshi, Y., 2011. Nitrate-nitrogen concentration in groundwater of in Kumamoto urban area and determination of its contribution using geographical information system and nitrogen stable isotope analysis. J. Japan Soc. Water. Env. 34, 1-9 (in Japanese with English Abst.).

Torrentó, C., Urmeneta, J., Otero, N., Soler, A., Viñas, M., Cama, J., 2011. Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. Chem. Geol. 287, 90–101.

Tuttle, M.L.W., Breit, G.N., Cozzarelli, I.M., 2009. Processes affecting $\delta^{34}\text{S}$ and $\delta^{18}\text{O}$ values of dissolved sulfate in alluvium along the Canadian River, central Oklahoma, USA. Chem. Geol. 265, 455–467.

Vengosh, A., Gill, J., Davisson, M.L., Hudson, G.B., 2002. A multi-isotope (B, Sr, O, H, and C) and age dating (3H – 3He and ^{14}C) study of groundwater from Salinas Vally, California: hydrochemistry, dynamics, and contamination processes. Water Resour. Res. 38, 2008. doi:10.1029/2001WR000517.

Vidal-Gavilan, G., Folch, A., Otero, N., Solanas, A.M., Soler, A., 2013. Isotope characterization of an *in situ* bionitrification pilot-test in a fractured aquifer. Appl. Geochem. 32, 153–163.

Vitòria, L., Soler, A., Canals, À., Otero, N., 2008. Environmental isotopes (N, S, C, O, D) to determine natural attenuation process in nitrate contaminated waters: Example of Osona (NE Spain). Appl. Geochem. 23, 3597–3611.

Warner, N.R., Kresse, T.M., Hays, P.D., Downa, A., Karr, J.D., Jackson, R.B., Vengosh, A., 2013. Geochemical and isotopic variations in shallow groundwater in areas of the Fayetteville Shale development, north-central Arkansas. Appl. Geochem. 35, 207–220.

Wellman, R.P., Cook, F.D., Krouse, H.R., 1968. Nitrogen-15: microbiological alteration of abundance. Science, 161, 269–270.

Widory, D., Kloppmann, W., Chery, L., Bonnin, J., Rochdi, H., Guinamant, J.-L., 2004. Nitrate in groundwater: an isotopic multi-tracer approach. J. Contam. Hydrol. 72, 165–188.

Yamanaka, M., Kumagai, Y., 2006. Sulfur isotope constraint on the provenance of salinity in a confined aquifer system if the southwestern Nobi Plain, central Japan. J. Hydrol. 325, 35–55.

Figure captions

721 Fig. 1: Conceptual $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ evolutionary groundwater model, with the progress
722 of both heterotrophic (reaction 2) and autotrophic (reaction 4) denitrification, based on field
723 observations under the specific condition where denitrification is the major anaerobic process
724 (Aravena and Robertson; 1998; Otero et al., 2009).

725 Fig. 2: Map of land-use and the sampling locations in the Kumamoto area.

726 Fig. 3: Relationships between (a) $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{13}\text{C}_{\text{DIC}}$ and (b) $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ in water samples
727 from the Kumamoto area. The $\delta^{15}\text{N}_{\text{NO}_3}$ data are after Hosono et al. (2013).

728 Fig. 4: Changes in NO_3^- , HCO_3^- , and SO_4^{2-} concentrations, and in $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$
729 values with A–A' and B–B' groundwater flows. The NO_3^- and $\delta^{15}\text{N}_{\text{NO}_3}$ data are after Hosono et
730 al. (2013). The plots correspond to groundwater samples collected from boreholes within the
731 solid lines in Fig. 2. The parallel axis indicates the sampling points in
732 groundwater-flow-direction order for A–A' and B–B'. The order was determined from the
733 connections between the A–A' (or B–B') lines and the perpendicular line from each site in Fig.
734 2.

735 Fig. 5: Relationships between (a) $\delta^{13}\text{C}_{\text{DIC}}$ and HCO_3^- and (b) $\delta^{34}\text{S}_{\text{SO}_4}$ and SO_4^{2-} in water samples
736 from the Kumamoto area. Three SO_4^{2-} concentration peaks for groundwater samples are shown
737 in gray color. The $\delta^{34}\text{S}_{\text{SO}_4}$ ranges for the possible sulfur sources are also shown for comparison.

738 Fig. 6: Developed model of the $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ evolutionary patterns in groundwater
739 according to combinations of major anaerobic bacterial reactions (case 1–4), in case that effect
740 of dissolution of carbonate minerals and mixing of different sources could be excluded. Rows
741 “a” and “b” show $\delta^{15}\text{N}_{\text{NO}_3}$ vs. $\delta^{13}\text{C}_{\text{DIC}}$ diagrams whereas row “c” shows $\delta^{15}\text{N}_{\text{NO}_3}$ vs. $\delta^{34}\text{S}_{\text{SO}_4}$
742 diagrams. Rows “a” and “b” represent groundwater in which DIC is depleted and enriched of
743 organic source C, respectively. The ideal compositional areas, showing sample plot distributions
744 for groundwater in environment where heterotrophic (gray zone) and autotrophic (dotted hatch
745 pattern) denitrification reactions could occur, are useful to distinguish between denitrification
746 types in aquifer systems.

747

748 **Appendixes**

749 Appendix 1: Hydrogeological cross sections between A–A' and B–B' in Fig. 2 (modified from
750 Hosono et al., 2013). Arrows represent the groundwater flow direction in each grid, obtained
751 from groundwater flow model calculations using a surface and subsurface coupling flow
752 simulator (GETFLOWS). The size of each arrow corresponds to the relative velocity of the
753 groundwater flow.

754 Appendix 2: ORP vs. DO diagram for water samples collected in the Kumamoto area.

755 Appendix 3: Comparison of the high and low groundwater potential seasons for (a) HCO_3^- , (b)
756 SO_4^{2-} , (c) $\delta^{13}\text{C}_{\text{DIC}}$, and (d) $\delta^{34}\text{S}_{\text{SO}_4}$.

757 Appendix 4: Distributions of (a) HCO_3^- , (b) SO_4^{2-} , (c) $\delta^{13}\text{C}_{\text{DIC}}$, and (d) $\delta^{34}\text{S}_{\text{SO}_4}$ in the study area.
758 Appendix 5: $\delta^{34}\text{S}_{\text{SO}_4}$ vs. Cl^- diagram for water samples collected in the Kumamoto area.
759 Appendix 6: $\delta^{34}\text{S}_{\text{SO}_4}$ vs. NO_3^- diagram for water samples collected in the Kumamoto area. The NO_3^-
760 data are after Hosono et al. (2013).

Appendix 1: Hydrogeological cross sections between A–A' and B–B' in Fig. 2 (modified from Hosono et al., 2013). Arrows represent the groundwater flow direction in each grid, obtained from groundwater flow model calculations using a surface and subsurface coupling flow simulator (GETFLOWS). The size of each arrow corresponds to the relative velocity of the groundwater flow.

Appendix 2: ORP vs. DO diagram for water samples collected in the Kumamoto area.

Appendix 3: Comparison of the high and low groundwater potential seasons for (a) HCO_3^- , (b) SO_4^{2-} , (c) $\delta^{13}\text{C}_{\text{DIC}}$, and (d) $\delta^{34}\text{S}_{\text{SO}_4}$.

Appendix 5: $\delta^{34}\text{S}_{\text{SO}_4}$ vs. Cl^- diagram for water samples collected in the Kumamoto area.

Fig. 1: Conceptual $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ evolutionary groundwater model, with the progress of both heterotrophic (reaction 2) and autotrophic (reaction 4) denitrification, based on field observations under the specific condition where denitrification is the major anaerobic process (Aravena and Robertson; 1998; Otero et al., 2009).

Appendix 6: $\delta^{34}\text{S}_{\text{SO}_4}$ vs. NO_3^- diagram for water samples collected in the Kumamoto area. The NO_3^- data are after Hosono et al. (2013).

Appendix 4: Distributions of (a) HCO_3^- , (b) SO_4^{2-} , (c) $\delta^{13}\text{C}_{\text{DIC}}$, and (d) $\delta^{34}\text{S}_{\text{SO}_4}$ in the study area.

Fig. 2: Map of land-use and the sampling locations in the Kumamoto area.

Fig. 3: Relationships between (a) $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{13}\text{C}_{\text{DIC}}$ and (b) $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ in water samples from the Kumamoto area. The $\delta^{15}\text{N}_{\text{NO}_3}$ data are after Hosono et al. (2013).

Fig. 4: Changes in NO_3^- , HCO_3^- , and SO_4^{2-} concentrations, and in $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ values with A–A' and B–B' groundwater flows. The NO_3^- and $\delta^{15}\text{N}_{\text{NO}_3}$ data are after Hosono et al. (2013). The plots correspond to groundwater samples collected from boreholes within the solid lines in Fig. 2. The parallel axis indicates the sampling points in groundwater-flow-direction order for A–A' and B–B'. The order was determined from the connections between the A–A' (or B–B') lines and the perpendicular line from each site in Fig. 2.

Fig. 5: Relationships between (a) $\delta^{13}\text{C}_{\text{DIC}}$ and HCO_3^- and (b) $\delta^{34}\text{S}_{\text{SO}_4}$ and SO_4^{2-} in water samples from the Kumamoto area. Three SO_4^{2-} concentration peaks for groundwater samples are shown in gray color. The $\delta^{34}\text{S}_{\text{SO}_4}$ ranges for the possible sulfur sources are also shown for comparison.

Case of Kumamoto

Fig. 6: Developed model of the $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ evolutionary patterns in groundwater according to combinations of major anaerobic bacterial reactions (case 1–4), in case that effect of dissolution of carbonate minerals and mixing of different sources could be excluded. Rows “a” and “b” show $\delta^{15}\text{N}_{\text{NO}_3}$ vs. $\delta^{13}\text{C}_{\text{DIC}}$ diagrams whereas row “c” shows $\delta^{15}\text{N}_{\text{NO}_3}$ vs. $\delta^{34}\text{S}_{\text{SO}_4}$ diagrams. Rows “a” and “b” represent groundwater in which DIC is depleted and enriched of organic source C, respectively. The ideal compositional areas, showing sample plot distributions for groundwater in environment where heterotrophic (gray zone) and autotrophic (dotted hatch pattern) denitrification reactions could occur, are useful to distinguish between denitrification types in aquifer systems.

Graphical Abstract

This study developed the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ tracing method to study denitrification mechanism with understanding of wide-range anaerobic bacterial processes in groundwater flow dynamics.