Highlights

- Dominant nitrate sources are chemical fertilizer and livestock wastes.
- It is difficult to distinguish pollution sources using $\delta^{18}O$ and $\delta^{15}N$ from NO_3.
- Coprostanol showed potential for source identification of nitrate pollution.
- A methodology using coprostanol is proposed to identify source of nitrate pollution.

On the use of coprostanol to identify source of nitrate pollution in groundwater

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Abstract

Investigation of contaminant sources is indispensable for developing effective countermeasures against nitrate (NO_3^-) pollution in groundwater. Known major nitrogen (N) sources are chemical fertilizers, livestock waste, and domestic wastewater. In general, scatter diagrams of $\delta^{18}O$ and $\delta^{15}N$ from NO_3^- can be used to identify these pollution sources. However, this method can be difficult to use for chemical fertilizers and livestock waste sources due to the overlap of $\delta^{18}O$ and $\delta^{15}N$ ranges. In this study, we propose to use coprostanol as an indicator for the source of pollution. Coprostanol can be used as a fecal contamination indicator because it is a major fecal sterol formed by the conversion of cholesterol by intestinal bacteria in the gut of higher animals. The proposed method was applied to investigate NO_3^- pollution sources for groundwater in Shimabara, Nagasaki, Japan. Groundwater samples were collected at 33 locations from March 2011 to November 2015. These data were used to quantify relationships between NO_3-N, $\delta^{15}N$-NO_3^-, $\delta^{18}O$-NO_3^-, and coprostanol. The results show that coprostanol has a potential for source identification of nitrate pollution. For lower coprostanol concentrations (<30 ng L^{-1}) in the nitrate-polluted group, fertilizer is likely to be the predominant source of NO_3^-. However, higher concentration coprostanol samples in the nitrate-polluted group can be related to pollution from manure. Thus, when conventional diagrams of isotopic ratios cannot distinguish pollution sources, coprostanol may be a useful tool.

Key words

Groundwater, Nitrate pollution, Stable isotopes, Coprostanol

1. Introduction

Nitrate contamination in groundwater as a consequence of intensive agricultural activities is a severe problem. In order to establish effective countermeasures against nitrate contamination identification of the nitrate source is crucial. Statistical methods such as correlation between nitrate and characteristic ions (e.g., SO_4^{2-}, Ca^{2+}, and Mg^{2+}) have been successfully used to locate and understand nitrate sources. Positive correlation with such ions means that the nitrate source originates from chemical fertilizer (Babiker et al., 2004). In a similar manner, stable isotopic ratios of nitrate ($\delta^{15}N$) have been applied as a powerful tool (e.g., Williams et al., 1998; Rivers et al., 1996). Although, $\delta^{15}N$ from nitrate sources shows a distinct range (e.g., -15 to +15‰ in atmospheric NO_3, -4 to +4‰ in inorganic fertilizer, +2 to +30‰ in organic fertilizer, and +10 to +20‰ in animal waste; Kendall, 1998), it is often difficult to distinguish pollution sources due to overlapping ranges. The $\delta^{18}O$ from nitrate, however, is an additional tool for determining nitrate source and reactions. Kendall (1998) illustrated the usefulness of scatter diagrams of $\delta^{18}O$ and $\delta^{15}N$ for interpreting dominant nitrate sources. He showed that different ranges could be explained by the diverse origins of nitrate (NO_3 in precipitation, desert NO_3 deposits, NO_3

55 fertilizer, NH_4 in fertilizer and rain, manure and septic waste, and soil N). Moreover, dual isotopic data
56 are useful for judging if denitrification occurs because this process increases the $\delta^{18}O$ and $\delta^{15}N$ of residual
57 nitrate. Although, nitrate originating from nitrate fertilizer or atmospheric sources are distinguishable
58 from ammonium fertilizer, soil N, and manure containing $\delta^{18}O$, it is still difficult to distinguish different
59 sources of chemical fertilizer and livestock waste sources because of overlapping $\delta^{15}N$ ranges. To
60 overcome this problem, isotopic data combined with a Bayesian mixing model is a reliable way for
61 quantifying proportional contributions of potential nitrate sources (Matiatos, 2016; Kim et al., 2015).
62 However, precaution is required because the model resolution is significantly affected by the temporal
63 variability of the isotopic composition of nitrate in the mixture and uncertainty of the isotopic
64 composition of different nitrate sources (Xue et al., 2012).

65 Shimabara City, Nagasaki, Japan, utilizes groundwater for agriculture, industry, and domestic
66 water including drinking water. However, due to intensive agricultural activities, the nitrate level in
67 groundwater has increased to above the Japanese drinking water quality standard (10 mg L^{-1}). According
68 to Nakagawa et al. (2016), 38% (15 out of 40 groundwater wells) exceed the permissible NO_3-N + NO_2-N
69 concentration. The nitrate pollution in groundwater has been shown to be related to chemical fertilizer
70 and livestock waste by use of the correlation matrix for major dissolved ion components. However, the
71 identification of specific nitrate sources could not be accomplished in the above study. For this reason, we
72 herein propose an easy-to-use approach involving coprostanol to identify the main nitrate source.

Coprostanol (5β (H)-Cholestan-3β-ol, CAS No. 360-68-9) is one of the sterols, which is produced by bacterial reduction of cholesterol in the gut of higher animals (Martins et al., 2007). It has been widely used as an indicator of fecal contamination in lagoons and estuaries (Martins et al., 2007; Reeves and Patton, 2005). In this paper, NO_3-N, coprostanol, $\delta^{18}O$, and $\delta^{15}N$ from nitrate were investigated to evaluate the feasibility of the proposed methodology to identify the source of nitrate groundwater pollution. For this purpose, three kinds of relationships were developed and analyzed; (i) $\delta^{18}O$ and $\delta^{15}N$ from nitrate derived from Kendall (1998), (ii) $\delta^{15}N$ from nitrate and coprostanol, and (iii) NO_3-N and coprostanol levels.

2. Study site

Shimabara City is located on the northeastern Shimabara Peninsula, covering 82.8 km^2 (Fig. 1). In the northern part of the city, an alluvial fan is formed from Mt. Fugen located on the apex center of the peninsula. Volcanic deposits such as tuff breccia, tuff, and volcanic conglomerate constitute and are distributed around the mountain. Upland areas and paddy fields are concentrated in the northern parts of the city. Areas above an altitude of 200 m are generally occupied by forest. Above an elevation of 300 m, hornblende-andesite is distributed. Due to the collapse of Mt. Mayu in 1792, Mayuyama avalanche debris deposits are distributed in the eastern area of the city. The urban area lies on these deposits. The climate is mild-humid with mean annual precipitation ranging between 1970 and 2476 mm and mean annual

91 temperature between 16.9°C and 17.2°C (2013-2015). Although it rains throughout the year, the rainfall is
92 particularly abundant from June to August.
93
94 **3. Materials and methods**
95 Groundwater samples were collected at 33 locations from March 2013 to November 2015 (Fig.
96 1). Sampling locations were constituted by 5 shallow wells, 21 deep wells, 1 unknown well depth, and 6
97 springs. Shallow well is defined as <30 m deep and deep well as >30 m deep. Collected water samples for
98 analysis of NO_3^- and coprostanol were filled in prewashed bottles and stored in refrigerator. Samples for
99 nitrate isotope ratios were filtered through 0.22 μm membrane filter and kept frozen until analysis. NO_3^-
100 was analyzed by ion chromatography of suppressor type (Metrohm 861 Advanced Compact IC). $\delta^{15}N$ and
101 $\delta^{18}O$ of nitrate were determined by the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001) for
102 samples collected on November 4 and 20 2014 and November 20 2015. Denitrifying bacteria lacking N_2O
103 reductase convert NO_3^- to N_2O. Analysis of $\delta^{15}N$ and $\delta^{18}O$ of induced N_2O were implemented
104 simultaneously. Dual isotopes of nitrate can be analyzed accurately for samples that are affected by
105 denitrification and with low nitrate level (1 μM) (Sigman et al., 2001; Hosono et al., 2011). Since
106 coprostanol is produced in the digestive tracts of mammals by microbial reduction of cholesterol,
107 livestock waste is likely to be a main contaminant source for samples with high coprostanol contents. The
108 extraction method of coprostanol for the groundwater samples was implemented according to the below

and referring to Hussain et al. (2010), though with some modification. In total, 800 mL of the water samples were acidified with 1.0 N HCl to pH 2-3. Groundwater samples were filtered through two borosilicate glass fiber filters with 0.7 and 2.7 μm. The coprostanol was extracted from the filters with methanol to collect adsorbed coprostanol on suspended particles such as organic matter and fine fractions of soil (coprostanol has low water solubility and tends to be associated with suspended particles (Hussain et al., 2010)). The extracted methanol was mixed with the water sample that passed through the filters. The coprostanol was extracted from the water samples by liquid-liquid extraction with dichloromethane under room temperature during three successive times. The extract was concentrated to near dryness (<1.0 mL) under pure nitrogen gas flow and dehydrated with anhydrous sodium sulfate. It was formed to trimethylsilyl ether using BSTFA (bis-trimethylsilyl trifluoroacetamide) at 80^{o}C during 60 min to increase resolution for chromatography, and then quantified by 7000A Triple Quadrupole GC/MS (Agilent Technologies).

For each sampling location, all measurement components (NO_3^-, coprostanol, $\delta^{15}N$, and $\delta^{18}O$) were averaged to describe the analytic results. Samples with undetected coprostanol were treated as 0. The detection limit was 1.4 ng L^{-1}. Analyses results were classified into four groups (Nakagawa et al., 2016) and plotted in the same diagram depending on nitrate concentration. The groups were determined through cluster analysis using major ion concentrations from our previous study (Nakagawa et al., 2016). According to this analysis, water samples can be classified into four spatial groups. The water chemistry

of Group 4 is influenced by nitrate pollution and ion dissolution. Most samples of Group 1 are influenced by only ion dissolution. Group 2 is influenced by mixed effects of the ion-dissolution and nitrate-pollution. There is no significant influence on Group 3 (Nakagawa et al., 2016).

4. Results and discussion

4. 1. NO_3-N pollution in groundwater

Averages and standard deviations for NO_3-N at respective location are shown in Fig. 2. The red line represents Japanese maximum permissible level of NO_2-N + NO_3-N for drinking (10 mg L^{-1}). NO_3-N levels ranged from 0.1 to 23.3 mg L^{-1}. Standard deviations varied from 0.02 to 4.4 mg L^{-1} with an average of 1.2 mg L^{-1}. NO_3-N concentration in shallow wells were relatively high as compared to the deep wells. Temporal variation was relatively small, although concentrations decreased due to dilution by rainfall, depending on the well (Nakagawa et al., 2016). About 39% of all locations displayed a higher concentration than the permissible level for drinking purposes. Shimabara City represents mainly three kinds of land use, namely forest, agricultural field, and urban area. NO_3-N levels for water samples collected from agricultural areas all tend to exceed the Japanese permissible level for drinking water (Nakagawa et al., 2016). NO_3-N contamination in the groundwater extended down to 50 m depth from the soil surface at the sampling sites O-1 and 2 (Amano et al., 2016). Therefore, identification of nitrate sources in groundwater is important in order to preserve water resources for the future.

145

146 **4. 2. Coprostanol in groundwater**

147 Averages and standard deviations of coprostanol concentrations for respective location are
148 shown in Fig. 3. Coprostanol levels ranged from 0.0 (N. D. = Not Detected) to 172.1 ng L^{-1}. Standard
149 deviations varied from 0.0 to 384.9 ng L^{-1} with an average of 79.1 ng L^{-1}. The highest coprostanol
150 concentration was found at site W-2. This site is located downstream of a potentially high nitrate loading
151 district of livestock waste. As coprostanol is mixed with organic colloids, it is highly likely to be
152 incorporated into sediments (Reeves and Patton, 2005). Coprostanol has a low solubility in water and
153 tends to adsorb to suspended particles and sediments (Hussain et al., 2010). In general, sterols are
154 hydrophobic, thus, coprostanol may be assumed to be associated with particles (Froehner et al., 2010).
155 These processes indicate that coprostanol levels in the groundwater may be lower than those in sediments.
156 Writer et al. (1995) suggested that sedimentary coprostanol concentrations higher than 100 ng g^{-1} should
157 be a result of sewage release. González-Oreja and Saiz-Salinas (1998) stated that coprostanol levels
158 greater than 500 ng g^{-1} may be an indication of sewage contamination. Considering adsorption
159 characteristics of coprostanol as mentioned above, the sediment contents in the study area might be higher
160 than these criteria. To confirm this hypothesis, contents of adsorbed coprostanol in the sediments should
161 be measured in future studies.

162

163 **4. 3. Nitrogen and oxygen isotopes of nitrate**

164 The method suggested by Kendall (1998) was used to investigate pollution sources. Thus, the
165 averaged concentrations of $\delta^{15}N$ and $\delta^{18}O$ from NO_3^- were plotted in a scatter diagram as shown in Fig. 4.
166 The data were classified into the four cluster groups according to the above and colored depending on
167 coprostanol level. All sampled data are confined between 3.3 and 8.4‰ for $\delta^{15}N$, and -0.4 and 3.1‰ for
168 $\delta^{18}O$ except for the site W-19. According to Hosono et al. (2013), the isotopic composition corresponds to
169 the range of chemical fertilizers which is a potential nitrate source in the study area. However, a large
170 number of livestock (approximately 1,000 milk cattle, 23,000 pigs, and 1,000,000 chickens in 2015) are
171 raised in the Shimabara study area. Although, the number of beef cattle and broilers is not known, there
172 are 62 and 2 livestock farmer associations for the respective livestock in the study area. The potential
173 nitrate load from livestock waste is thus much higher than that of chemical fertilizer (Nakagawa et al.,
174 2015). For this reason, livestock waste is expected to be an important nitrate source in the study area. As
175 mentioned above, except for the sampling site (W-19) where denitrification occurred, the plotted results
176 are concentrated to an overlapping region of both chemical fertilizer and livestock waste sources. The site
177 W-19 shows at least 1.5 times the concentration for HCO_3^- induced by denitrification processes as
178 compared to the other sites (Nakagawa et al., 2016). The samples with higher levels of coprostanol did
179 not display a relatively high isotopic constituent from livestock waste but instead showed a low isotopic
180 level. Samples with no detected coprostanol are located in the higher isotope area. Therefore, it is still

difficult to distinguish the contaminant source. Some samples classified into non-polluted groups 1–3 (lower nitrate and coprostanol levels) are also located in this overlapping region. These results indicate that the source of nitrate in non-polluted groups are soil NH_4^+ and/or septic waste sources because the location of these groups corresponds to forested and urban area.

4. 4. Relationship between $\delta^{15}N$ of nitrate and coprostanol

As a further analysis, we plotted averaged coprostanol and $\delta^{15}N$ in a scatter diagram (Fig. 5). The classification of the groups and coprostanol levels are the same as in Fig. 4. As can be seen, also here no clear relationship can be observed. However, coprostanol concentrations can be used to divide the polluted samples from the non-polluted group. This indicates that heavily polluted groundwater samples are related to livestock waste in the study area. Relatively high level coprostanol ($\geqq$30 ng L^{-1}) samples correspond to the polluted sample group 4 (Nakagawa et al., 2016). These results correspond to the potential nitrate load from livestock waste load (Nakagawa et al., 2015), which is much higher than that of chemical fertilizers, based on calculations from the Census of Agriculture and Forestry (Ministry of Agriculture, Forestry and Fisheries, Minister's Secretariat Statistics Bureau, 2012).

4. 5. Relationship between NO_3-N and coprostanol

According to the above, it appears difficult to identify nitrate sources using isotopes only. For

this reason, averaged nitrate and coprostanol concentrations were plotted in a scatter diagram (Fig. 6). The four characteristic water quality groups according to Nakagawa et al. (2016) are also plotted in the same diagram. As seen from the diagram, high coprostanol concentrations coincide with the polluted group 4. However, also sampling locations with high NO_3-N concentration (10 mg L^{-1}) and classified into polluted group 4 include samples containing lower levels of coprostanol (<30 ng L^{-1}). Chemical fertilizers are likely to be the predominant source of NO_3^- for this lower level area. In recent sampling campaigns, it was difficult to detect coprostanol in our study area. The predominant nitrate source may therefore be shifting from livestock waste to chemical fertilizer. Some sampling sites that showed lower levels of both coprostanol and NO_3^- were located in the urban area, indicating that coprostanol originates from septic waste (human excrement). In any case, coprostanol has a clear potential for source identification of NO_3^- pollution for nitrate-polluted samples. As indicated in the figure, most samples containing lower level of coprostanol (<30 ng L^{-1}) drop below 10 mg L^{-1} NO_3-N concentration. Probably, these samples do not contribute to the pollution. Therefore, we propose this value as a criteria of identify predominant pollutant source. On the other hand, according to the distribution of sampling locations (Fig. 1), sampling points with a concentration below 70 ng L^{-1} coprostanol appear to gather into a specific location. This means that 70 ng L^{-1} is another possibility for the criteria. In any case, the above suggested criteria should be further elaborated on in future research.

5. Conclusion

In this study, a methodology based on coprostanol concentrations was tested to identify the source of nitrate pollution in groundwater. Using the method proposed by Kendall (1998), the data were seen to be concentrated in an overlapping region of chemical fertilizer and livestock waste sources. Therefore, it is difficult to distinguish pollution sources based on stable isotopes alone. To arrive at a clearer picture, we plotted the relationship between coprostanol concentration and $\delta^{15}N$. Also, in this case it was difficult to discern clear relationships. The relationship between nitrate and coprostanol concentrations displays a clearer picture. Higher concentration coprostanol samples (>30 ng L^{-1}) corresponded to the polluted sample group obtained from cluster analysis (Nakagawa et al., 2016). However, this polluted-cluster also included samples containing low levels of coprostanol. Chemical fertilizer is likely to be the predominant source of nitrate in these low coprostanol concentration samples (Hosono et al., 2013). According to the above analysis, coprostanol has potential for source identification of nitrate pollution. When pollution sources cannot be distinguished by conventional diagrams of isotopic ratios proposed by Kendall (1998), coprostanol analysis may be a useful tool, even if results do not correspond to the isotopic analysis. More feasibility studies are necessary to refine the use of coprostanol as an identifier of nitrate source.

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Figure Captions

Fig. 1 Location of sampling sites.

Fig. 2 NO_3-N concentration depending on sampling location; *Shallow well, **Deep well, ***Unknown well depth.

Fig. 3 Coprostanol concentration depending on sampling location. N. D. denotes not detected;

307 *Shallow well, **Deep well, ***Unknown well depth.

308 **Fig. 4** Relationship between $\delta^{15}N$ nitrate and $\delta^{18}O$ nitrate concentrations. The isotopic range

309 identifying the source was organized according to Kendall et al. (1998).

310 **Fig. 5** Relationship between $\delta^{15}N$ nitrate and coprostanol concentrations. The isotopic range

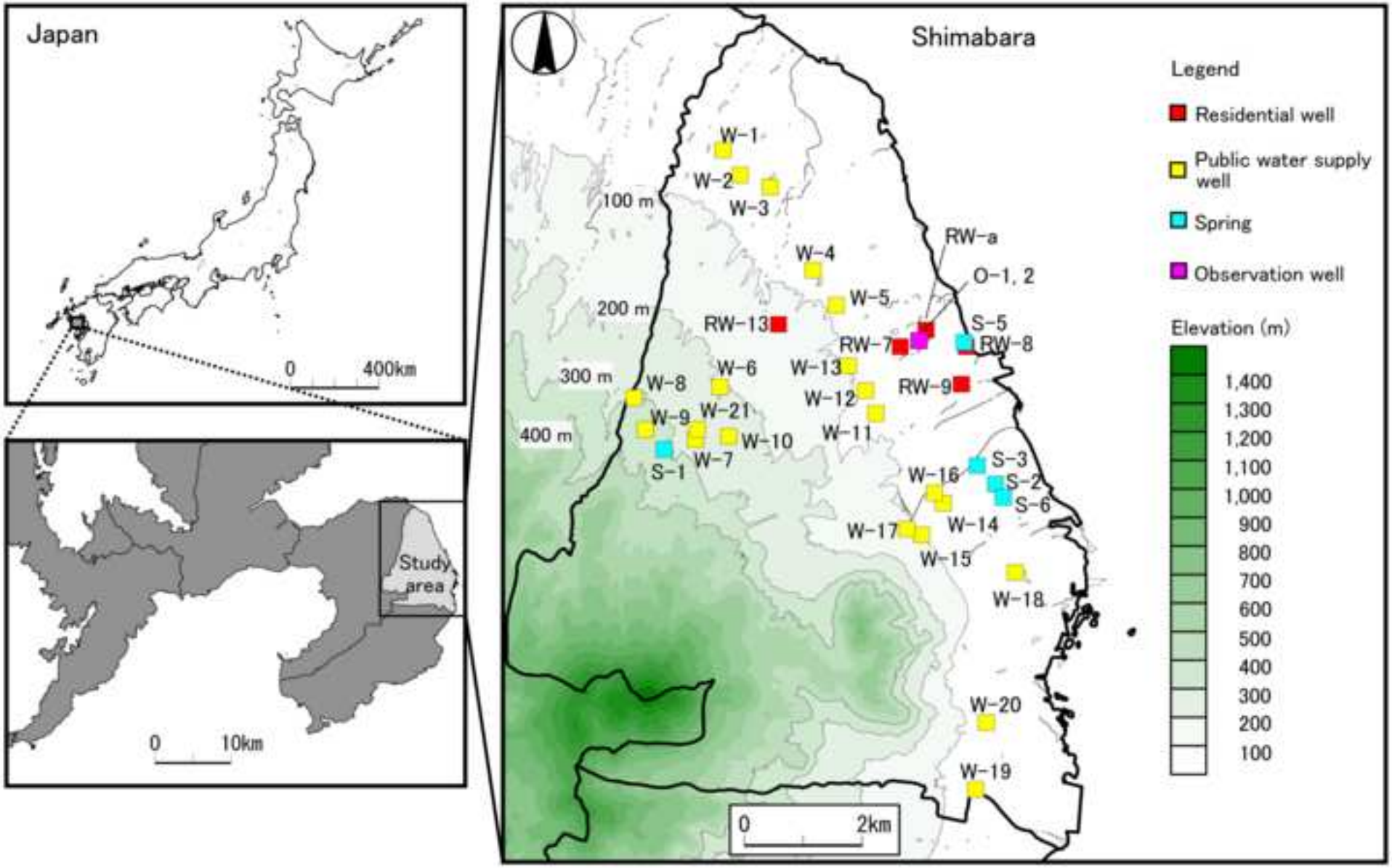
311 identifying the source was organized according to Kendall et al. (1998). Groups were organized

312 according to Nakagawa et al. (2016).

313 **Fig. 6** Relationship between coprostanol and NO_3-N concentrations. Groups were organized

314 according to Nakagawa et al. (2016).

Figure 1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400km
Study area
0
10km
Shimabara
Legend
Residential well
Public water supply well
Spring
Observation well
Elevation (m)
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1,300
1,200
1,100
1,000
900
800
700
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W-1
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W-3
W-4
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W-16
W-17
W-18
W-19
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W-21
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RW-9
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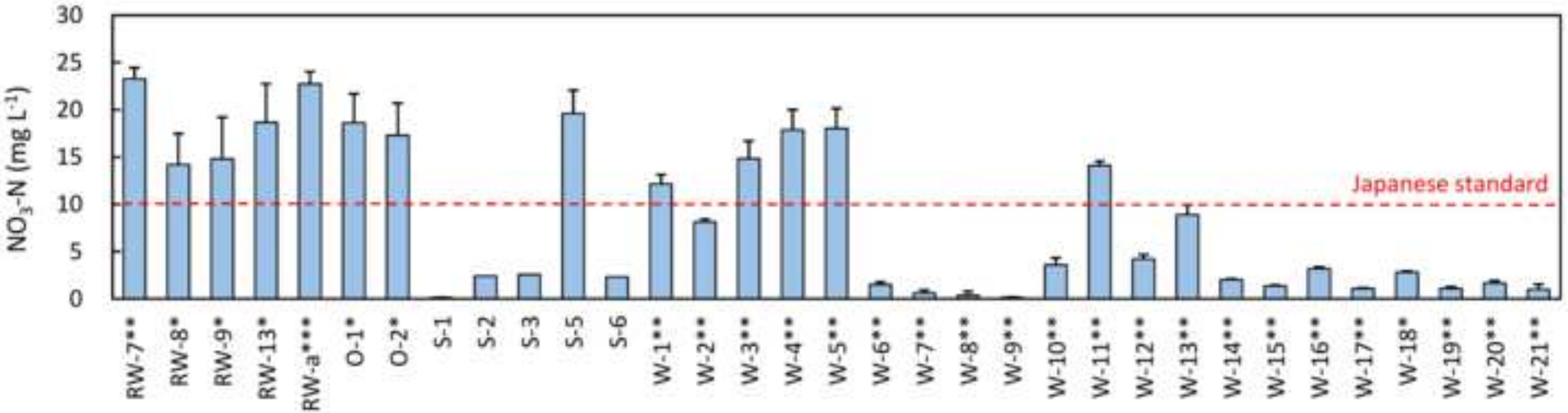
Figure 2


30
25
20
15
10
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0
NO_3-N (mg L^{-1})
Japanese standard
RW-7**
RW-8*
RW-9*
RW-13*
RW-a***
O-1*
O-2*
S-1
S-2
S-3
S-5
S-6
W-1**
W-2**
W-3**
W-4**
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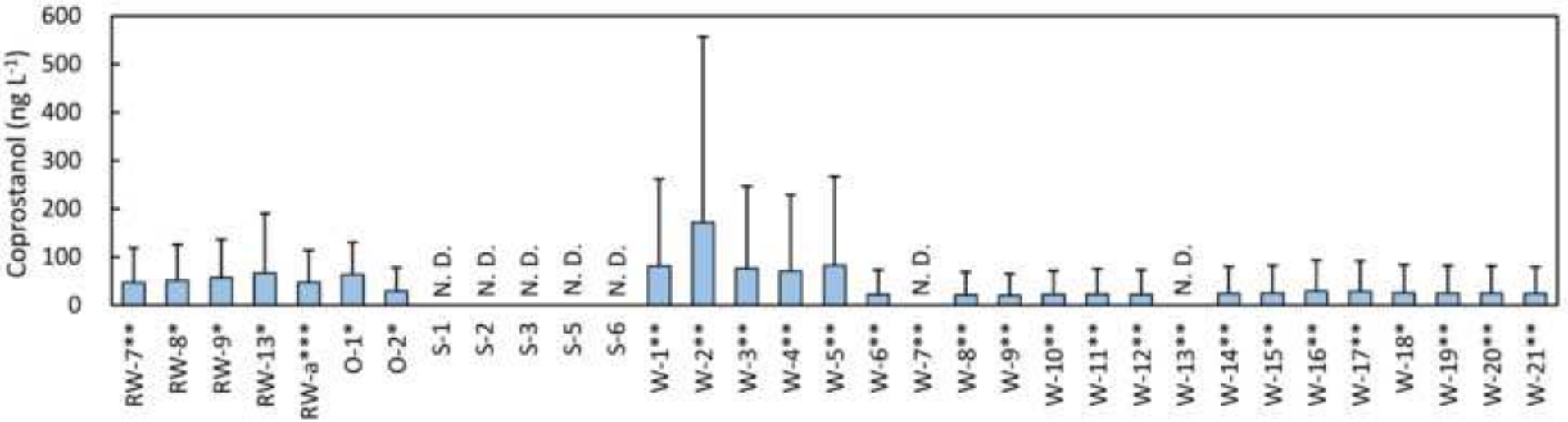
W-21**

Figure 3


Coprostanol (ng L-1)
600
500
400
300
200
100
0
N. D.
RW-7**
RW-8*
RW-9*
RW-13*
RW-a***
O-1*
O-2*
S-1
S-2
S-3
S-5
S-6
W-1**
W-2**
W-3**
W-4**
W-5**
W-6**
W-7**
W-8**
W-9**
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W-16**
W-17**
W-18*
W-19**
W-20**
W-21**

Figure 4

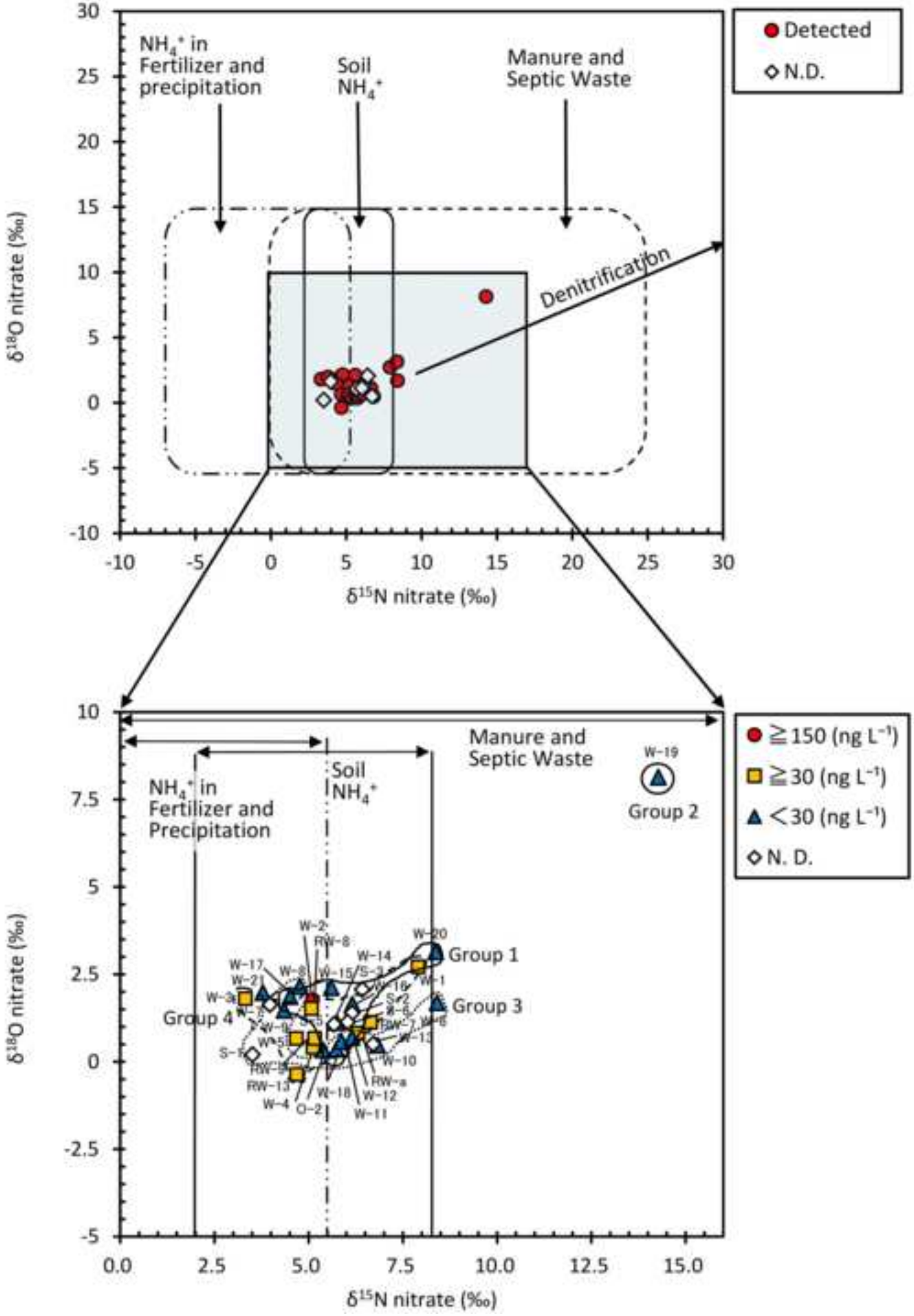

Detected
N.D.
NH4+ in Fertilizer and precipitation
Soil NH4+
Manure and Septic Waste
Denitrification
δ18O nitrate (‰)
δ15N nitrate (‰)
≧150 (ng L-1)
≧30 (ng L-1)
<30 (ng L-1)
N. D.
W-19
Group 2
Group 1
Group 3
Group 4

Figure 5

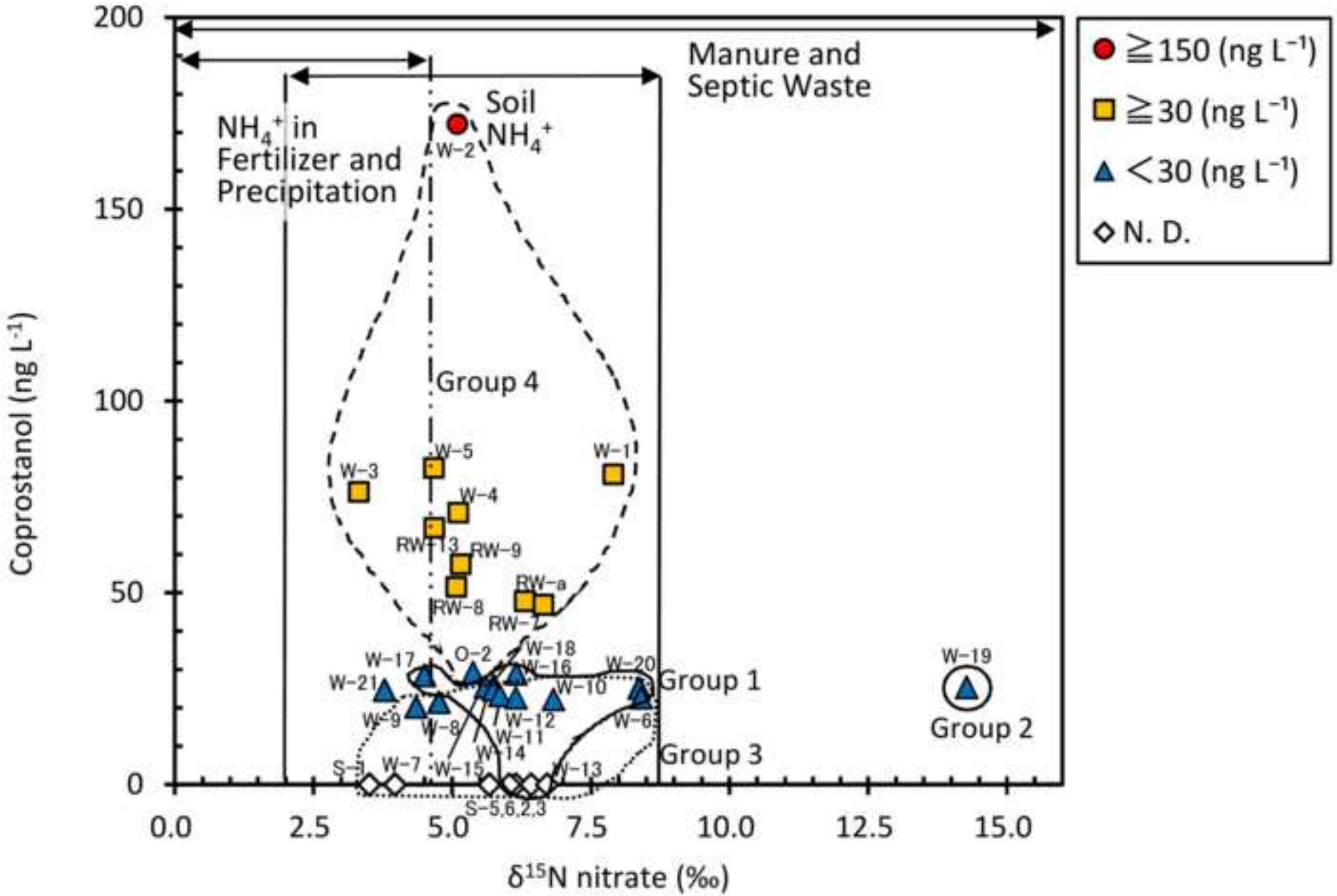
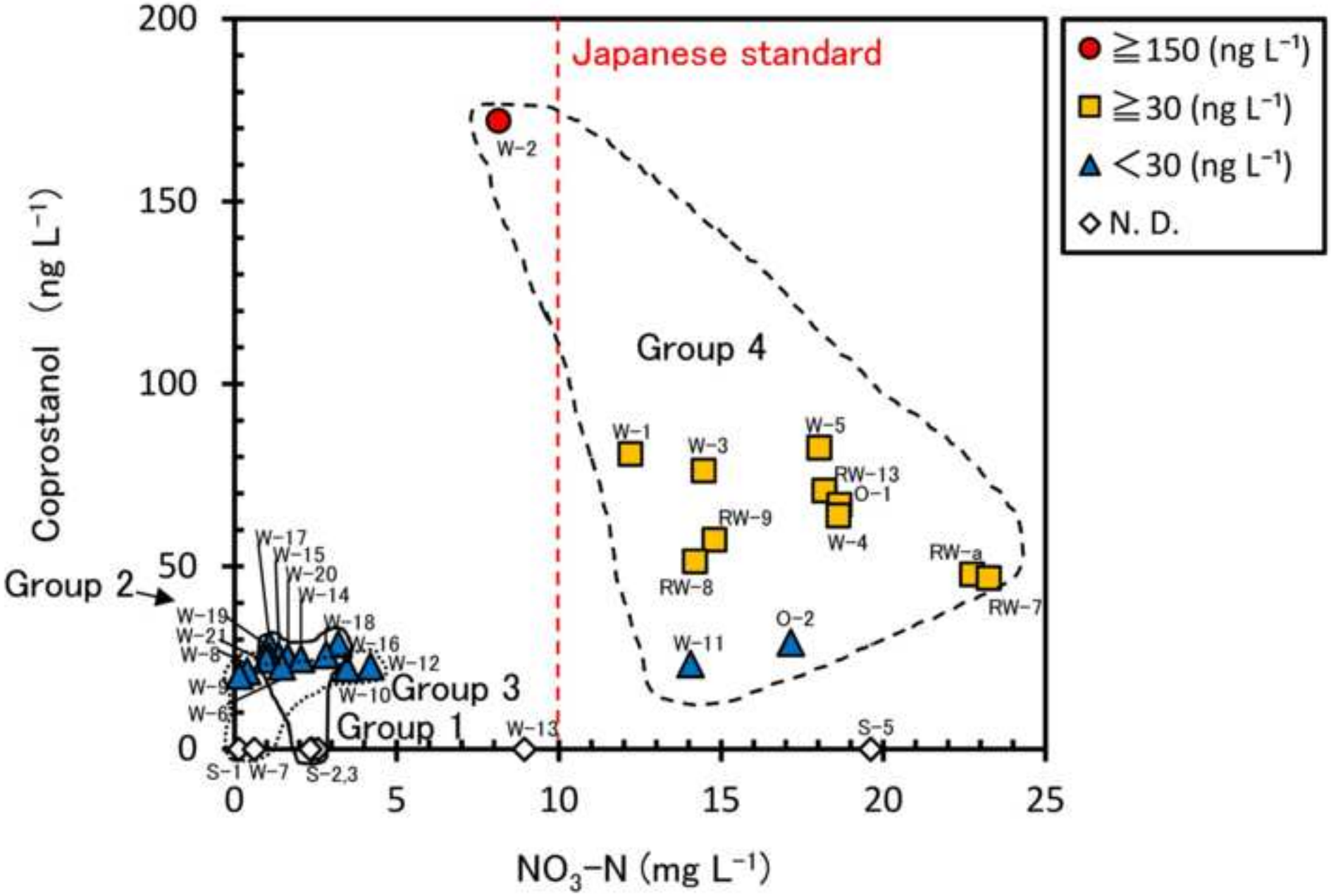

Manure and
Septic Waste
NH4+ in
Fertilizer and
Precipitation
Soil
NH4+
W-2
Group 4
W-5
W-1
W-3
W-4
RW-13
RW-9
RW-8
RW-a
RW-7
W-17
O-2
W-18
W-16
W-20
W-21
W-10
W-9
W-8
W-12
W-11
W-14
W-6
W-7
W-15
W-13
S-1
S-5,6,2,3
Group 1
Group 2
Group 3
W-19
≧150 (ng L⁻¹)
≧30 (ng L⁻¹)
＜30 (ng L⁻¹)
N. D.
Coprostanol (ng L-1)
δ15N nitrate (‰)
200
150
100
50
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0

Figure 6


Japanese standard
≧150 (ng L⁻¹)
≧30 (ng L⁻¹)
＜30 (ng L⁻¹)
N. D.
Coprostanol (ng L⁻¹)
NO3–N (mg L⁻¹)
Group 1
Group 2
Group 3
Group 4
W-1
W-2
W-3
W-4
W-5
W-6
W-7
W-8
W-9
W-10
W-11
W-12
W-13
W-14
W-15
W-16
W-17
W-18
W-19
W-20
W-21
O-1
O-2
RW-7
RW-8
RW-9
RW-13
RW-a
S-1
S-2,3
S-5
0
50
100
150
200
0
5
10
15
20
25