Use of sterols to monitor surface water quality change and nitrate pollution source

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25 **Abstract**

26 Coprostanol was tested as ecological indicator to trace domestic and manure effluents and to investigate
27 possible pollution sources in surface water. Pollution assessment was performed by analysing NO_3^-, NO_2^-,
28 coprostanol (5β(H)-Cholestan-3β-ol), and cholestanol (5α(H)-Cholestan-3β-ol) in water samples from 42
29 sites along rivers in Shimabara and Unzen City, Japan. NO_2-N concentration exceeded 0.04 mg L^{-1} at two
30 sampling sites during winter and six sampling sites during summer. (NO_3+NO_2)-N concentration
31 exceeded 10 mg L^{-1} at 19 sampling sites during winter and 7 sampling sites during in summer. The
32 highest concentration was 82.4 mg L^{-1} in summer. Detectable NO_3-N concentration was observed in
33 northern parts of the study area. Coprostanol concentration exceeded 700 ng L^{-1} (Australian Drinking
34 Water Standard) at 8 sampling points during winter and 6 sampling sites during summer. At 10 and 5% of
35 the sampling sites, both nitrate and coprostanol concentration exceeded drinking water standard during
36 winter and summer, respectively. The percentage of sampling sites where either concentration was above
37 drinking water standard was 45% during winter and 22% during summer season. However, depending on
38 sampling site, the relationships between nitrate and coprostanol concentrations showed different patterns.
39 The sterol ratio exceeded 0.5 at 17 sampling sites during winter and 14 sampling sites during summer.
40 Thus, it was confirmed that fecal pollution is present in the studied surface water. A method to distinguish

between principal pollution sources was developed by separating four areas in a nitrate concentration and sterol ratio plot. Results show that sampled data could be reasonably classified into appropriate polluted/non-polluted groups. Thus, coprostanol and sterol ratio can be used as indicators to distinguish between different nitrate pollution sources in surface water.

Keywords

Surface water, Nitrate pollution, Coprostanol, Sterol ratio

1. Introduction

Many areas in the world experience nitrate pollution in surface and groundwater supplies (e.g., Nakagawa et al., 2016; Amano et al., 2018; Górsk et al., 2019; Chitsazan et al., 2017; Sorensen et al., 2015; Hansen et al., 2012; Chandna et al., 2011; Ribbe et al., 2008; Liu et al., 2005). Nitrate pollution is related to significant health threats known as cause of blue baby syndrome for infants and cancer occurrence for adults. Thus, World Health Organization (WHO, 2011) has set a maximum nitrate level in drinking water at 50 mg L^{-1}. Eckhardt and Stackeberg (1995) considered nitrate concentration above 13 mg L^{-1} in groundwater as indicative of pollution by human activity.

A common problem is to establish sources for the nitrate pollution. A common method for this is to use isotopes of nitrogen and oxygen in the nitrate (Kendall, 1998). However, when many sources of nitrate overlap, it may still be difficult to separate between them. For this reason, we propose to use coprostanol to improve the source separation (Nakagawa et al., 2017). Coprostanol is a sterol, which is produced by bacterial reduction of cholesterol in gut of higher animals such as humans and livestock.

Coprostanol and related sterols can thus, be used as a biomarker of pollution from domestic and manure effluent discharge (Reeves and Patton 2005). Coprostanol has been widely used as an indicator of fecal pollution in the water environment such as river, lagoons, and estuaries (He., 2018; Costa et al., 2018; Rada et al., 2016; Adnan et al., 2010; Froehner et al., 2009; Martins et al., 2007; Reeves and Patton, 2005). This, has been confirmed by comparison with biological indicators such as *E. coli*. (Albuquerque de Assis Costa et al., 2018). Consequently, coprostanol may be used as a proxy for ecological indicators. Although, problems remain to establish relationships in different environments.

For groundwater, the concentration of coprostanol is often quite low. A possible reason for this is adsorption to soil and rock material. Sterols are hydrophobic and thus, coprostanol may be assumed to be associated with particles (Froehner et al., 2010). Thus, it may be expected that surface water displays higher concentrations and more clear relationships between coprostanol and nitrate concentration. However, to improve the pollution source classification, the sterol ratio between cholestanol and coprostanol can be used. Cholestenol is also an isomer sterol that is formed from cholesterol reduction to cholestanol. This occurs preferentially in natural environments. In view of this, we firstly investigated the spatial variation of nitrate, coprostanol, and sterol ratio for the study area. Secondly, we plotted relationships between $\delta^{15}N$ and $\delta^{18}O$ from nitrate as suggested by Kendall (1998). Thirdly, we analyzed relationships between $\delta^{15}N$ from nitrate and sterol ratio to validate initial results. Finally, we propose a general methodology to distinguish between main sources of nitrate pollution in surface water by use of sterol ratio and nitrate. In other words, this study assessed the nitrate pollution in rivers and possibilities to separate between different sources of pollution using several biochemical indicators.

2. Experimental study area

Shimabara is one of the cities on Shimabara Peninsula (**Fig. 1**) in Nagasaki prefecture, Japan. It is adjacent to the Unzen and Minamishimabara Cities. The city area is 82.8 km^2, which occupies 18% of the peninsula. Land use is concentrated to forest, upland fields, and urban areas. In the southern part of the city, urban areas are situated on the sloping flat land between Mt. Mayuyama and the Ariake Sea. The sloping flat land is constituted by debris avalanche deposits from the part of Mt. Mayuyama that collapsed in 1792. The northern and central parts of the study area are constituted by agricultural areas.

The city is well-known for volcanic activities. The most recent eruptive activity of Mt. Fugendake in the center of the peninsula occurred from 1990 to 1995. Due to the frequent volcanic activities, the geology is mainly constituted by volcanic rock such as Pre-Unzen and Unzen volcanic rocks (Sugimoto, 2006). Pre-Unzen volcanic rock is covered by Unzen volcanic rock. The former is composed of olivine basalt and two pyroxene andesite. The latter is mainly constituted by hornblende andesite to dacite.

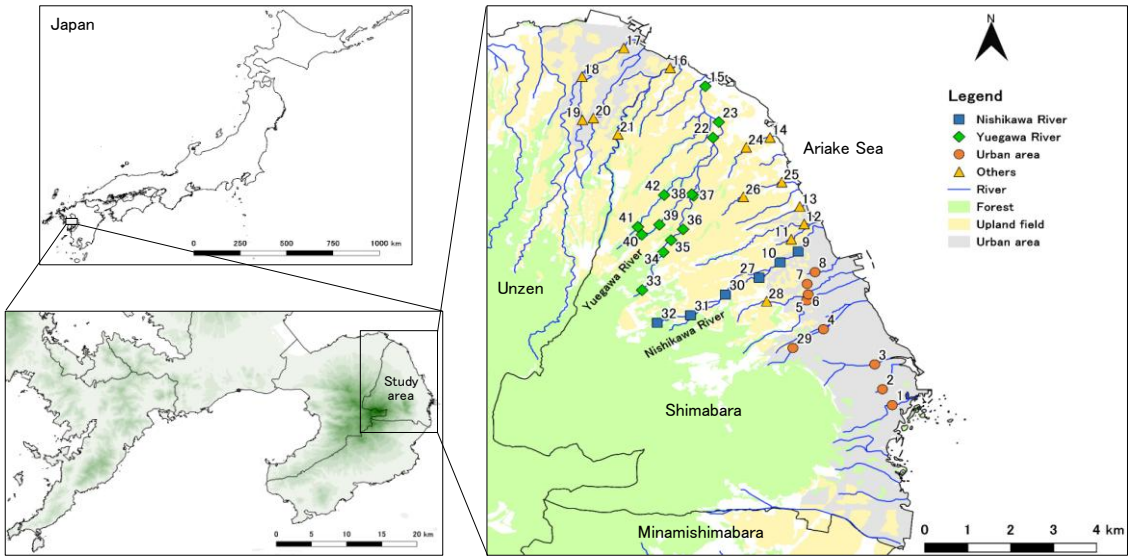


Fig. 1 Location of water sampling sites.

The climate is humid temperate with a mean annual temperature of 17.1°C and annual precipitation of 1989 mm. The precipitation in January, February, and August for each sampling campaign was 56.5, 125.5, and 196.5 mm, respectively. About 41% of the population of 46,437 people are connected to waste water treatment (Nagasaki Prefecture, 2017). Most of the treatment (97.6%) is constituted by septic tanks due to lacking sewage system connections in the city. It is estimated that about 224 kg T-N/day are released to the river system through discharge of treated waste water (treated septic tank effluent). Nitrate load to the groundwater from treated waste water is, however, only about 5% of the load from fertilizers and livestock waste (Committee on Nitrate Reduction on Shimabara Peninsula, 2016).

3. Materials and methods

3.1. Water samples

Water samples were taken at 42 locations in Shimabara City including a part of Unzen City during winter (January 17 and 24 and February 6) and summer (August 21 and 22) 2017. However, during the summer campaign, sampling at site 27 (**Fig. 1**) was not possible due to dried up river conditions. The reason for sampling during both summer and winter was to better understand the seasonal variation of the biochemical indicators. Water samples of 500 mL and 1000 mL were collected directly from the center section of the river (except for sampling sites 6 and 27) in pre-washed bottles and then stored in refrigerator for analysis of NO_3^-, NO_2^-, NH_4^+, coprostanol, and cholestanol. At sites 6 and 27, we used a bailer sampler due to the difficulty to access the water surface. Samples of 30 mL for nitrate isotope ratios were filtered through 0.22 μm membrane filter and kept frozen until analysis.

3.2. Analysis procedure

Dissolved oxygen (DO) was determined in situ by use of a luminescence based sensor (HACH HQ30d). NO_3^-, NO_2^-, and NH_4^+ were analyzed by ion chromatography (Metrohm 861 Advanced Compact IC). Nitrate $\delta^{15}N$ and $\delta^{18}O$ were determined by the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001; Hosono et al., 2014; Hosono et al., 2013). Analysis of $\delta^{15}N$ and $\delta^{18}O$ of induced N_2O was conducted simultaneously. The nitrate isotopes were analyzed in deionized water extracted samples from the cattle manure and excrement of milk and beef cattle. Cattle manure is composed by cattle excrements and sawdust. The cattle manure comes from both milk cattle (cow milking raw milk for dairy products) and beef cattle (cow raised for meat production). Animal waste was collected at Nagasaki Agricultural

and Forestry Technical Development Center on September 17 2015. In the extraction, 30 mL of deionized water was added to 3.0 g of dried samples (manure and excrement) in a centrifuge tube and shaken during 30 min. The extract was separated using centrifugal separation at 3000 rpm x 10 min. Supernatant liquid was added to a 50 ml volumetric flask through a filter paper. For further details, see Oyanagi et al. (2004).

As mentioned earlier, we focused on two sterols: coprostanol and cholestenol. These sterols are isomer and originate from different processes. Coprostanol is produced by bacterial reduction of cholesterol in gut of higher animals (Martins et al., 2007). On the other hand, cholesterol reduction to cholestanol occurs preferentially in natural environment. Coprostanol (5β(H)-Cholestan-3β-ol) and cholestenol (5α(H)-Cholestan-3β-ol) were extracted from the water samples according to Hussain et al. (2010) with some modification (Nakagawa et al., 2017). At start, a surrogate was added to 800 mL of water samples to monitor the performance of preprocessing. 1 M HCl was added to samples to modify pH from 2 to 3. After suction filtration of water samples using 0.7 mm and 2.7 mm borosilicate glass fiber filters, the sterols on the filters were extracted with methanol using an ultrasonic bath during three successive times. The extracts with methanol were mixed with the water sample after filtration with 0.20 μm membrane filter. The sterols were extracted from water samples by liquid-liquid extraction with 60 mL dichloromethane in room temperature during three successive times. The extracts were concentrated to near dryness (<1.0 mL) under pure nitrogen gas flow and dehydrated with anhydrous sodium sulfate. The extract was formed to trimethylsilyl using BSTFA (bis-trimethylsilyl trifluoroacetamide) at 80^{o}C for 60 min after concentration and dehydration, then quantified by use of 7000A Triple Quadrupole GC/MS (Agilent Technologies). The detection limit for sterols ranged between 0.2 and 3.2 ng L^{-1}. The mean

149 recovery of surrogates was 85.9% with a standard deviation ±15.3%.

150

151 **3.3. Spatial variation of nitrate, coprostanol, and sterol ratio**

152 To accomplish the first aim of the study, to improve the understanding of the spatial variation
153 of nitrate, coprostanol, and sterol ratio, geographical information system (GIS) was applied. Thus, QGIS
154 2.18.21 'Las Palmas' (QGIS development team 2019) was used to map the results. The QGIS is a Free
155 and Open Source Geographic Information System licensed under the GNU General Public License.

156 Maximum permissible nitrate concentration in Japan for drinking water is 10 mg L^{-1}. Therefore,
157 this concentration was used as a criteria for nitrate pollution. To evaluate fecal pollution, several
158 coprostanol concentrations have been proposed. In Australia, a maximum of 700 ng L^{-1} has been proposed
159 for drinking water (Hussain et al., 2010). In the natural water bodies, according to the relationship
160 between BOD and coprostanol concentration, 500 ng L^{-1} has been proposed for indicating fecal pollution
161 (Itoh and Tatsukawa, 1978). In this study, we evaluated fecal pollution based on drinking water standard
162 (proposed in Australia) of coprostanol concentration as same as nitrate concentration.

163 Another method to evaluate fecal pollution is to use the sterol ratio (Matić et al., 2016):

164 $$S_r = \frac{5\beta}{5\alpha + 5\beta}$$

165 where S_r is sterol ratio, 5β is concentration of coprostanol (ng L^{-1}), and 5α is concentration of cholestanol
166 (ng L^{-1}). Depending on sterol ratio, samples are often divided into three pollution classes "certain (> 0.5)",
167 "uncertain (0.3 – 0.5)", and "no pollution (< 0.3)" (Matić et al., 2016). Consequently, these thresholds
168 were used to indicate fecal pollution in the current study.

3.4. Relationship between nitrate $\delta^{15}N$ and $\delta^{18}O$

Kendall (1998) suggested a method to investigate pollution sources that can be used together with groundwater sampling (Nakagawa et al., 2017). It builds on a scatter plot using $\delta^{15}N$ and $\delta^{18}O$ concentrations. The scatter plot will display distinctive ranges depending on nitrate source (e.g., ammonium fertilizer, soil N, and manure and septic waste). Thus, several studies have used this method to elucidate nitrate pollution source (Baily et al., 2011; Hosono et al., 2011; Kaown et al., 2009).

3.5. Relationship between nitrate $\delta^{15}N$ and sterol ratio

The relationship between nitrate $\delta^{15}N$ and sterol ratio is plotted in a scatter diagram. The nitrate $\delta^{15}N$ indicates the nitrate source while the sterol ratio displays the risk for fecal pollution. Nitrate originating from manure or septic waste would have a heavier isotopes range compared to ammonium fertilizer and nitrate from soil with a high sterol ratio (> 0.5).

3.6. Relationship between NO_3+NO_2-N and sterol ratio

We suggest a general methodology to distinguish between main pollution sources of nitrate by using a scatter plot between NO_3+NO_2-N (x-axis) and sterol ratio (y-axis). In such a plot, four main groups may be distinguished. These groups correspond to drinking water standard regarding NO_3+NO_2-N and fecal pollution criteria (group I, NO_3+NO_2-N: < 10 mg L^{-1}, sterol ratio: < 0.3; group II, NO_3+NO_2-N: < 10 mg L^{-1} , sterol ratio: > 0.3; group III, NO_3+NO_2-N: >10 mg L^{-1}, sterol ratio: < 0.3; group IV,

189 NO_3+NO_2-N: > 10 mg L^{-1}, sterol ratio: > 0.3). Group I indicates no pollution or close to no pollution

190 while other groups indicate pollution. However, pollution sources might be different, as domestic

191 wastewater in group II, chemical fertilizer in group III, and livestock waste in group IV.

192

193 **4. Results**

194 **4.1. Spatial variation of nitrate, coprostanol, and sterol ratio**

195 The spatial variation of nitrate plus nitrite as nitrogen (NO_3+NO_2-N, hereafter referred to as

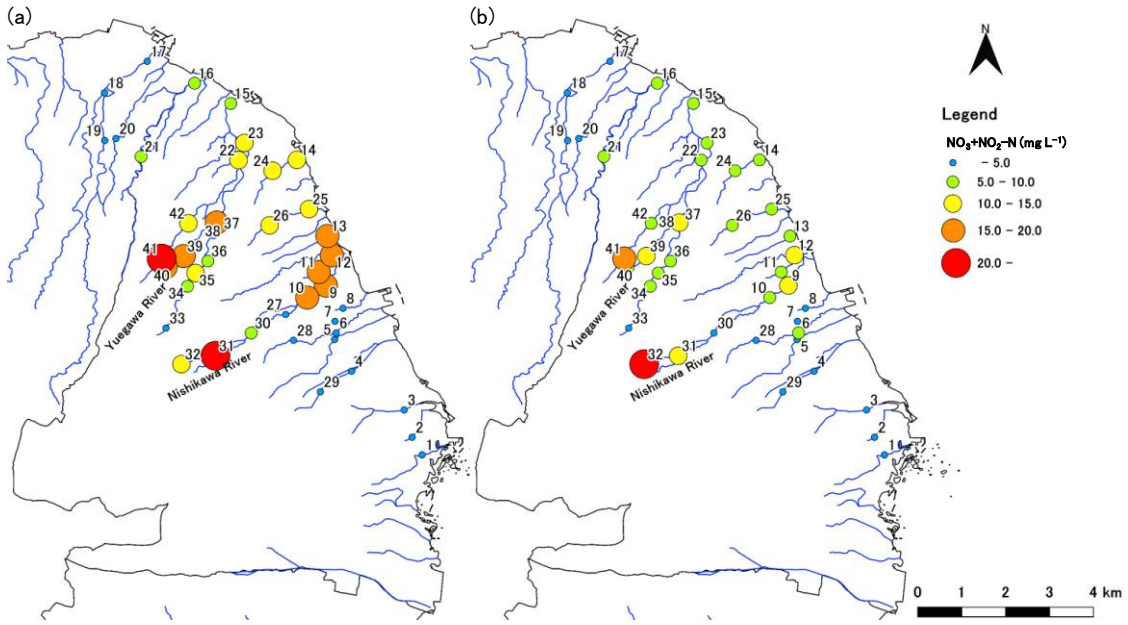
196 nitrate) during winter and summer 2017 is shown in **Fig. 2**. Descriptive statistics of chemical components

197 are summarized in **Table 1**. Nitrate concentration exceeded 10 mg L^{-1} at 19 sampling locations during

198 winter and 7 sampling locations during summer. Highest concentrations were 27.5 mg L^{-1} (site 31) in

199 winter and 82.4 mg L^{-1} (site 32) in summer. High nitrate concentration was observed in northern parts of

200 the study area, especially for Yuegawa and Nishikawa River.



201

202 **Fig. 2** Spatial variation of NO_3+NO_2-N concentration (a)Winter (Jan.-Feb. 2017), (b) Summer

(August 2017).

Table 1 Descriptive statistics of analysed chemical components

		winter season				summer season			
		Average	SD	Maximum	Minimum	Average	SD	Maximum	Minimum
NO_3-N	mg L^{-1}	9.7	6.9	27.5	1.0	7.9	11.3	74.8	1.3
NO_2-N	mg L^{-1}	0.2	0.7	3.5	0.0	0.2	1.2	7.6	0.0
NO_3+NO_2-N	mg L^{-1}	9.9	6.8	27.5	1.0	7.9	12.4	82.4	1.3
NH_4-N	mg L^{-1}	2.1	7.3	37.0	0.0	0.0	0.1	0.6	0.0
Coprostanol	ng L^{-1}	2064.7	10509.2	68340.3	17.9	330.9	438.7	1826.1	2.5
Cholestanol	ng L^{-1}	883.3	3267.4	21196.6	30.6	299.0	346.2	1654.7	37.5
Sterol ratio		0.47	0.17	0.77	0.14	0.41	0.20	0.76	0.06
DO	mg L^{-1}	10.14	2.49	11.58	0.70	8.08	0.85	10.42	5.26

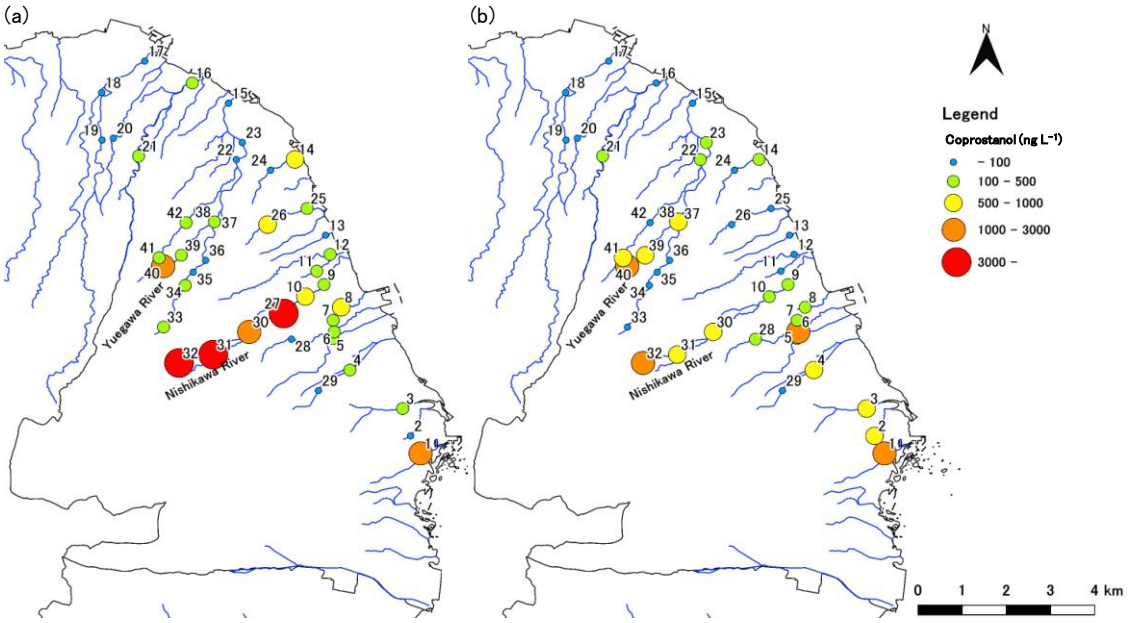
Nitrite (NO_2-N) was detected at only two locations during winter (site 30 and 32). For summer, nitrite was detected at six locations (site 1, 4, 7, 8, 11, and 32). Although nitrate concentrations meet drinking water standard (10 mg L^{-1}) except for site 32 (**Fig. 2**(b)), nitrite exceeds Japanese drinking water standard at these sites (0.04 mg L^{-1}). Very high nitrite and nitrate concentration (7.6 mg L^{-1} and 82.4 mg L^{-1}, respectively) was detected at site 32 in summer.

Ammonium (NH_4-N) was detected in water samples collected at locations 26, 27, 31 and 32 (winter season) and 30 (winter and summer season). The ammonium concentrations ranged between <9.5 to 37.0 mg L^{-1} for most of the sampling locations, within Nishikawa River Basin (locations 27 and 30-32) and <1.8 mg L^{-1} for location 26 in winter. The maximum concentration (37.0 mg L^{-1}) was detected at site 32 in winter. In summer (location 30), ammonium was detected with concentration of 0.6 mg L^{-1}, which is significantly lower than in winter season.

The spatial variation of coprostanol concentration in winter and summer 2017 is shown in **Fig.**

3. A total number of 8 and 6 sampling points exceeded the Australian standard of 700 ng L^{-1} during winter and summer, respectively. Site 32 (upstream Nishikawa River) displayed the highest coprostanol concentration, 68340 and 1247 ng L^{-1} during winter and summer, respectively. The Nishikawa River is highly affected by fecal pollution from livestock waste. Similarly, as for the nitrate concentration, coprostanol concentration decreased during summer. However, some sites like 2, 3, 4, and 6 (urban area) and 22, 23, 37, 39, and 41 (Yuegawa River) displayed increasing coprostanol concentration during summer.

According to the above, sterol ratios > 0.5 represent "certain" fecal pollution. Values between 0.3 and 0.5 suggest "uncertain" sewage and natural sterol inputs. Values < 0.3 indicate "no pollution". Spatial variation of sterol ratio during winter and summer in 2017 is shown in **Fig. 4**. Based on these criteria, 17 sampling points in winter and 14 sampling points in summer exceeded 0.5, indicating certain fecal pollution. In total, 10 sampling points in winter and 15 sampling points in summer showed a ratio of less than 0.3.



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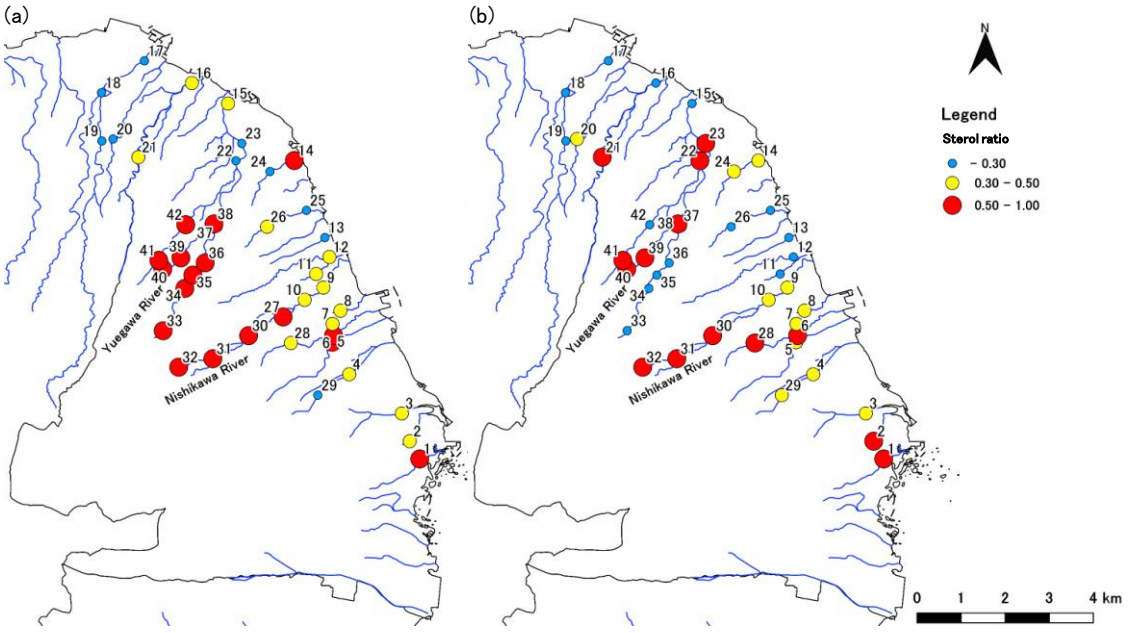
234 **Fig. 3** Spatial variation of coprostanol concentration (a) Winter (Jan.-Feb. 2017), (b) Summer (August

235 2017).



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237 **Fig. 4** Spatial variation of sterol ratio (a) Winter (Jan.-Feb. 2017), (b) Summer (August 2017).

238

239 Concentrations of NO_3+NO_2-N and coprostanol are shown in **Fig. 5** to give a direct comparison

of concentrations of these components. 19 sampling sites displayed higher nitrate concentration than drinking water standard during winter season. Within these sites, 14, 31, 32, and 40 showed high coprostanol concentration, above the Australian drinking water standard. Thus, 10 % of all sampling sites showed high concentrations for winter season. The percentage that displayed high concentration for nitrate or coprostanol was 45 % for winter season. During summer season, both nitrate and/or coprostanol concentration decreased at sites 14, 31, and 40, and only site 32 displayed high concentrations for both chemicals during both seasons. In addition, site 39, where only nitrate was high during winter season, showed high concentrations of nitrate and coprostanol during summer season. The percentage of sites where both chemical concentrations were high was only 5 % for summer season. The percentage of sampling sites where either concentration was above drinking water standard was 45% during winter and 22% during summer season.

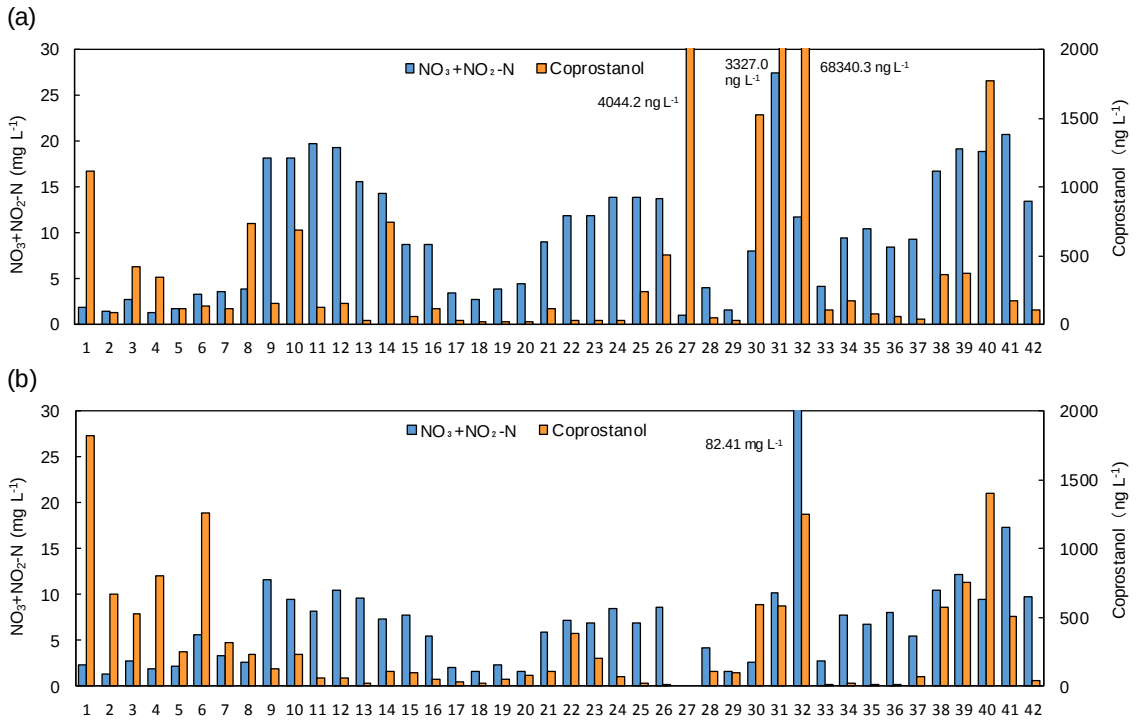

Fig. 5 NO_3+NO_2-N and coprostanol concentration depending on sampling site (a) Winter (Jan.-Feb. 2017), (b) Summer (Aug. 2017).

4.2. Relationship between nitrate $\delta^{15}N$ and $\delta^{18}O$

The average concentration of nitrate $\delta^{15}N$ and $\delta^{18}O$ was plotted in a scatter diagram as shown in **Fig. 6**. Corresponding analyses from livestock waste and manure are also plotted in the figure. From winter to summer, most of the samples somewhat moved towards higher concentrations for both isotopes. It is seen that summer samples are more affected by livestock waste. In other words, winter samples seem to be more affected by nitrate from chemical fertilizers. Yet, most of the samples and especially samples from Nishikawa and Yuegawa River are located in the vicinity of manure and septic waste (**Fig. 6)**.

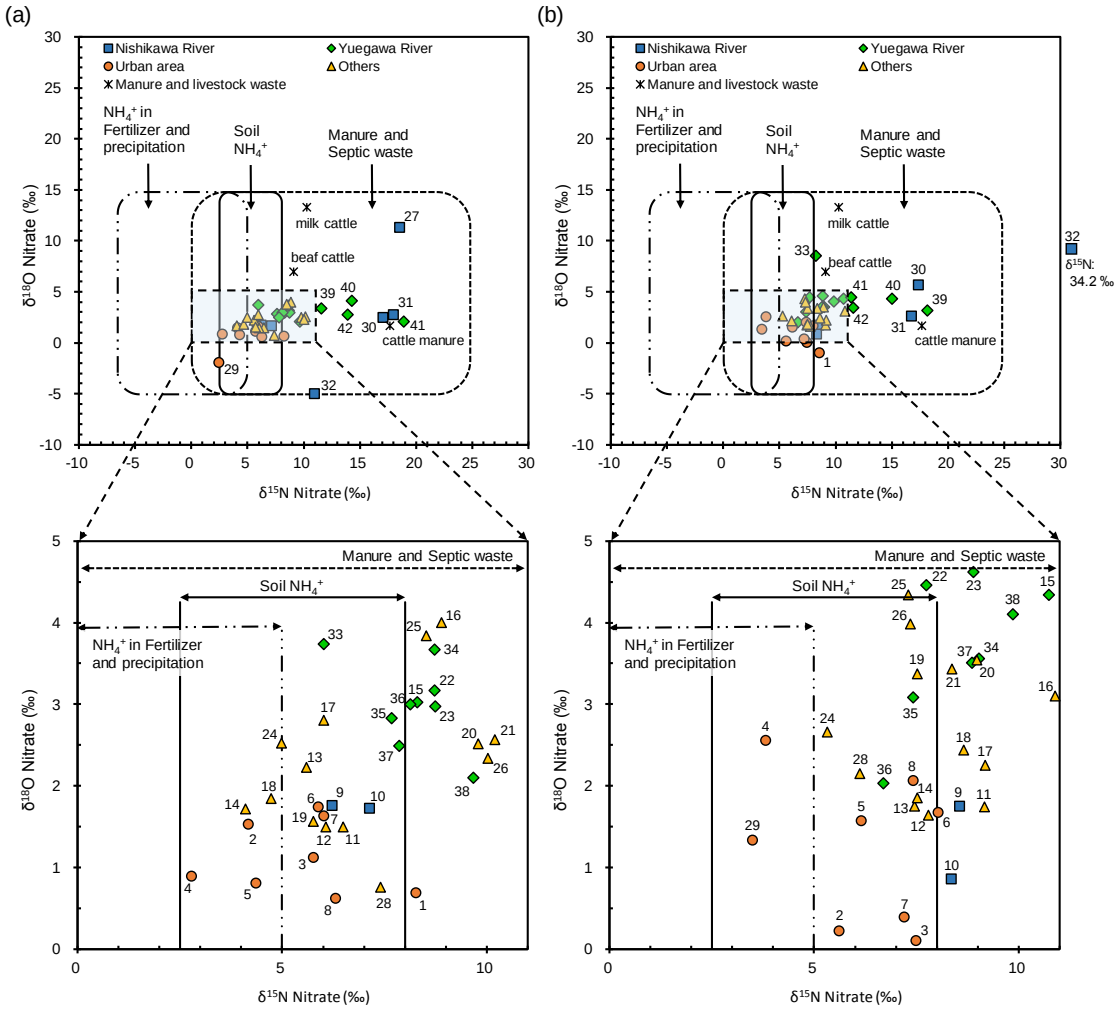

Fig. 6 Relationship between nitrate $\delta^{15}N$ and $\delta^{18}O$ concentrations (a) Winter (Jan.-Feb.2017), (b) Summer (August 2017). The isotopic range identifying the source was organized according to Kendall (1998).

4.3. Relationship between nitrate $\delta^{15}N$ and sterol ratio

In order to confirm the increasing tendency for both nitrate $\delta^{15}N$ and sterol ratio, the relationship between them was plotted in **Fig. 7**. Symbols that are encircled with a red line indicate that coprostanol concentration exceeded 700 ng L^{-1}. In the case of Nishikawa River, at upstream site 30, 31,

32, and 27 (winter only), the sterol ratio was higher than 0.5 and nitrate $\delta^{15}N$ was higher than 10‰. On the other hand, downstream sites 9 and 10 were below 0.5 for the sterol ratio and below 10‰ for the nitrate $\delta^{15}N$. In the case of Yuegawa River, most of the sampling sites, except for 22, 23 (winter) and 33-36 (summer), displayed a relatively high sterol ratio (> 0.3) and nitrate $\delta^{15}N$ (> 5‰). Also, the urban area displayed an increasing tendency for both nitrate $\delta^{15}N$ and sterol ratio.

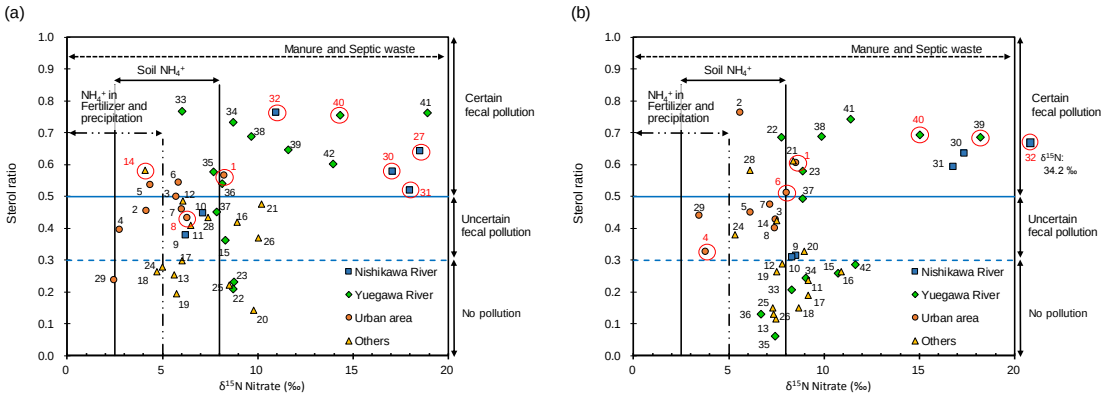

Fig. 7 Relationship between nitrate $\delta^{15}N$ and sterol ratio (a) Winter (Jan.-Feb. 2017), (b) Summer (August 2017).

4.4. Relationship between NO_3+NO_2-N and sterol ratio

The relationship between nitrate concentration (NO_3+NO_2-N) and sterol ratio is plotted in **Fig. 8**. According to the above, a methodology to distinguish principal pollution sources by separating four principal fields in the plot is proposed. For example, the first field with (group I) indicates small ammounts of pollution because nitrate concentration meets drinking water standard and sterol ratio is below 0.3. Other groups are classified as polluted, but the pollution source may be different. Coprostanol concentration of samples marked in red exceeds 700 ng L^{-1}. According to this classification, most samples

display reduced nitrate concentration from winter to summer, and tend to end up in group I or II. Sites located in the upstream of Yuegawa and Nishikawa River such as 31, 32, 38, 39, 40, and 41 are classified into group IV both for winter and summer. All samples from urban areas were classified into group I or II with lower nitrate concentration in both seasons. As shown in **Fig. 3**, nitrate concentrations in urban areas were less than that of other areas, which is obvious in the winter season. Group III obtained a small number of samples for both seasons. In winter season, only five samples (site 13, 22, 23, 24, and 25) were classified into group III. Only site 12 belonged to group III for the summer season.

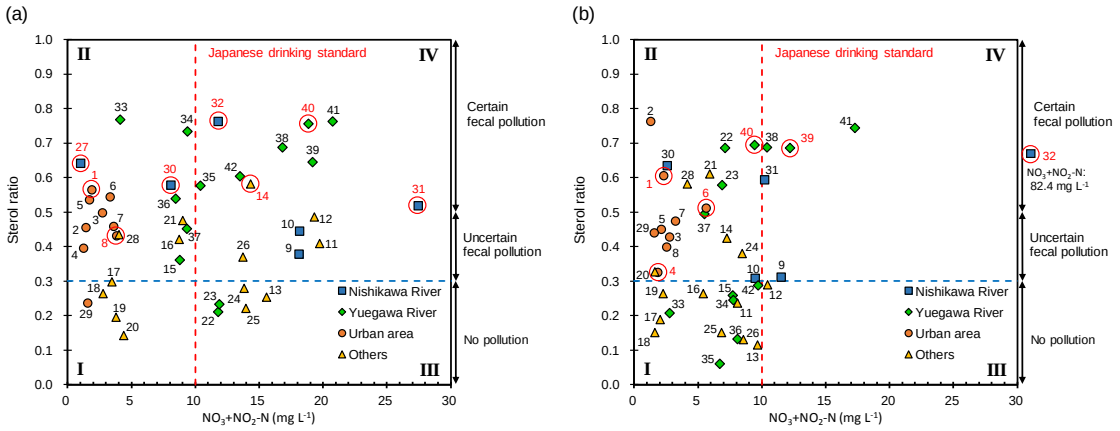

Fig. 8 Relationship between NO_3+NO_2-N concentration and sterol ratio (a) Winter (Jan.- Feb. 2017), (b) Summer (August 2017).

5. Discussion

5.1. Spatial variation of nitrate, coprostanol, and sterol ratio

The Yuegawa and Nishikawa Rivers are part of an area with intense agriculture. In general, spatial variation of nitrate in surface water displayed similarity to that of the groundwater (see Nakagawa

et al., 2016). Surface and groundwater were generally not polluted in forest and urban areas. Most of the summer samples, except for sites 6 and 32, displayed smaller concentration as compared to samples from the winter period. A reason for this is dilution of river water by precipitation during the rainy season.

Site 32 is located at the upstream of Nishikawa River. Here, a pig farm is located close to the sampling site. In winter, although nitrate concentration met drinking water standard ($8.61 < 10$ mg L^{-1}) at this location, nitrite greatly exceeded the standard ($3.19 > 0.04$ mg L^{-1}). Probably, nitrite was produced by denitrification during the biological treatment process for wastewater, because low DO (1.51 mg L^{-1}) was observed as nitrification bacteria need more oxygen as compared to heterotrophic bacteria. Ammonium was detected at sites 27, 30, 31, and 32. These sites are located at the upstream of Nishikawa River and probably affected by effluents from the pig farm.

A livestock waste water treatment plant is located upstream of the Yuegawa River. Probably, the effluent of this plant affects pollution levels in the river. High coprostanol concentrations were detected at site 1 in the urban area and this is most probably due to domestic waste water. The coprostanol level is usually correlated with occurrence of *E. coli.*. According to this, a coprostanol concentration of 60 ng L^{-1} has been proposed for as threshold of fluvial water quality (Albuquerque de Assis Costa et al., 2018). In this sense, most of the investigated sites in this study relate to a significant health threat. Measurement of coprostanol concentration makes it possible to detect serious river pollution, even if nitrate concentrations are not high. Thus, observations of coprostanol concentration have an important role to fill in terms of pollution source investigations.

The sterol ratio indicates that relatively many sampling points are affected by fecal pollution in

the study area. In summer, sites 2, 20, 21, 22, 23, 28, and 29 showed increased sterol ratio. In the urban area (site 2 and 29), domestic waste water caused increased ratio during summer similar to the coprostanol concentration. Downstream Yuegawa River (site 22 and 23) as well displayed increased ratio. On the contrary, southern upstream area of Yuegawa River (site 33, 34, 35, and 36) displayed a decreased ratio. This is probably due to the fact that fecal pollutants are transported from upstream to downstream along the river during flood season. With sterol ratio of 0.7, the coprostanol concentration even higher than 700 ng L^{-1} can be classified as "uncertain" or close to no pollution indicators. Consequently, as described by Matić et al. (2016), the ratio of 0.5 should be more secure for fecal pollution detection.

5.2. Relationships between nitrate $\delta^{15}N$ and $\delta^{18}O$

According to documented cultivation procedures of major crops in the study area (Amano et al., 2016), fertilizers are applied on seven out of nine crops from September to January. This is an essential reason why winter samples displayed less isotopic content of $\delta^{15}N$. However, the general tendency for both plots was a small difference between winter and summer. Thus, it appears that it is difficult to separate between different pollution sources using these isotopes. The same difficulty has been experienced for groundwater samples (Nakagawa et al., 2017).

The results for Nishikawa and Yuegawa Rivers indicate that samples are clearly affected by livestock waste according to the isotopic analysis using the Kendall (1998) plot. Additionally, three kinds of isotopic observations from livestock waste and manure correspond to samples from Nishikawa and Yuegawa Rivers. These facts are consistent with the assumption that the main nitrate source in the study

area is the livestock waste. A large number of livestock is raised in this area (Nakagawa et al., 2015). It is estimated that the potential nitrate load from livestock waste is much higher as compared to that from chemical fertilizers.

5.3. Relationship between nitrate $\delta^{15}N$ and sterol ratio

According to the above discussion, both Yuegawa and Nishikawa Rivers are affected by livestock waste. In these rivers, results for both nitrate $\delta^{15}N$ and sterol ratio are quite consistent with fecal pollution. For the urban areas, the results indicate pollution originating domestic sewage water.

5.4. Relationship between NO_3+NO_2-N and sterol ratio

As mentioned above, group I does not indicate pollution. In group II, although nitrate concentration meets the drinking water standard, sterol ratio is larger than 0.3. Thus, this group is considered to be polluted mainly by livestock waste or domestic waste water discharge with a low T-N. Site 1-8 and 29 are located in the urban area, indicating that some of these are affected by domestic waste water discharge. In group III, although nitrate concentration exceeded drinking water standard, sterol ratio is below 0.3. In this case, chemical fertilizers are considered to be the main source of pollution. As mentioned above, fertilizer application on main crops in the study area is conducted from September to January. This is one of the reason that larger number of samples were classified into group III for the winter season. Finally, for group IV, samples exceeded both criteria and livestock waste is considered to be a main source of nitrate pollution. Some samples from the Yuegawa and Nishikawa River indicate that

363 group IV samples are affected by livestock waste. This is confirmed by the close location of livestock
364 waste water treatment plant and pig farm.
365
366 **Conclusions**
367 In this study, we examined nitrate pollution in two rivers and possibilities to distinguish
368 between different sources of pollution by the use of several biochemical indicators. As a case study, and
369 to clarify the surface water pollution in the Shimabara study area, we firstly investigated the distribution
370 of nitrate, coprostanol, and sterol ratio as biochemical indicators. Secondly, we plotted the relationship
371 between nitrate $\delta^{15}N$ and $\delta^{18}O$ according to Kendall (1998) to elucidate the general tendency of the
372 samples. Then, we plotted the relationship between nitrate $\delta^{15}N$ and sterol ratio to confirm identified
373 pollution sources. Finally, we proposed a methodology to indicate the main sources of pollution in the
374 surface water of the study area. As a conclusion, we can summarize major results of our analysis
375 according to the following: 1) Nitrite (NO_2-N) concentration exceeded 0.04 mg L^{-1} at 2 sampling
376 locations during winter and 6 sampling points during summer. 2) Nitrate (NO_3+NO_2-N) concentration
377 exceeded 10 mg L^{-1} at 19 sampling points during winter and 7 sampling points during summer. The
378 highest concentration was 82.4 mg L^{-1} in summer. 3). Coprostanol concentration exceeded 700 ng L^{-1} at 8
379 sampling points in winter and 6 sampling points in summer. Thus, the study confirmed that fecal pollution
380 of the water occurs. 4) The sterol ratio exceeded 0.5 at 17 sampling points in winter and 14 sampling
381 points in summer. According to the above analysis, coprostanol and sterol ratio have a potential to be used
382 to distinguish between different nitrate pollution sources in surface water.

To summarize this study, we showed a potential use of coprostanol and sterol ratio to evaluate pollution sources as biochemical indicators. Also, we proposed a concrete methodology to separate between pollution sources based on these biochemical indicators. Additionally, the use of these indicators, can not only separate between source of nitrate pollution but also display hidden health threats caused by fecal pollution.

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