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Modified Multiple Antibiotic Resistance Analysis for the Nonpoint Source Tracking of Fecal Pollution

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Abstract

Although Multiple Antibiotic Resistance Analysis (MARA) has been adopted for the source tracking of bacterial contamination in natural waters, the accuracy and experimental cost of MARA still have the potential to be improved. Therefore, a process of modified MARA using turbidity was developed, and its feasibility and reliability were evaluated for Fecal Streptococci (FS) in this study. The experimental results are as follows. The development of turbidity via the aesculin hydrolysis by FS could occur in 2-3 hours, and the turbidity of the stock solutions and the number of FS had a proportionate relationship ($R^2 = 0.991$). Thus, the modified MARA could exclude several incubation steps as well as reduce experimental errors by inoculating the same amount of the bacteria into the culture media mixed with antibiotics. The Average Rates of Correct Classification (ARCCs) of the established database based on the modified MARA technique for two-way division and three-way division showed much higher ARCCs (89%) than those of previous studies (61~84%), implying that the modified MARA technique had relatively higher reproducibility than conventional MARA. Also, reliability of the modified MARA was verified by source tracking of stream samples that could be predicted and the real source tracking was carried out by applying the modified MARA in the stream (upstream, midstream, and downstream), which showed reasonable prediction results. The results conclusively demonstrated the modified MARA to be more efficient tool/phenotypic method than conventional MARA for source tracking of fecal contamination in water.

Keywords: *modified multiple antibiotic resistance analysis, fecal streptococci, nonpoint pollution, source tracking, turbidity*

1. Introduction

The water quality management of water flowing through a city, especially near residential areas, is important due to increasing requirements of higher environmental quality for citizens. However, the contamination of surface water and groundwater with untreated manure and sewage continues to be a serious environmental problem (Sinton *et al.*, 1993; Lee, 2011). For the water quality management of streams, the management of nonpoint source pollution as well as point sources must be considered. At present, pathogens are the most frequently reported category among factors causing water damage in the water system. The causes resulting in fecal non-point pollution include pollutants from livestock, pets, wild animals, and human feces from sewer breakages and sewer overflow (Nagel *et al.*, 2002). Since fecal pollutants include pathogenic microorganisms, when such polluted water is used for drinking water or leisure activities, infection or diseases can occur (Webster *et al.*, 2004).

Understanding the origin of fecal pollution is paramount in assessing associated health risks as well as the actions necessary to remedy the problem while it still exists. The concept that the origin of fecal pollution can be traced using genotypic and phenotypic has been termed microbial source tracking (Scott *et al.*, 2002; Carroll *et al.*, 2009). It has been considered that the genotypic method is expensive and needs a complex process for one outcome resulting in limiting the number of analysis (Gordon, 2001; Gallagher *et al.*, 2008). On the other hand, the use of Multiple Antibiotic Resistance Analysis (MARA), which is one of phenotypic methods, is based on the underlying principle that the bacterial flora present in the gut of various types of animals are subjected to different types, concentrations, and frequencies of antibiotics. Over time, selective pressure within a specific group of animal selects for flora that possess specific "fingerprints" of antibiotic resistance (Hagedorn *et al.*, 1999; Harwood *et al.*, 2000). The MARA technique has been shown to be successful in discriminating *E. coli* or fecal streptococci isolated from specific animal species, including wildlife, various livestock (cattle, pigs, horses, and chickens), and humans and considered as inexpensive and simple as compared with genotypic approaches to source tracking (Hagedorn *et al.*, 1999; Harwood *et al.*, 2000; Scott *et al.*, 2002; Ebdon and Taylor, 2006). In recent years, the conventional MARA technique has been used in the process of establishing

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Total Maximum Daily Loads (TMDL) of fecal bacteria in the USA (Booth *et al.*, 2003; Maryland Department of the Environment, 2009). The need for current and future source tracking technologies is increasing, and these methods are certain to play a pivotal role in identifying point and non-point sources of fecal pollution in impaired water systems (Scott *et al.*, 2002; Gallagher *et al.*, 2008).

However, previously developed experimental methods for MARA still require a relatively large library, consisting of as many isolates as possible to ensure adequate representativeness (Wiggins *et al.*, 2003). Therefore, the MARA method needs to be modified to reduce the experimental costs and time requirements. Furthermore, during the conventional MARA procedure, inconsistent numbers of bacteria transferred into the plate can reduce the reliability of conclusions. Measuring the turbidity of the stock solution containing the bacteria could ensure the number of transferred bacteria is consistent, and reduce the time of incubation; in this way MARA could be more reliable and economical. In this study, a modified MARA, using turbidity, was developed using potentially known sources of fecal pollution in the city of Ansan, Korea. The purpose of this work is to examine whether the modified MARA has potential use as a phenotypic method for determining sources of fecal pollution in water.

2. Materials and Methods

2.1 Sample Collection

2.1.1 Known Sources Used to Establish Database

All animal fecal samples and sewage samples (designated as "human") were collected in Ansan, Korea. During each sampling event, feces from multiple animals at the same source were collected. Pet fecal samples (*i.e.*, dog) were obtained from an animal shelter, livestock fecal samples (*i.e.*, cow) were obtained from livestock farms, and sewage samples were obtained from influent into the Ansan sewage treatment plant, where the majority of isolates would be of human origin. The known sources were processed following the method explained in section 2.2 and used for establishing database following the modified MARA technique explained in section 3.1.2.

2.1.2 Unknown Sources for Applying Modified MARA Technique

To verify the established database and perform real-world source tracking using the modified MARA technique, this study collected unknown sources in Ansan Stream in Ansan, Korea. Ansan Stream can be divided into upstream, midstream, and downstream areas. A number of farming and stock farming houses are located in the upstream area. In the midstream and downstream areas, are concentrations of residential and commercial areas. The midstream area is an old part of town in which Ansan installed intercepting sewers due to concerns over the incorrect connection of storm water sewers with sanitary sewers because the effluents from the storm water sewers during dry weather have a similar characteristics to sanitary sewers. In contrast, the downstream area is a new part of town and has a relatively good arrangement of sewer pipes.

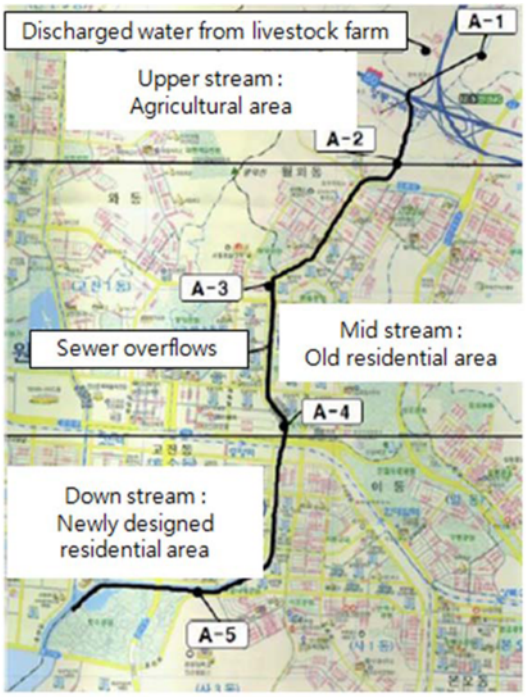

Fig. 1. Ansan Stream and Sampling Points

To verify the reliability of the modified MARA technique through the source tracking of samples that enabled the prediction of results, we selected and sampled the water discharged from livestock farm near the upstream area and the water discharged from storm water sewers located in the midstream area. Furthermore, in order to perform real source tracking, five sampling points were established in Ansan Stream. Sampling was carried out at two points in the upstream area (A-1 and A-2), two points in the midstream area (A-3 and A-4), and one point downstream (A-5). The sampling was performed during the rainy season and a test was performed within 8 hours after the sampling. Fig. 1 shows Ansan Stream in Ansan, Korea and the sampling points.

2.2 Sampling Process for the Number of Fecal Streptococci and the Determination of Antibiotic Kinds/Concentrations

The indicator microorganisms used in this study were Fecal Streptococci (FS) and their analysis was carried out following standard methods (APHA, 2005). The samples were transported to the laboratory on ice and were processed within 2 hours of collection. First, 10 g of fecal material were placed into 200 mL of buffer solution (Hardy Diagnostics, Orcutt, CA, USA) and blended. Then, the blended fecal matter and the sewage samples were further diluted, depending on the source of the sample. The dilutions were filtered through membranes with 0.45-μm pores on absorbent pads (Gelman, Ann Arbor, MI, USA). After the

Table 1. Types and Concentration of Antibiotics

Antibiotics	Concentrations in Enterolert medium (μg/mL)
Amoxicillin (AMX)	2.5, 5, 10
Cephalothin (CEP)	25, 50, 75
Erythromycin (ERY)	10, 25, 50
Neomycin (NEO)	10, 25, 100
Oxytetracycline (OTC)	25, 50, 100
Streptomycin (STR)	10, 25
Tetracycline (TET)	50, 100

filtration, one set of absorbent pads was saturated with 2 mL of KF Strep Broth media (Hach Company, Loveland, Co, USA) for FS and incubated at 35°C for 48 hours. Individual red colonies of FS on membrane filters were counted to enumerate the number of FS (CFU/100 mL).

A screening test was conducted to select both the kinds and the concentrations of antibiotics that would be used in this study. Eight antibiotics were tested by the modified MARA procedure (as explained in Section 3.1.2.): Amoxicillin (AMX), Cephalothin (CEP), Erythromycin (ERY), Neomycin (NEO), Oxytetracycline (OTC), Rifampicin (RIF), Streptomycin (STR), and Tetracycline (TET), which were diluted to various concentrations (2.5, 5, 10, 25, 50, 75, and 100 μg/mL in Enterolert medium, as explained in section 3.1.2.). The purpose of the screening test was to exclude the types and concentrations of antibiotics showing an extreme pattern in which injected indicator microorganisms clearly resisted the antibiotic or died by failing to resist, and to select the kinds and concentrations of antibiotics showing certain levels of patterns. Accordingly, as shown in Table 1, seven types of antibiotics with mutually differentiated concentrations were selected, and based on these, 19 combinations were selected.

2.3 Statistical Analysis

Discriminant analysis using SPSS v. 12.0 (SPSS Inc., Chicago, IL, USA) was used to classify the isolates by source. The table generated by the SPSS software displays the numbers and percentages of isolates from each known source and the unknown sources that are classified in each source category. The analysis was performed by two approaches, two-way (human and animal) and three-way (human, livestock, and pet). The number of isolates from a given source that were placed in the correct source category by discriminant analysis is termed the rate of correct classification. The Average Rate of Correct Classification (ARCC) for the database is obtained by averaging the correct classification percentages for all sources.

3. Results and Discussion

3.1 Development of Modified MARA Technique Based on Turbidity Measurement

In this study, a new experimental method was introduced by excluding and modifying a few of the analysis steps of the conventional MARA to reduce the experimental errors and running time. The conventional MARA has several significant disadvantages. The number of cultured bacteria (dots on the filter paper) to be transferred into the well plates and then dishes may be very different because both the sizes of the dots and the amounts to be transferred are always different, which results in less reliable conclusions. In addition, transferring errors might occur when inoculating bacteria from well plates to the dishes containing mixed antibiotic mediums, because the transferring prongs could be contaminated by the antibiotics and not be maintained as completely sterile. In addition, the conventional method requires two steps for incubation; thus, the total duration of the experiments is relatively long (at least 80 hours).

To solve these problems, we exploited a characteristic of aesculetin. Specifically, FS hydrolyzes aesculin in the culture medium (enterococcosel broth, Difco, Sparks, Md. USA) to aesculetin, and it then produces a dark brown complex by reacting with ferric ammonium citrate; this complex can be measured as a turbidity (MacFaddin, 2000). The turbidity generated by the aesculin hydrolysis by FS can occur in 2-3 hours. Thus, the total time of our modified MARA method (maximum 35 hours) could be shorter than that of the conventional method (at least 80 hours). Therefore, if the relationship between turbidity and the FS concentration is linear, this method could exclude several incubation steps as well as reduce experimental errors by transferring the same amount of the bacteria.

3.1.1 Correlation between Turbidity and Fecal Streptococci Concentration

This study examined whether stock solutions that exhibited a certain value of turbidity included the same number of FS. All the cultivated isolates in 96 well plate, which had changed to black through the hydrolysis of aesculin by FS, was mixed. Several stock solutions with various amount from the mixed culture was prepared and then the amounts of the culture media and the turbidities of the solutions were examined. As shown in Fig. 2, a linear relationship was identified ($R^2 = 0.999$). Likewise, the amount of culture media and the number of FS had a linear relationship ($R^2 = 0.993$). Therefore, the turbidity of the stock solution was confirmed to be linearly proportionate to the number of FS ($R^2 = 0.991$). From these results, the same amounts and conditions of FS could be inoculated into the culture media mixed with antibiotics so that this new method using turbidity could be used as a new modified MARA.

3.1.2 Experimental Procedure of Modified MARA Technique

Figure 3 shows the modified MARA analytical procedure. A diluted sample is filtered with filter paper (No. 4, membrane with 25-μm pore size, Whatman, Little Chalfont, UK) to exclude any turbidity material in the sample. Next, 0.1 mL of the filtered sample are transferred into the wells of a 96-microwell plate containing 0.2 mL of enterococcosel broth and incubated at 35°C for 2-3 hours. All FS isolates in the wells that turn a dark brown color, indicating aesculin hydrolysis, after incubation are mixed,

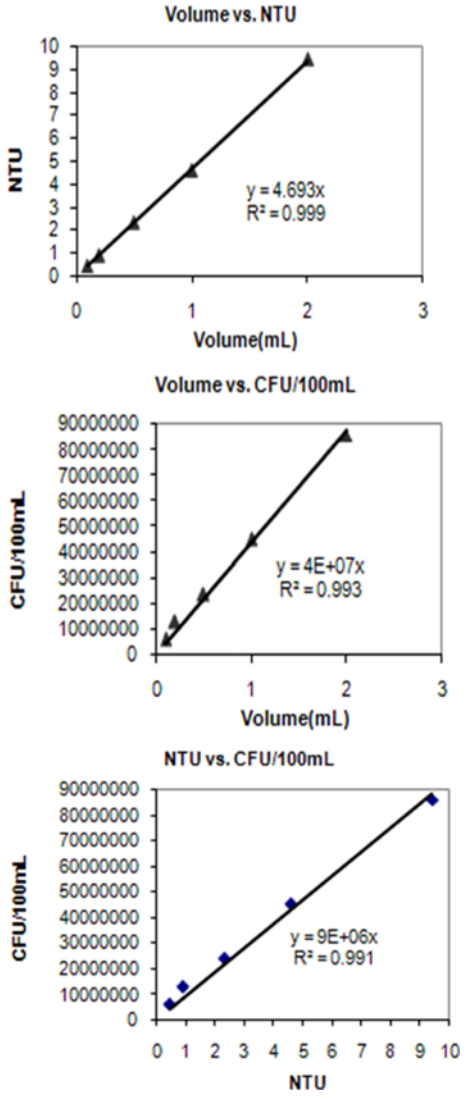

Fig. 2. Correlation of Turbidity and Numbers of Fecal Streptococci

and 0.1 mL of the mixed FS isolate is diluted with 15 mL of buffer solution (also referred to as stock solution). It is necessary to decide the designated turbidity, because different numbers of transferred bacteria may result in variations in MARA results. The turbidity of the stock solution is measured by a turbidimeter (2100N Turbidimeter, Hach, Loveland, Co, USA). Either more of the isolate or more buffer solution is added into the stock solution to achieve the designated turbidity of 0.4 NTU (in this study, 0.1 mL of the FS isolate with 15 mL of the buffer solution produced a turbidity of 0.4 NTU). Each 0.5 mL of the stock solution is transferred into a 96-microwell plate containing 0.1 mL each of Enterolert media (IDEXX, Westbrook, ME, USA) and the pre-selected antibiotics. Then, the 96-microwell plate is incubated at 35°C for 24 hours. After incubation, the wells that fluoresce under a UV lamp at a wavelength of 365 nm are recorded for growth. The data collected from the known sources are used for establishing database and the data collected from the unknown sources are compared with the established database of

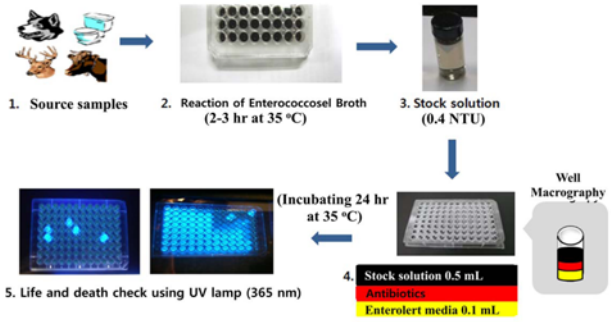

Fig. 3. Procedure of Modified MARA Analysis

the known sources using statistical analysis for source tracking.

3.1.3 Evaluation of the Database Established Based on Modified MARA

The database that was established based on the modified MARA method was first divided into two categories (humans and animals) and then the classification of samples into these categories was evaluated. Table 2(a) shows the results of the modified MARA using the two-way method. The category of humans had a classification accuracy of 99.7%, whereas the category of animals had a classification accuracy of 84.1%. The ARCC of the overall database was 89.6%. While the identification of human sources exhibited a high level of accuracy, the classification of animal sources showed a relatively lower level of accuracy. When the database was divided into three categories (humans, livestock, and pets), the level of correctness for each category was observed again. Table 2(b) shows the results of the modified MARA based on the three-way classification method. These results showed that humans, livestock (cattle), and pets had classification accuracy levels of 82.4%, 90.5%, and 89.2%, respectively. The ARCC of the overall database was 87.3%.

Our study also compared the ARCCs of databases established by various researchers and institutions using conventional MARA and the ARCC of the database established by this study using a

Table 2. Modified MARA of the Two-way and Three-way Databases

(a) Two-way

Source	Predicted membership (%)		Total
	Human	Animal	
Human	335 (99.7)	1 (0.3)	336 (100)
Animal	99 (15.9)	525 (84.1)	624 (100)
ARCC	89.6%		

(b) Three-way

Source	Predicted membership (%)			Total
	Livestock	Pet	Human	
Livestock	304 (90.5)	31 (9.2)	1 (0.3)	336 (100)
Pet	15 (5.2)	257 (89.2)	16 (5.6)	288 (100)
Human	0 (0.0)	59 (17.6)	277 (82.4)	336 (100)
ARCC*	87.3%			

*ARCC: average rate of correct classification

Table 3. Comparison of ARCCs from this Study and Other Studies

Source category	Isolation Number of Database	Varieties of Antibiotics used	ARCC (%)	Reference
Human, Cow, Poultry, Wild	1,435	16	84.0	Wiggins, 1996
Human, Cow, Poultry, Wild	2,844	40	65.0	Wiggins *et al.*, 1999
Human, Livestock, Pet	995	20	61.0	Gallagher 2008
Human, Livestock, Pet, Wild	2398	32	69.1	Whitlock *et al.*, 2002
Human, Livestock, Pet, Wild	1,074	37	81.0	Price *et al.*, 2006
Human, Livestock, Pet	960	19	89.6	This Study

modified MARA (Table 3). Our study showed a relatively higher ARCC than those of other studies. A mere numerical comparison of the ARCC could lead to incorrect conclusions about reliability, because each research study had differences in the types of source categories, the size of the database, and the types of antibiotics used (Wiggins *et al.*, 2003). However, a database from an existing study (Gallagher, 2008) with relatively similar conditions to those of this study (regarding the size of the database and the types and diversity of source categories) had an ARCC of 61.0%, whereas this study's ARCC was much higher at 87.3% (the rows given in boldface in Table 3). This result suggests that the modified MARA technique increases reliability by revising or removing the procedures that could have introduced errors during the test process.

3.2 Verification of the Modified MARA by Source Tracking of Predictable Fecal Contamination

This study was intended to verify the reliability of the modified MARA technique through the source tracking of samples that enabled the prediction of results. Accordingly, source tracking was performed in the downstream area of Ansan Stream near livestock farming houses and the water discharged from storm water sewers during dry weather, which is suspected to contribute human sources of fecal matter due to the incorrect connections of sanitary and storm water sewers (Lee *et al.*, 2012).

The results of the three-way source tracking for the verification of the MARA database are shown in Table 4. As expected, the results show that livestock sources accounted for 93.8% of the samples collected from the downstream area near the stock farming houses and human sources accounted for 100% of the samples collected from the sewer overflow, which verifies that the modified MARA has a high level of reliability.

3.3 Source Tracking of Fecal Pollution in Ansan Stream Using Modified MARA

After the verification of the modified MARA, a case study was conducted for the source tracking of fecal pollution using relatively predictable samples from Ansan Stream. Table 5 shows the results of source tracking in the upstream, midstream, and downstream areas. It was expected that point A-1 would be polluted by livestock feces, because a livestock farmhouse is located at the upstream area of Ansan Stream. However, the influence of livestock is almost absent from the results. It appears that a storm water runoff with the livestock feces has been managed well and could not reach the stream, because the livestock farmhouse routes the waste far away from the stream. In contrast, the influences of humans and pets showed high impacts of 54.2% and 43.8%, respectively. This may be due to the contamination with wastewater effluent by the damaged or misconnected sewers and the many pets around the upstream area. Point A-2 is the boundary point between the ending of the upstream section and the beginning of the midstream area. Therefore, the results could include the characteristics of both upstream and midstream sections. In the results, the highest value (93.8%) was human and the impact of pets decreased to 6.3%. The human influence was high due to the increase in residences from upstream to midstream areas. The influence of humans (100%) was the main factor at points A-3 and A-4 (the midstream portion of Ansan Stream). The dense residences around these points may result in the absolute influence of humans. Even though the sewer system in Ansan is a separated sewer system, the effluents from the sewers were delivered to the sewage treatment plant by intercepting sewers, especially around the midstream area, due to the misconnection between the storm water sewers and sanitary sewers. However, incomplete interceptions seemed to occur, and heavy sewer overflow was also shown during wet weather periods. Thus, the stream appears to be contaminated by human sources. The highest influence of the 60.4% by pets was shown at point A-5, in the downstream section of Ansan Stream, while the human impact at that point

Table 4. Source Tracking for Discharged Water from Livestock Farms and Sewer Overflows

Source	Predicted membership (%)			Total
	Livestock	Pet	Human	
Discharged water from livestock farms	45 (93.8)	0 (0.0)	3 (6.3)	48 (100)
Sewer overflows	0 (0.0)	0 (0.0)	48 (100)	48 (100)

Table 5. Fecal Contamination Source Tracking for Ansan Stream

Source		Predicted membership (%)			Total
		Livestock	Pet	Human	
Upstream	A-1	1 (2.1)	21 (43.8)	26 (54.2)	48 (100)
	A-2	0 (0.0)	3 (6.3)	45 (93.8)	48 (100)
Midstream	A-3	0 (0.0)	0 (0.0)	48 (100)	48 (100)
	A-4	0 (0.0)	0 (0.0)	48 (100)	48 (100)
Downstream	A-5	0 (0.0)	29 (60.4)	19 (39.6)	48 (100)

decreased to 39.6%. The human influence was less than that of the midstream area because the downstream section is a newly constructed area; thus, the possibility of human sources of waste entering the stream in this area could be very low. However, many parks and esplanades are located near A-5, where pet sources might inflow into the stream, and may have resulted in the high influence of pets.

4. Conclusions

This study was conducted to develop a new method based on the measurement of turbidity to address the problems and limitations of the conventional MARA technique. Our conclusions are as follow:

1. The turbidity generated via the aesculin hydrolysis by FS could occur in 2-3 hours, and the turbidity of the stock solutions and the number of FS showed a linear relationship (R^2 = 0.991). Thus, the modified MARA could exclude several incubation steps as well as reduce experimental errors by inoculating the same amount of the bacteria into the culture media mixed with antibiotics.
2. The ARCCs of the database established based on the modified MARA technique were 89.6% in the case of the two-way method (classification into human and animal sources) and 87.3% in the case of the three-way method (classification into human, livestock, and pet sources). When compared with the ARCCs of previous studies, the results from this study showed much higher ARCCs, implying that the modified MARA technique has relatively higher reproducibility than the conventional MARA technique.
3. The reliability of the modified MARA was verified by performing the source tracking of Ansan Stream samples that could be predicted. The source tracking of the samples suspected to be of livestock and human origin showed ARCCs of 93.8% and 100%, respectively, verifying that the modified MARA procedure has high levels of reliability.

The results of this study demonstrate that the modified MARA technique is simpler, more economical and more reliable than the conventional MARA procedure. Therefore, the modified MARA technique is likely to be effective in identifying fecal nonpoint source pollutants and diagnosing the causes of pollution that can worsen the quality of domestic streams, so that they can be reasonably managed under the policy of total maximum daily load.

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