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The investigation of the bacterial indicators and point sources of pollution for the Nanshih River, Taiwan: a case study

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ABSTRACT

The objective was to evaluate representative bacterial indicators found in the Nanshih River, a resource having multiple uses including recreation, water supply, and agriculture. Human activities were investigated in order to clarify the relationship between bacterial indicators and point source pollution discharged into this river. The optimal bioindicator was evaluated using four approaches by two-dimensional principal component analysis (PCA), which included ten water parameters, the median river pollution index parameters, bacterial numbers and specific fluorescence *in situ* hybridization indicators. The results indicate that *Bifidobacterium* spp. with the range of 1.80 ± 0.96–$14.14 \pm 1.24\%$ were identified as the best bioindicator for the Nanshih River and these bacteria are able to identify four characteristic groups of point pollution sources using PCA. It is suggested the specific bacterial indicator need to be used for the regular monitoring of the Nanshih River in addition to the present regulation requirements of total coliforms counts. The major contributor to the biological pollutants was determined to be hot spring resort activity, which implies that tourists may be vulnerable to waterborne recreational illnesses. An effective strategy aimed at controlling point source pollution should be able to reduce drinking water resource and recreational activity public health risks associated with the Nanshih River.

Keywords: Bacterial indicators; Median river pollution index (RPI-M); Fluorescence *in situ* hybridization (FISH); *Bifidobacterium* spp.; Total coliforms (TC)

1. Introduction

The Nanshih River is a famous river located within New Taipei City in northern Taiwan and it flows through the Wulai District and the southern part of the Xindian District of Taipei City and Yilan County for 45 km. Furthermore, it is one of tributaries of Xindian River; in the Guishan area of Xindian District, the river joins the Beishih River to form the Xindian River. The Nanshih River has the multiple uses in the Taipei area, specifically as a drinking water resource and for recreational activity. Many pollutants from human activity, such as water sports,

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hot spring resorts, and aquaculture farms, affect this river. Significant amounts of microorganisms are generated and discharged into this river. These microorganisms can threaten the water supply system and public health if the drinking water resource is contaminated [1]. In addition, tourists in contact with the surface water may get waterborne illnesses during recreational activity. In this context, a total maximum daily load should be established in order to safeguard human health.

Many bacteria found in the intestines of warm-blooded animals can be used as biological indicators for drinking or recreational water when grown on selective and differential media [2,3]. Total coliforms (TC) are defined as the total number of bacteria that are members of the family Enterobacteriaceae, which includes *E. coli* and members of the various other genera, namely *Enterobacter*, *Klebsiella* and *Citrobacter*; these are aerobic and facultatively anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose producing gas and acid over 24 h at 35˚C. The TC count in water is usually regarded as a measure of the potential danger of waterborne diseases caused by pollution. Taiwan's Environmental Protection Agency (EPA) regulations state standards in terms of TC counts for surface water (rivers or ponds) as a measure of quality and as an assessment of fecal contamination. Fecal coliforms (FC) are defined as the TC count plus a requirement that the isolates grow and ferment lactose with the production of gas and acid at 44.5˚C. FC has been found to have a positive correlation with fecal contamination from warm-blooded animals. Fecal streptococci (FS) occur in the digestive systems of warm-blooded animals, including humans. In addition to TC and FC counts, FS counts can be also used to evaluate water samples and act as supplement information when a more precise determination of the source or sources of contamination is required. The above bioindicators selectively are used to monitor surface water in many developed counties such as the US, EU, and Austria. However, TC, FC, and FS counts still have many limitations as predictors of risk of waterborne diseases because of the short half-life of the relevant organisms in water and because these organisms are more susceptible to water treatment.

Alternative biological indicators of river pollution have been investigated and their advantages and limitations have been reviewed many times. For example, *E. coli* is a normal inhabitant of the intestinal tract of humans and other warm-blooded animals, where it is part of the normal flora. Studies have demonstrated *E. coli* and *Enterococcus* isolates are better indicators of (1) tropical drinking water quality and (2) health risk from water contact with recreational waters [4]. The US EPA has established a limit for *E. coli* in recreational freshwater, namely a monthly geometric mean of 126/100 mL. Bifidobacteriaceae are commensal intestinal microbiota of warm-blooded animals. Clostridiaceae, some species of which are pathogenic, occur in water, soil, and the intestinal tract of humans and lower animals. Diseases are associated with *Clostridia* include diarrhea, tetanus, botulism, and gas gangrene. Some FS strains, such as *Streptococcus faecalis* and *Streptococcus faecium* in particular, have persistence patterns that are similar to those of a range of potential waterborne pathogenic bacteria. Nevertheless, most of the widely used fecal indicators are found in both human and animal feces and thus do not allow source differentiation. Thus, no single organism can serve as an adequate indicator for all types of water and all routes of exposure. Recently, molecular fingerprinting has been applied to identify fecal contamination [5]. One example is a rapid PCR assay that is able to detect and differentiate human and ruminant sources of fecal pollution in natural water samples in order to identify specific pollution points [6]. Alternatively, the PCR-DGGE method is able to evaluate strain level differences in *E. coli* from cattle, poultry and humans [7]. 16S rRNA-targeted oligonucleotide probes have been designed, validated, and tested with specificities that allow the quantification of predominant groups of anaerobic bacteria in human fecal samples by dot blot hybridization and fluorescent *in situ* hybridization (FISH) [8]. These types of information allow bacterial indicators to be used on a case-by-case basis if there are different pollutant sources present on different rivers. To carry out monitoring of water borne pollution, a reliability and relevant measure is required that is both reasonable in cost and feasible logistically.

The chemical and physical parameters of a water body usually are usually able to reflect water quality in many polluted rivers [9]. However, the characterization of the water quality within the water supply protected watershed of the Nanshih River is not the same as with other rivers in Taiwan. Water quality parameters such as COD, BOD, SS, DS, NH_4^+–N, DO, total phosphate and PO_4^{3-} are always present in small amounts and remain relatively constantly throughout the year. This makes the tracing and identifying of point-source pollution more difficult. As an alternative, the presence of various bacteria can act as good bioindicators because their numbers or physiological characteristics are related to specific types of artificial pollution. The objective in this study was to develop bioindicators that allow the various different pollutants in the Nanshih River to be distinguished. The numbers of fecal bacteria and other representative

species that are present were measured as a means of monitoring the seasonal incidence of pollution. For the first time in Taiwan, FISH was used to measure the bacterial number of three Gram-positive bacterial genera, namely Bifidobacteriaceae, Streptococcaceae, and Clostridiaceae. Human activities such as land use and various possible point-sources of pollution were investigated in detail in order to clarify the relationship between the bioindicators and possible pollution of the Nanshih River.

2. Materials and methods

2.1. Site description and sampling

Fig. 1 shows the eight sampling locations on the Nanshih River with their GPS coordinates that were used for this study during 2006. The standard of surface water quality in this river is regulated and should be class A at seven sampling locations, namely No. 1 Fushan; No. 2 upstream of Tunho River; No. 3 Tunho River; No. 4 Wulai Weir; No. 5 the SunMoon-Light Hot Spring; No. 6 Quchi Weir; and No. 7; Qingtan Weir. These river sections are part of the Taipei Water Supply Protected Watershed, which is managed by the Taipei Water Management Office of the Water Resources Agency, Ministry of Economic in Taiwan. The other sampling location, No. 8; Bitan, is regulated as class B in terms of the standard of surface water quality. In this study, the samples used for counting bacterial numbers were collected once each month. The bacterial community of FISH was assessed every two months. The collected samples were transported to the laboratory in a 4℃ portable refrigerator, which took 4–6 h. In addition, each location was surveyed carefully for possible pollutant sources and this survey covered peripheral areas, and locations that were adjacent to natural drains that would discharge into the river. The investigation was assisted by the use of a GIS satellite map of Taiwan from the EPA and the Google earth website.

2.2. Bacterial number by growth on media

Microbiological examination of each sample was started immediately on arrival at the laboratory. The counts for each bacterial indicator were obtained by serial dilution and spread plating on four different media using 30–300 colonies per plate as acceptable statistically. ChromoCult® coliform agar (Merck, Germany) was used to obtain the TC count and *E. coli* colonies were identified by the fact that they were β-galactosidase positive and cytochrome oxidase negative. The FC count was carried out on M-FC-Agar (Merck, Germany) at 44.5℃ with incubation for 24 h. The FS count involved incubated on FS KF-streptococcus-agar (Merck, Germany) at 37℃ for 48 h. Selective and differential MacConkey-Agar

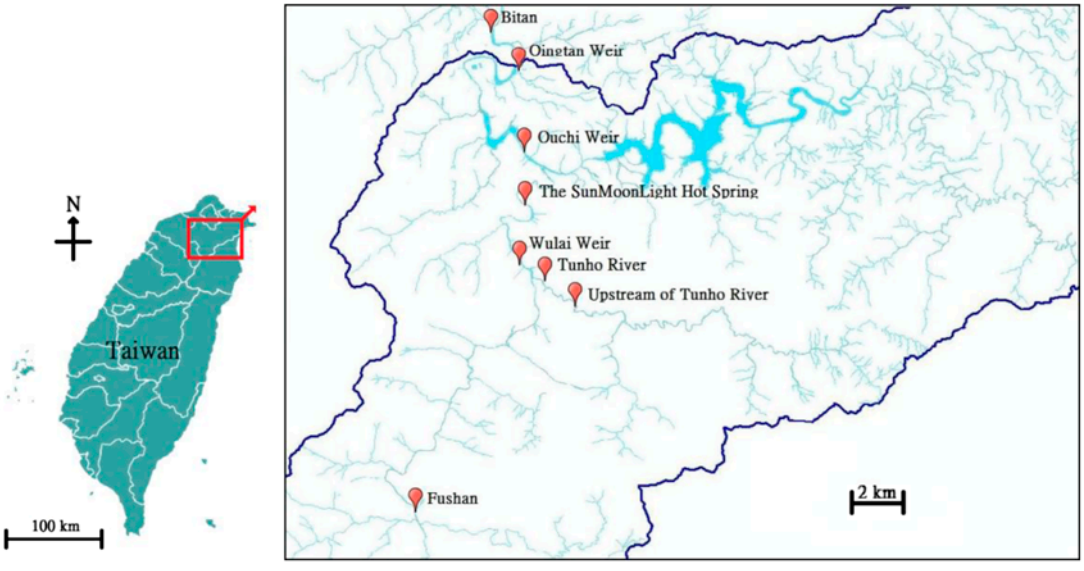

Fig. 1. Sampling locations in this study (yellow) using the UTM projection system. These range from the upstream to downstream along the Nanshih River: No.1: Fushan ($X = 300{,}905$, $Y = 2{,}741{,}596$); No.2: upstream of Tunho River ($X = 307{,}386$, $Y = 2{,}749{,}818$); No.3: Tunho River ($X = 305{,}895$, $Y = 2{,}750{,}822$); No.4: Wulai Weir ($X = 305{,}263$, $Y = 2{,}751{,}684$); No.5: the SunMoonLight Hot Spring ($X = 305{,}259$, $Y = 2{,}754{,}012$); No.6: Quchi Weir ($X = 305{,}312$, $Y = 2{,}756{,}185$); No.7: Qingtan Weir ($X = 305{,}087$, $Y = 2{,}759{,}579$); No.8: Bitan ($X = 304{,}110$, $Y = 2{,}761{,}042$).

supplied by the Becton, Dickinson and company (USA) was used to count non-lactose fermenting bacteria (NLFB), which is colorless colonies, after growth on the agar at 37°C for 24 h.

2.3. The bacterial community by FISH

The FISH technique, which is a rapid and sensitive bio-molecular diagnosis tool, was used to evaluate bacterial indicators during this study [9]. The sample was filtered firstly through a 1.2 μm pore-size glass microfibre filters (GF/C, Whatman®) to remove any auto-fluorescent impurities or by-products that might confuse the experiments. Cells from the samples were then collected by filtration through a 0.22-μm pore-size polycarbonate filter (Critical®, USA) and transferred to gelatin-coated slides by the addition of 5 μL of distilled deionized water to each well after which the filter was gently pressed onto the gelatin for a few seconds. These air-dried filters fall onto the slides very easily. After filtration, the samples were fixed in 2 ml of paraformaldehyde-PBS (4%) for 60 min at 4°C. Hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris–HCl, 5 ng L^{-1} HPSF-grade probes, pH 7.2) was added to dilute the sample, and then that sample was hybridized at 46°C for 3 h. The probes used in the present study were selected to include a range of representative species that might be present in the Nanshih River. The probes were labeled with a CY3™ flourescent tag (MWG-Biotech, Germany) at the 5′ end. Formamide was added to the probe buffer if there was a requirement for more stringent hybridization conditions. Table 1 describes the selected 16S or 23S rRNA oligonucleotide probes and the hybridization conditions used. The probes used included (1) two phyla/subclasses probes (HGC and LGC) [12,13], (2) three specific-species probes for *Bifidobacteria* spp., *Clostridium perfringens* and *Streptococcus* spp. [8,14,15] and (3) NONEUB338, which acts as a negative control probe by detecting nonspecific hybridization [16].

After hybridization, the cells were washed twice in washing buffer (variable NaCl, 20 mM Tris–HCl, pH 8.0, 0.01% SDS, pH 8.2) at 48°C for 20 min. They were then stained with polyphosphate for 30 min using 1 μg mL^{-1} DAPI (4′-6′-diameidino-2-phenylindole)/ddH2O. Since DAPI reacts with both DNA and RNA from the cells, the DAPI-positive cells represent the total bacterial number in the sample. Cells were then visualized using an Olympus BX41 microscope (Japan) equipped with two fluorescent filters for detecting DAPI and Cy3™. The microscope was attached to a charged coupled camera (SamBa EZ140, Isuzu Optics Corp., Taiwan). The total cell counts ranged from 500 to 1,500 bacteria per sample. The microscopic analysis included manual counting of the cells from at least ten photographs of duplicate samples by the Image-Pro Plus software (Version 6.0) as experimental quality control.

2.4. Water parameter analysis

Samples for water parameter analysis were acquired at the same sampling locations and at the same time as the microbial samples. Ten water parameters, including temperature, pH, conductively, SS, DS, DO, BOD, COD, NH_4^+–N and total phosphate were analyzed and obtained from the website of Taiwan EPA. All experimental measurements followed the procedures of the standard methods for the examination of water and wastewater [17].

Table 1
Oligonucleotide probes and hybridization/washing conditions used in this study

Probe name[a]	Target group[b]	Target site (rRNA positions[c])	Probe sequence from 5′ to 3′	FA%[d] *in Situ*	NaCl[e] (M)
HGC69a	Actinobacteria	23S(1901–1918)	TAT-AGT-TAC-CAC–CGC-CGT	25	0.11
LGC354A	Firmicutes	16S(354−371)	TGG-AAG-ATT-CCC-TAC-TGC	35	0.046
BIF1278	Bifidobacteriaceae	16S(1278–1,294)	CCG-GTT-TTC-AGG-GAT-CC	0	0.9
CP2	*Clostridium perfringens*	16S(199–218)	GCT-CCT-TTG-GTT-GAA-TGA-TG	20	0.165
STRC493	Most *Streptococcus* spp. and some *Lactococcus* spp.	16S(493–511)	GTT-AGC-CGT-CCC-TTT-CTG-G	0	0.9
NONEUB338	Negative control	–	ACTCCTACGGGAGGCAGC	20	0.17

Notes: a: From probe bases [10]. b: Specificity checked using the probe match function at RDPII website. c: *E. coli* numbering [11].
d: Hybridization buffer formamide concentration. e: Washing buffer NaCl concentration.

2.5. Principal component analysis (PCA)

PCA was carried out to identify the optimal bioindicators for the Nanshih River and this was done using Statistica 10.0 (StatSoft Inc., 2011). Each point in the PCA represents a pattern and the distances between points approximate to pattern similarities. The PCA method consists of the following steps: (1) normalization calculated using the bacterial numbers, the bacterial percentages or the ten water parameters and (2) factor analysis using Ward's minimum-variance method. The minimum eigenvalue in the PCA was set to 0.000 in order to examine all principal component (PC) variances. The explanation of the two PCs should be >70% of the two dimension profile.

3. Results and discussion

3.1. Bacterial number of TC, FC and FS

Fig. 1 shows that average TC numbers in the Nanshih River for every month at each of sampling locations. All of data from sampling locations No. 1 to No. 8 are higher than the standard of surface water quality classification A (50 CFU/100 mL) or classification B (5,000 CFU/100 mL). Bacterial TCs are commonly used as an indicator of water quality in a water body. For drinking water purpose, TC numbers are the standard test because their presence indicates contamination of a water supply by an outside source. In this study, TC numbers were in the average range 1.14–4.76 $\times 10^4$ CFU/100 mL, and did not change obviously at any of the sampling locations. This means that TCs as a bioindicator are not suitable for explaining in detail the presence of biological pollutants in the Nanshih River. The greatest variation in TC numbers was found upstream of the Tunho River and included the lowest and highest TC numbers in this river (4.5×10^2 and 2.43×10^5 CFU/100 mL, respectively).

Fig. 2 shows the FC counts in the Nanshih River at every location for each month. There were significant changes in FC counts over the year. About 25% data of FC counts are negative (0 CFU/mL). FC bacteria were found in the highest numbers upstream of the Tunho River and in the Tunho River during most months and ranged from 2.77 to 3.07×10^3 CFU/100 mL. The greatest number of FC bacteria was 9.00×10^3 CFU/100 mL during December in the Tunho River. FC bacteria are a subgroup of the TCs and can be considered to represent specifically bacteria from the gut and feces of warm-blooded animals. Strict growth conditions are required for FCs to grow in an aqueous environment, namely BOD >14 mg/L, temperature >13°C and no chlorination [2]. In this study, the effluent from hot spring resort recreational activity deposits organic pollutants into the sediment of the Tunho River and this allows FCs to survive when the river is warmer (the temperature of the Tunho River ranges as 15.0–28.5°C). The greatest FC count was 7.55×10^3 CFU/100 mL at the SunMoonLight Hot Spring during May, which demonstrates the effect of growth conditions.

Fig. 3 shows that the FS counts ranged from 2.96 to 8.54×10^3 CFU/100 mL at sampling locations No. 3 Tunho River, No. 4 Wulai Weir, No. 5 the SunMoonLight Hot Spring, No. 6 Quchi Weir and No. 8 Bitan. The presence of FS is able to identify faecal pollution and provide information concerning the range and probable origin of any pollution. The greatest numbers of FS bacteria, 1.40–8.50 $\times 10^4$ CFU/100 mL, were detected at these locations during the same month, April. However, about 12.5% of the samples were negative for FS (0 CFU/mL), which shows that the pollution is episodic in nature.

3.2. E. coli and NLFB

E. coli and possible enteric pathogens form a major group within the FC bacteria. Fig. 3 shows that *E. coli* numbers reached 1.05–2.50 $\times 10^4$ CFU/100 mL at various sampling locations, namely upstream of the Tunho River, in the Tunho River and at the SunMoonLight Hot Spring resort. Average bacterial number ranged from 1.35 to 5.42×10^4 CFU/100 mL at six sampling locations. Importantly, the river section of the Tunho River and at Wulai Weir had 2.50×10^2 to 1.54×10^4 CFU/100 mL over the whole year. *E. coli* generally survives longer in water than various intestinal pathogens. The fact that 6% of the samples gave positive results for *E. coli* suggests that human recreational activity might increase the risk of disease due to water contact. Some *E. coli* bacteria can cause gastroenteritis, while others can cause urinary tract infections.

Fig. 4 shows that the highest numbers of NLFBs were reached at Fushan and Bitan (1.13–2.50 $\times 10^5$ CFU/100 mL). The other sampling locations are in the range 2.83–7.15 $\times 10^4$ CFU/100 mL. One major contributor to the presence of *E. coli* and NLFBs might be activity at the hot spring resorts. Negative results (0 CFU/mL) were obtained from 23% of the sites over the year. One group of NLFB are members of the Enterbacteriaceae and include the genera *Proteus*, *Morganella*, *Providencia*, *Edwardsiella*, *Salmonella*, *Shigella* and *Yersenia*. These bacteria can cause waterborne diseases such as dysentery, typhoid and paratyphoid fevers, which are a threat to public health.

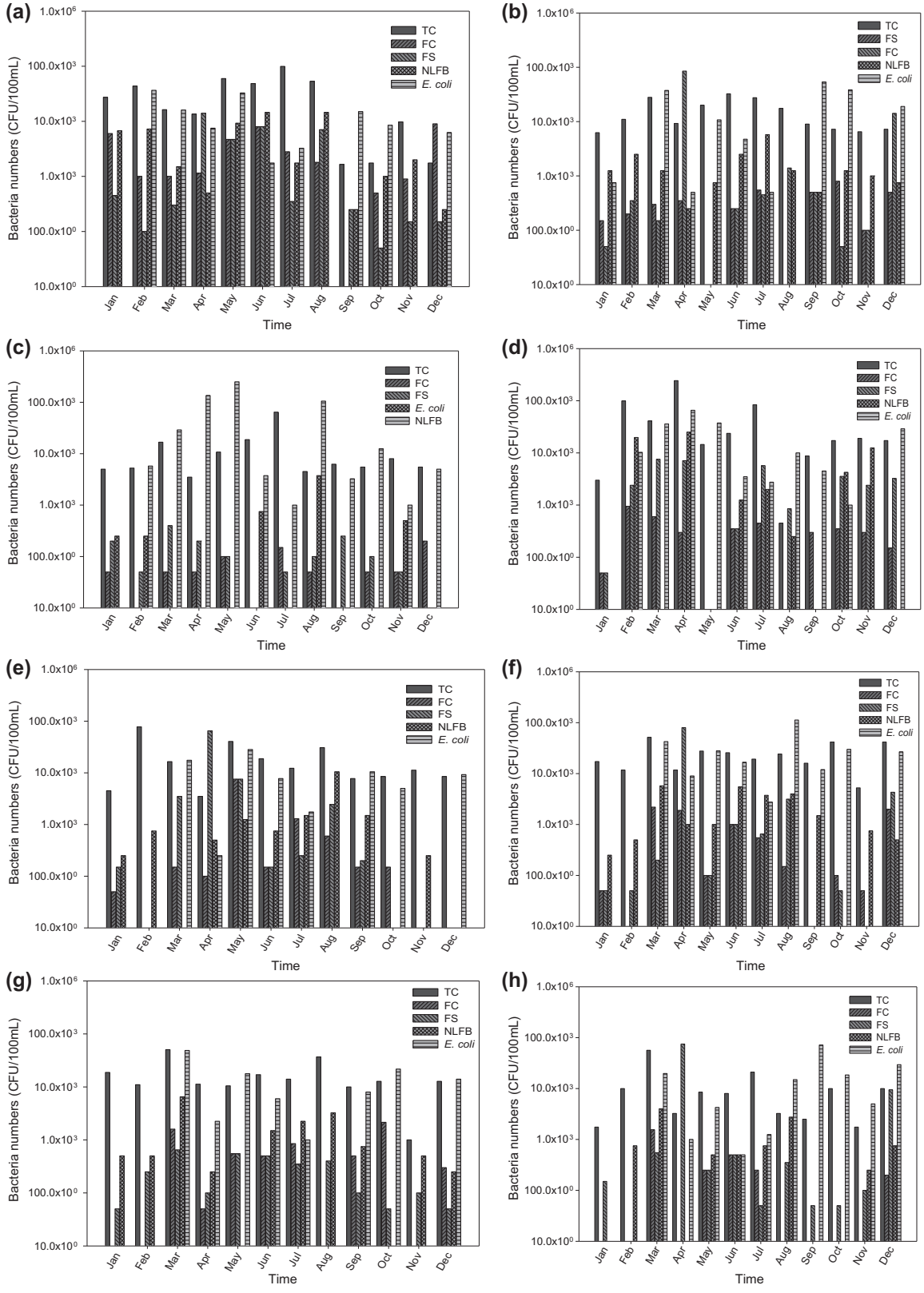

Fig. 2. Variation in bacterial numbers, including TC, FC, FS, *E. coli* and NLFB in the Nanshih River: (a) Fushan; (b) upstream of Tunho River; (c) Tunho River; (d) Wulai Weir; (e) the SunMoonLight Hot Spring; (f) Quchi Weir; (g) Qingtan Weir; and (h) Bitan.

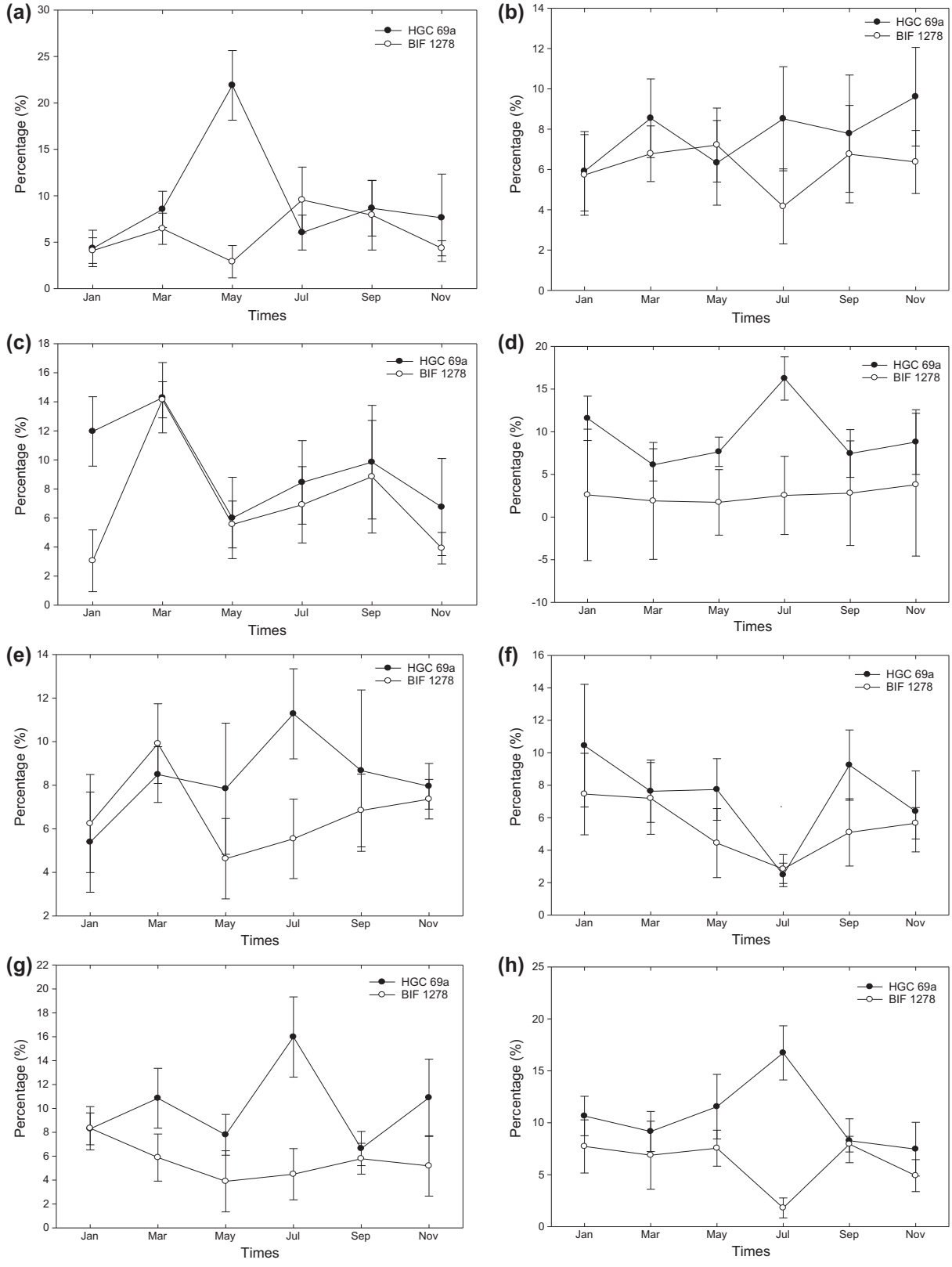

Fig. 3. Variation in the percentage of selected Gram-positive HGC bacteria in the Nanshih River: (a) Fushan; (b) upstream of Tunho River; (c) Tunho River; (d) Wulai Weir; (e) the SunMoonLight Hot Spring; (f) Quchi Weir; (g) Qingtan Weir; and (h) Bitan.

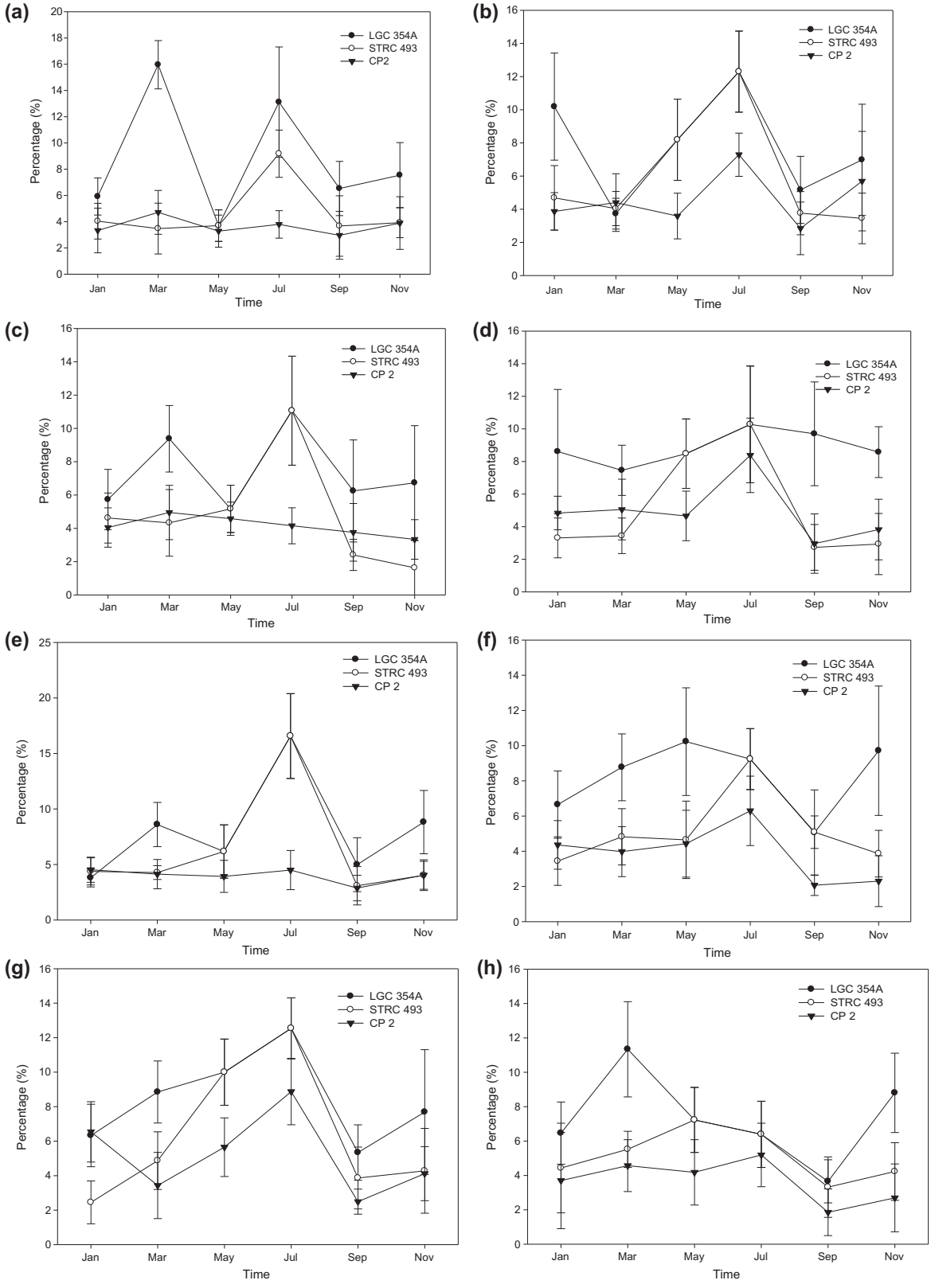

Fig. 4. Variation in the percentage of selected Gram-positive LGC bacteria in the Nanshih River (a) Fushan; (b) upstream of Tunho River; (c) Tunho River; (d) Wulai Weir; (e) the SunMoonLight Hot Spring; (f) Quchi Weir; (g) Qingtan Weir; and (h) Bitan.

3.3. Bacterial community

3.3.1. Phylum

High G + C Gram-positive bacteria (HCG) are classified in the phylum *Actinobacteria*, which includes the representative Order Bifidobacteriaceae in this study. Fig. 3 shows significant differences in Actinobacterial numbers with a range from 7.32 to 10.64% over the sampling locations. The highest percentage for the HCG phylum ranked as follows: 21.89 ± 3.75% at Fushan in May; 15.98 ± 3.36% at Qingtan Weir in July; and 14.28 ± 2.42% at Bitan in July. The low G + C Gram-positive bacteria (LGC) are members of the phylum *Firmicutes*, which is divided into three classes: *Mollicutes*, *Clostridia* and *Bacilli*. Fig. 4 shows that the percentage of LGC ranged from 7.31 to 8.85% over the year. The highest percentage of LGC was 16.57 ± 3.82% at the SunMoonLight Hot Spring in July. The next highest percentages of LGC were 15.96 ± 1.84% and 13.11 ± 4.21% at Fushan in March and July, respectively.

3.3.2. Selection of species as bioindicators

Fig. 3 shows that the percentage of bacteria hybridizing to the BIF1278 probe, which is used to determine the population of *Bifidobacterium* spp. present, including *B. breve, B. bifidum, B. pseudolongum*, *B.bifidum and B. longum* in human fecal samples, [14,18–20] ranged widely in the Nanshih River, with the lowest value being 1.80 ± 0.96% and the highest being 14.14 ± 1.24%. The highest count was for the Tunho River in March. The average percentages for BIF1278 probe hybridizing bacteria ranged from 5.45 to 7.07%. The *Bifidobacteria*/HGC ratio ranged from 55.57 to 80.20%. The higher percentages of *Bifidobacterium* spp. were measured at specific times over several sampling locations, which suggest that human sources are associated with point or nonpoint pollution. Point source of instant fecal pollution in this river may be ascribable to human domestic sewage in this river. Such point-source pollution was investigated and could be due to the present of 553 hot spring resorts, hotels and guest houses, 180 restaurants and several campsites in the area.

C. perfringens is usually a good water quality indicator, and it has been used as an indicator of recreational water quality when determining the proximity and nature of sources of fecal pollution. *C. perfringens*, which can survive a very long time in polluted river water, is used to identify point sources of sewage pollution. *C. perfiringens* bacterial counts are the most suitable indicator for the inactivation and removal of viruses during drinking water treatment [21] and they also appear to have a positive relationship to the inactivation of cysts and oocysts. Fig. 3 shows that *C. perfringens*, as measured by the CP2 probe, is present and the proportion of these bacteria range from 1.85 ± 1.36 to 8.87 ± 1.92%. *C. perfringens* reached >8% at two sampling sites; these were the Qingtan Weir and Wulai Weir during July. The average percentage for CP2 positive bacteria ranged from 3.66% to 5.18% over all sampling sites. The CP2/LGC ratio showed that CP2 positive bacteria were dominant at 41.64–61.28%.

Members of the genera *Streptococcus* and *Enterococcus* in the Streptococcaceae family cause major human infections. The STRC493 probe is able to detect most *Streptococcus* spp. and in this study was used to pinpoint various *Streptococci* and *Enterococci* [8]. *Streptococcus* spp. such as *S. faecalis* and *Streptococcus druran* are good bioindicators of short term human fecal pollution [2,3]. *S. faecalis* is an opportunistic pathogen that causes urinary tract infections and endocarditis. Fig. 4 shows that bacteria positive for the STRC493 probe in the Nanshih River. *Streptococcus* spp. can be seen to be widely distributed in some river sections. The highest percentage of *Streptococcus* spp. at 16.65%, which was measured at the SunMoonLight Hot Spring during July, while the lowest percentage at 1.63% was measured in the Tunho River during November. The average percentage for all river sections was calculated to the range from 4.67 to 6.42%. The STRC493/LGC ratio showed that STRC493 positive bacteria were dominant at 53.05–78.24%.

3.4. Evaluation of the optimal point-source bioindicators

The optimal bioindicators for the Nanshih River were evaluated using water parameters, bacterial numbers and the percentage of specific bacteria present at the various sampling points. Statistical PCA analysis was applied to explain the data trends because the raw experimental data is highly complex. Human activities were investigated in order to clarify the possible sources of biological pollution.

3.4.1. Evaluation on water parameters

Fig. 5(a) shows the two-dimension PCA profile created using the presence of ten physical/chemical water parameters at the various sampling points. Four groups are presented on the PCA profile and these explain 97.76% of the variation. Group 1 (a#1) seems to represent nonpollution control at the No. 1 Fushan site. Group 2 (a#2) covers the major point source of pollution caused by hot spring resort activity, namely

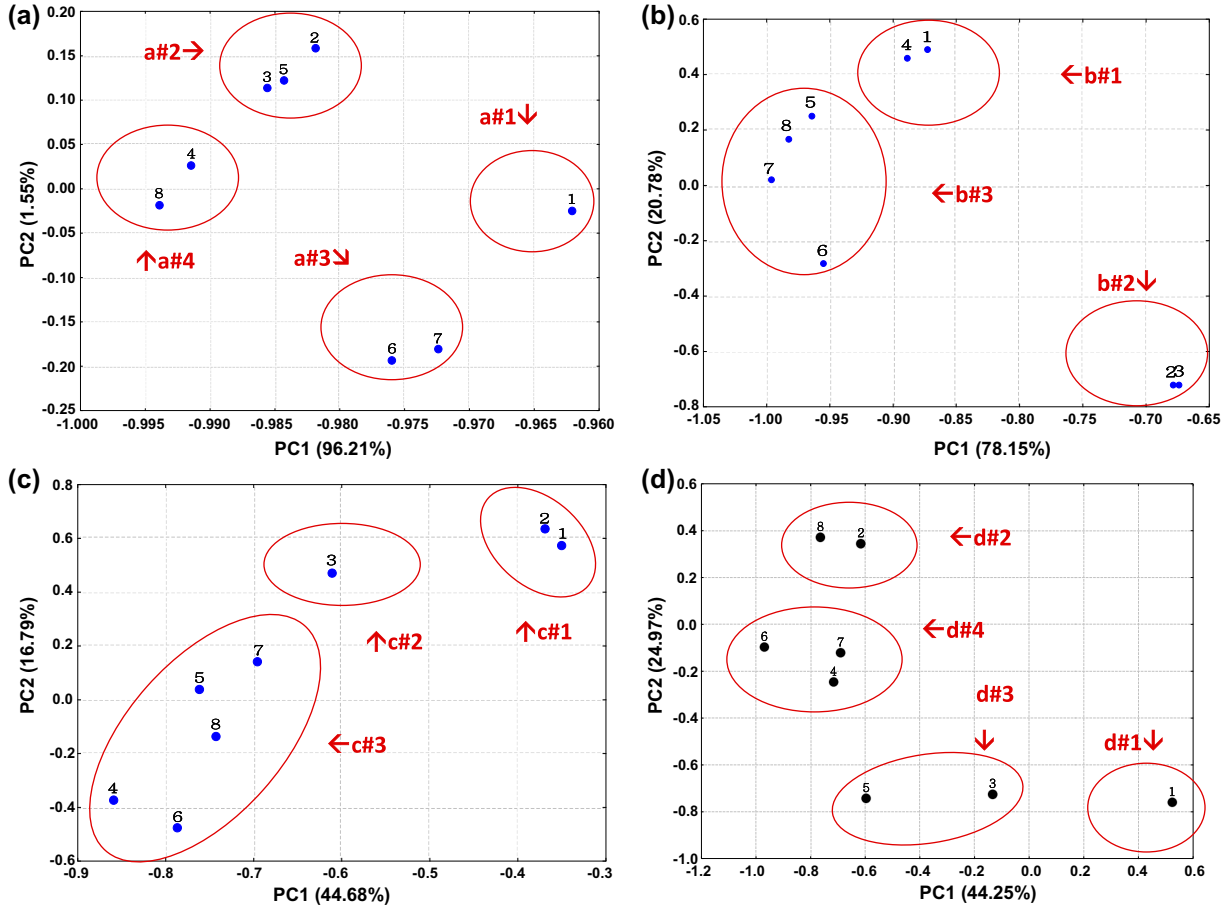

Fig. 5. Two-dimensional PCA profile based on (a) ten water parameters (PC1 + PC2 = 97.76%); (b) the RPI-M parameters (98.93%); (c) bacterial numbers (PC1 + PC2 = 61.47%); (d) *Bifidobacterium* spp. (PC1 + PC2 = 69.22%) sampling location numbers are marked on the profile: No.1: Fushan; 2: upstream of Tunho River; 3: Tunho River; 4: Wulai Weir; 5: the SunMoonLight Hot Spring; 6: Quchi Weir; 7: Qingtan Weir; and 8: Bitan.

at No. 2 upstream of the Tunho River, No. 3 the Tunho River and No. 5 the SunMoonLight Hot Spring. If the other two groups are examined, group 3 (a#3) consists of No. 6 Quchi Weir and No. 7 Qingtan Weir, while group 4 (a#4) is made up of No. 4 Wulai Weir and No. 8 Bitan. Statistical analysis of these groups does not provide enough information to explain the relationships between point-source pollution and our dataset. Most water parameters remain steady over the various different river sections and some experimental values are below the method detection limits.

The current assessment of river quality used by the Taiwan EPA is based on a comprehensive index known as the river pollution index (RPI). The RPI is an integrated indicator that can be used to determine the level of pollution of a river. Table 2 selects water parameters and carries out an RPI calculation for the Nanshih River. The DO ranges from 4.5 to 9.8 mg/L, which kept high for all months. The other three water parameters, SS, BOD and NH_4^+–N, have relatively low values. No significant change in these parameters is identifiable. The median river pollution index (RPI-M) is calculated as the median of the above water parameters in river and is less than 2.0 at all sampling locations. This means that the Nanshih River is classified as being a nonpolluted or slightly polluted river over all river sections. Fig. 5(b) shows the PCA profile of the RPI-M, which explains 98.93% of the variation. This cannot be used to try and identify parts point sources of pollution based on geographic distribution. Three groups made up the PCA profile. Group 1(b#1) consists of the upstream part of the Nanshih River, namely the sites No. 1 Fushan and No. 4 Wulai Weir. Group 2 (b#1) consists of the branch river, namely the sites on the Tunho River, including No. 2 upstream of the Tunho River and No. 3 Tunho River. Group 3 (b#3) covered all sampling locations downstream on the Nanshih River, namely No. 5 the SunMoonLight Hot Spring, No. 6 Quchi Weir; No. 7 Qingtan Weir and No. 8 Bitan.

Table 2
Selected water parameters and median RPI in the Nanshih River

Sampling location number	DO (mg/L)		BOD (mg/L)		SS (mg/L)		NH_4^+–N (mg/L)		(RPI-M)
	M	*R*	*M*	*R*	*M*	*R*	*M*	*R*	
1	8.10	5.20–9.40	0.70	0.30–2.20	3.10	0.5–51.9	0.0150	0.00019–0.060	A
2	7.80	4.50–9.80	0.80	0.30–1.30	1.20	0.6–3.40	0.0195	0.00086–0.089	A
3	7.95	6.20–9.10	0.70	0.20–1.70	1.50	0.6–5.20	0.0305	0.00116–0.089	A
4	7.90	5.20–9.20	0.75	0.50–3.10	4.35	1.4–47.8	0.0225	0.0040–0.0700	A
5	7.80	6.20–9.10	0.70	0.50–2.60	2.20	0.35–29.7	0.0200	0.0033–0.0580	A
6	7.85	5.10–8.80	0.80	0.50–2.80	2.50	0.60–31.6	0.0180	N.D.-0.073	A
7	7.90	5.60–9.10	1.70	0.60–2.40	3.50	0.40–34.4	0.0240	0.00021–1.050	A
8	7.20	6.20–8.50	0.80	0.50–3.40	2.70	0.80–24.9	0.0335	0.00306–0.1550	A

Note:1: Values are the results of 18 test samples from March to December, 1995–1996, which are published on the website of the Taiwan EPA. 2: *M* = median; *R* = range. 3: Sampling Location, No. 1: Fushan; No. 2: upstream of Tunho River; No. 3: Tunho River; No. 4: Wulai Weir; No. 5: the SunMoonLight Hot Spring; No. 6: Quchi Weir; No. 7: Qingtan Weir; and No. 8: Bitan. 4: RPI-M: Median river pollution index.

3.4.2. Evaluation by bacterial numbers

Fig. 5(c) shows the PCA profile calculated using all bacterial numbers obtained by growth on selective and differential media and these explain 61.47% of the variation. Three groups, ranging from upstream to downstream locations, make up the two-dimensional PCA profile. Group 1 (c#1) covers the two upstream sampling locations, namely No. 1 Fushan and No. 2 Upstream of Tunho River. Most of the area that makes up this group is restricted for ecological protection purposes and is covered by original forest. Amount of pollutant effluent reaching the river in this area is small because there are few human activities. Previous research has shown that there are two types of the point-source wastewater affecting the Nanshih River; these are domestic sewage and hotspring resort wastewater. In 2000, about 61% of all domestic sewage was collected in this area. About 71% of domestic sewage effluent (about 580 CMD) is treated by the PinLin wastewater treatment plant, which was set up in this river basin in 2001. Group 2 (c#2) consists of No. 3 Tunho River and No. 5 the SunMoonLight Hot Spring sampling sites and the pollution in this river section can be mostly ascribed to hotspring resort wastewater. Hotspring operators usually combine domestic wastewater with hotspring wastewater when releasing it into the river. The qualitative and quantitative characteristics of the water body and ecosystem are obviously affected. Group 3 (c#3) cover of the remaining five sampling locations (Nos. 4–8). Many residential buildings are located in these river sections and an incomplete wastewater collection system allows some domestic wastewater to be released into the river. In addition, some tourists stay in home stay accommodation, which generate extra domestic wastewater, which is calculated as 50 L/d per person.

3.4.3. Evaluation using specific species probes

Three specific probes were used individually to evaluate whether one or more can be used as bioindicators for the Nanshih River. Fig. 5(d) identifies four groups within the BIF probe two-dimensional PCA profile and these explain 69.23% of the variation. The STRC493 and CP2 PCA profiles are not shown. This PCA profile clearly identifies specific sources of pollution. *Bifidobacterium* spp. make up a major part of the intestinal microflora of humans, as well as that of other warm blooded animals. *Bifidobacterium* spp. have been shown to be a better indicator of recent fecal contamination in tropical freshwaters than either *E. coli* or FCs [18]. A simple and specific protocol based on the detection of certain *Bifidobacterium* spp. is able to discriminate between human and animal fecal pollution [20]. The behaviour of the four PCA groups is able to pinpoint specific pollutants. Group 1 (d#1) consists of only the No. 1 Fushan sampling that is located upstream of the Nanshih River. Water quality is identified as the background level. This is true even though a few Taiwanese aborigines inhabit the Fushan Area. Artificial pollution includes some small scale waste water treatment plants and limited agriculture activity such as farms growing tea, aquaculture businesses and a livestock (chicken) farm. Group 2 (d#2) consists of two sampling locations,

namely No. 2 Upstream of Tunho River and No. 8 Bitan. The factors affecting the characteristics of these two locations involve recreational activity. Recreational activity began to use the Nanshih River and her riversides after the Taiwan government began two day weekends. Tourists take part in eco-tourism, cultural activities, and water sports along this river and these activities are increasing year by year. It was found that 2,713 tourists per day entered the Taipei Water Supply Protected Watershed in 2002. Recreational wastewater is estimated to have 135.6 CMD, which will contribute about 6% of the wastewater effluent. Many activities, such as whitewater rafting, stream trekking, snorkeling, swimming, fishing and camping, involve contact with the water body and these involve the risk of recreational water illnesses. The definition of contacting the water body includes swallowing, breathing or having contact with contaminated water from the river.

Group 3 (d#3) covers the hot spring water resort facilities at sampling locations No. 3 the Tunho River and No. 5 the SunMoonLight Hot Spring. There are three hot spring outcrops in the Nanshih River basin that allow hot spring resort activities. Taiwan EPA has estimated each person used 0.57–0.62 CMD per person when hot spring bathing (2004). A large amount of hot spring sewage is generated by hot spring facilities along the riverside, which include resorts, hotels, guest houses, spas and camps. An investigation of the pollution found 43 hot spring facilities in the area of the No. 3 Tunho River sampling point and 35 hotspring facilities in the area of the No. 5 SunMoonLight Hot Spring. In addition there were 28 restaurants within the group 3 area. Hot spring wastewater and tourist sewage are collected together and therefore effluent with possible pollutants is discharged directly into the Nanshih River. According to an investigation by the Taiwan EPA in 2003, the wastewater effluent consists of 46% domestic wastewater and 48% hot spring wastewater in the Nanshih River area. The pollution in the Wulai hot spring area, which includes sampling locations Nos. 3 and 5, was estimated to be 86% domestic wastewater, 11% hot spring wastewater, 2% restaurant wastewater and 1% recreational wastewater. Group 4 (d#4) consists of three similar hydraulic entities, namely weirs; these are No. 4 the Wulai Weir, No. 6 the Quchi Weir and No. 7 the Qingtan Weir. Weir barriers across the Nanshih River are designed to alter the flow characteristics of the river water to allow the generation of electricity by hydroelectric power plants. The water quality characteristics are not the same as at other locations along the river. For example, the weirs artificially reduce the upstream water velocity and river volume, which leads to an increase in silt deposition. Lower DO values are usually measured for the Group 4 areas. Moreover, river water is collected at Qingtan Weir for use as drinking water as part of the Taipei Water Supply Protected Watershed. The results of this study imply that there may be an increase in risk to human health because more coagulant and disinfectant will be required in order to process the water passing through the water supply treatment plant.

4. Conclusions

The Nanshih River has many point source biological pollutants due to human activity; these are present both upstream and downstream and include hotspring resorts and agricultural activity. Best management practice for the river catchment area is to develop approaches that are able to effectively decrease the risk of recreational water illnesses. Some river sections are used as a drinking water resource and these need more restrictions on them in terms of human development. Wastewater and sewage effluent release into the river water body should be strictly controlled by constructing a complete system of wastewater collection and treatment.

The statistical approach embodied in two-dimensional PCA is a powerful and effective method and was able to pinpoint the best bioindicator by evaluating different water parameters in the Nanshih River. In this study, it is obvious that the ten physical and chemical water parameters (presented by RPI-M) cannot be used to specifically identify point-source pollution along the Nanshih River. Even bacterial TC numbers are often above the requirement needed for public health safety at specific times and at specific locations in the Nanshih River. Other bacterial measures, such as FC, FS, NLFB, and *E. coli* counts can be a regular part of the environmental monitoring of water quality. Three areas of the river, from upstream to downstream, were classified by PCA profiling using the bacterial numbers. Moreover, the use of 16s rRNA probes and FISH provides an easy and rapid biomolecular approach that can replace traditional incubation growth on media. In this context, measurement of specific Gram-positive bacterial species, including Bifidobacteriaceae, Streptococcaceae and Clostridiaceae from warm blood human and animals counts is able to provide a good genetic fingerprinting. This allows the monitoring of the health risk to tourists in this and similar rivers where there are multiples uses of the water body, such as a drinking water resource, as a hotspring resort input/output and for recreational activity. *Bifidobacterium* spp. was found to be a

representative bioindicator in the Nanshih River and was able to classify the river into four pollutant-characterized areas based on the PCA profiles.

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