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An alternative method to quantify 2-MIB producing cyanobacteria in drinking water reservoirs: Method development and field applications

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ABSTRACT

2-Methylisoborneol (2-MIB) is a commonly detected cyanobacterial odorant in drinking water sources in many countries. To provide safe and high-quality water, development of a monitoring method for the detection of 2-MIB-synthesis (*mib*C) genes is very important. In this study, new primers MIBS02F/R intended specifically for the *mib*C gene were developed and tested. Experimental results show that the MIBS02F/R primer set was able to capture 13 2-MIB producing cyanobacterial strains grown in the laboratory, and to effectively amplify the targeted DNA region from 17 2-MIB-producing cyanobacterial strains listed in the literature. The primers were further coupled with a TaqMan probe to detect 2-MIB producers in 29 drinking water reservoirs (DWRs). The results showed statistically significant correlations between *mib*C genes and 2-MIB concentrations for the data from each reservoir ($R^2 = 0.413–0.998$; $p < 0.05$), from all reservoirs in each of the three islands ($R^2 = 0.302–0.796$; $p < 0.01$), and from all data of the three islands ($R^2 = 0.473–0.479$; $p < 0.01$). The results demonstrate that the real-time PCR can be an alternative method to provide information to managers of reservoirs and water utilities facing 2-MIB-related incidents.

1. Introduction

Reservoirs are important drinking water sources in Taiwan and many other countries. Due to high nutrient concentrations, many of these reservoirs are facing serious cyanobacterial problems. Some cyanobacterial species may produce secondary metabolites such as cyanotoxins or off-flavor compounds, posing additional health risk and esthetic problems to consumers. 2-Methylisoborneol (2-MIB) and trans-1,10-dimethyl-trans-9-decalol (geosmin) are the two earthy/musty odorants of cyanobacteria most commonly detected in drinking water sources in Taiwan and many other countries (Jüttner and Watson, 2007; Lin et al., 2012; Tsao et al., 2014; Tung et al., 2008; Watson, 2003; 2004). The two chemicals can be detected by humans at as low as ~10 ng/L (Cook et al., 2001; Watson et al., 2008). Although there is no evidence for a serious health threat of 2-MIB and geosmin to humans or aquatic animals (Dionigi et al., 1993; Jüttner and Watson, 2007), many consumers view the earthy/musty 2-MIB/geosmin laden water to be unsafe to drink. Therefore, a method to quantify the potential 2-MIB and geosmin producers in drinking water sources is warranted to manage the water sources and the off-flavor problems.

2-MIB and geosmin may be produced by cyanobacteria (Izaguirre et al., 1982; Jüttner, 1987; Wu and Jüttner, 1988), actinomycetes (Gerber, 1979; Pollak and Berger, 1996; Schöller et al., 2002), myxobacteria (Dickschat et al., 2007), and fungi (Boerjesson et al., 1993; Mattheis and Roberts, 1992). However, cyanobacteria are considered as the most important microorganisms for the production of the two odorants (Butakova, 2013; Jüttner and Watson, 2007; Li et al., 2012; Sun et al., 2013; Tung et al., 2008; Watson et al., 2008). More than 40 species of cyanobacteria, both benthic and planktonic strains, have been confirmed as 2-MIB producers; these include *Lyngbya*, *Oscillatoria*, *Phormidium*, *Planktothricoides*, *Planktothrix* and *Pseudanabaena* (Giglio et al., 2011; Izaguirre and Taylor, 2004; Jüttner and Watson, 2007; Kakimoto et al., 2014; Sun et al., 2013; Suurnäkki et al., 2015; Wang et al., 2011, 2015a). Because of the difficulty to remove 2-MIB and geosmin by the conventional drinking-water treatment process (Lin et al., 2002; Srinivasan and Sorial, 2011), it is essential to control their production in the source water. Conventionally, the cyanobacterial species in water samples are quantified by microscopy, and the concentrations of the two odorants are analyzed by gas chromatography (GC). The former process is time-consuming as well as requiring specially trained personnel. The method also disallows one to separate odorous from non-odorous cyanobacteria. Meanwhile, although the GC method speciates and quantifies the odorants in water, it offers no information about the

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producers in the source water, and requires specialized equipment.

The genetic information regarding the 2-MIB synthesis has been developed for both actinomycetes (Komatsu et al., 2008) and cyanobacteria (Giglio et al., 2011; Wang et al., 2011). 2-MIB is known to be a methylated monoterpene synthesized by terpene synthases (Brock et al., 2013; Oldfield and Lin, 2012). The biosynthesis of 2-MIB includes a conversion of geranyl diphosphate (GPP) to methyl-GPP by GPP C-methyltransferase, and a cyclization of methyl-GPP to 2-MIB by 2-MIB synthase (Brock et al., 2013; Komatsu et al., 2008).

Recently, bio-molecular methods for synthesis genes have been used to detect toxin- and odor-producing cyanobacteria in freshwater bodies (Dittmann et al., 2013; Giglio et al., 2011; Michinaka et al., 2012; Rasmussen et al., 2008; Sivonen and Börner, 2008; Suurnäkki et al., 2015; Tsao et al., 2014; Wang et al., 2011, 2015b; Yen et al., 2012). A real-time polymerase chain reaction (PCR) has been developed to quantify the monoterpene cyclase gene produced by 2-MIB-producing cyanobacteria in laboratory systems (Giglio et al., 2011; Wang et al., 2011) and in reservoir samples (Wang et al., 2015b). In these studies, a good correlation was established between the concentrations of 2-MIB and its synthesis gene. However, only limited field samples were analyzed for one reservoir (Wang et al., 2015b).

In this study, a genetic method to quantify the 2-MIB-synthesis gene of cyanobacteria was developed and applied to 29 drinking water reservoirs to determine its utility as a surrogate measure of 2-MIB occurrence. Specific primers were designed for the 2-MIB-synthesis (*mib*C, referred as mic in Wang et al., 2015) gene sequences based on 14 monoterpene cyclase gene sequences (Fig. 2). The primers were checked against 17 cyanobacterial *mib*C gene sequences, and then integrated into a real-time PCR scheme and applied in the quantification of the genes in the samples collected from 29 reservoirs in Taiwan. The real-time PCR results of *mib*C gene concentrations were then compared and correlated with 2-MIB concentrations measured with GC.

2. Material and methods

2.1. Isolation and identification of 2-MIB producing cyanobacterial strains

Cyanobacterial samples with earthy-musty odorants were collected from a reservoir in Siju Island, Matsu, Taiwan in 2012. Individual filaments of cyanobacteria were selected by use of the capillary pipette method (Andersen and Kawachi, 2005) under a microscope (BX51, Olympus, Japan). The filaments were washed 10 times with the ASM medium (Rippka, 1988) to remove other microorganisms; they were then placed into 25-cm^2 cell-culture flasks preloaded with ASM and fitted with a vent cap (No. 707003, Nest Biotechnology, China). The flasks were incubated in ASM at 25 °C and under a 12 h/12 h light/dark cycle with a 19.8 μmol m^{-2} s^{-1} light intensity for 30–60 days.

The isolates were initially checked for the ability to produce 2-MIB by a GC coupled with a mass spectrometry (MS) (6890/5973, Agilent, USA). After the 2-MIB producing capability was confirmed, the isolated strains were then identified based on their morphology using a microscope (BX51, Olympus, Japan) and by 16S rDNA sequencing. The 16S rDNA was amplified with 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1494R (5′-GTACGGCTACCTTGTTACGAC-3′) as the forward and reverse primers (Taton et al., 2003), coupled with amplification on a 96-well thermal cycler (C1000™, Bio-Rad Corp., California, USA). A PCR mixture includes 5 μL of the extracted DNA solution, 2 μL of 10 × Ex Taq™ buffer, 200 μM of dNTP mix, 0.2 μM of the forward and reverse primers, and 0.1 μL of TaKaRa Ex Taq™ DNA polymerase (Code No. RR01A, Takara Biotechnology, Japan), with the volume being made up to 20 μL using sterile deionized water. The PCR mixture was applied for the PCR experiment. The PCR experiment was executed with a pre-incubation at 94 °C (for 5 min), a 30-cycle denaturation at 98 °C (10 s), an annealing at 55 °C (30 s), and an extension at 72 °C (1 min), and a final extension at 72 °C for 10 min. The PCR product was purified using Amicon Ultra-0.5 Centrifuge Filter Device (Millipore, USA) and then sent to Mission Biotech Company (Taipei, Taiwan) for sequencing.

2.2. Cyanobacterial strains and mibC genes

To test the specificity of the selected primers, 17 cyanobacterial strains and 13 sequences of the *mib*C gene were used in this study, as listed in Supplementary information (SI) Tables S1 and S2. Among the cyanobacterial strains used, nine were isolated in Taiwan, six were purchased from the Japan NIES Collection (the Microbial Culture Collection, National Institute for Environmental Studies, Japan), two were isolated by the Australian Water Quality Center (AWQC) (Adelaide, Australia), and one was purchased from the Pasteur Culture Collection Center (Paris, France). All of the cyanobacterial strains were incubated in ASM medium at 25 °C with a 12 h/12 h light/dark cycle at a light intensity of 19.8 μmol m^{-2} s^{-1}. For the sequences of *mib*C gene, only 13 sequences were available from the National Center for Biotechnology Information (NCBI) database; they were all used in the specificity analysis.

2.3. Study sites

Water samples were collected from 29 reservoirs in Taiwan. Sample sources include 10 drinking water reservoirs (DWRs) in Taiwan main island, and 9 DWRs in each of Taiwan's two off-shore islands, Kinmen islands and Matsu islands. The sampling locations for the reservoirs were all near the water intakes of the associated water treatment plants. The locations of all studied DWRs are shown in Fig. S1 (SI).

2.4. Extraction of DNA from cyanobacterial cells

Cyanobacteria-laden water samples (10 mL) were first concentrated by centrifugation before DNA extraction. A desktop centrifuge with a 24-well rotor (Hettich Mikro 20 Microfuge, Biotech Equipment, California, USA) rotating at 16,060 rcf for 5 min was used to separate the cyanobacterial cells from the samples. After centrifugation, the supernatant was removed and the cyanobacterial pellets remained were re-suspended in 90 μL of 1 × TE buffer and 10 μL of Lysozyme (50 mg/mL, Invitrogen, Life Technologies, California, USA) and transferred to a 2 mL TruCool® cryovials (BCS-2401, Biocision®, California, USA). To lyse the cyanobacterial cells, the sample cryovials were placed into liquid nitrogen for 30 s, followed by placing them into a water bath at 37 °C for 1 min. After this freezing-thawing procedure was repeated 3 times, the samples were incubated at 37 °C in a water bath for 10 min. Then, DNA was extracted from the lysed cyanobacterial samples using Illustra™ triplePrep kit (GE Healthcare, UK) according to the suggested protocol. The extracted DNA was finally eluted with 100 μL of the elution buffer for subsequent real-time PCR quantification.

2.5. Detection and quantification of mibC gene

The primers and their target DNA segments for this study are summarized in Table 1. Quantification of *mib*C gene was performed with a real-time PCR device (Smart Cycler®II; Cepheid, California, USA). The PCR solution was a mixture of 2 μL of the

Table 1
The list of oligonucleotides employed in this study for 2-MIB-producing cyanobacteria.

Primer/Probe name	Sequence (5′–3′)	Product size (bp)	Tm (°C)
MIBS02F	ACCTGTTACGCCACCTTCT	307	63.2
MIBS02R	CCGCAATCTGTAGCACCATG		63.1
MIB	FAM/ ACGACAGCTTCTACACCTCC/ BHQ_1		63.1

extracted DNA solution, 12.5 μL of 2 × qPCR BIO Probe Mix No-ROX kit (PCR Biosystems, UK), and 0.1 μM of each primer and probe, with the volume being made up to 25 μL using sterile deionized water. The real-time PCR protocol includes pre-incubation at 95 °C (for 300 s), a 40-cycle denaturation at 95 °C (10 s), and an annealing/extension at 60 °C (20 s). At the end of the extension, the fluorescence intensity was recorded at 530 nm from the FAM channel. The threshold cycle (Ct) values were determined using Smart Cycler® software version 2.0 (Cepheid, California, USA).

2.6. Sequence analysis of mibC gene

The 96-well C1000™ thermal cycler was used to perform PCR, with a mixture consisting of 5 μL of the extracted DNA solution, 5 μL of 10 × Ex Taq™ buffer, 200 μM of dNTP mix, 0.2 μM of the two primers, and 0.25 μL of TaKaRa Ex Taq™ DNA polymerase, with the volume being made up to 50 μL using sterile deionized water. The PCR protocol includes a pre-incubation at 94 °C (for 5 min), a 30-cycle denaturation at 98 °C (10 s), an annealing at 56 °C (30 s), and an extension at 72 °C (1 min), and a final extension at 72 °C for 10 min.

The PCR products were cloned by use of a commercial kit (TOPO® TA Cloning®, Invitrogen, Life Technologies, California, USA) following the suggested protocol. The DNA sequencing of cloned inserts was determined by Mission Biotech Company, Taipei, Taiwan. Similarity searches were conducted using the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) with the NCBI database.

2.7. Measurement of 2-MIB

2-MIB was measured for both the laboratory cultures and the water samples collected from the DWRs. Before the analysis, the samples were filtered using a glass fiber filter (Grade GF-75, Advantec, Japan) to separate dissolved 2-MIB from cell-bound 2-MIB. Both the unfiltered and filtered samples were analyzed, with the former representing the total 2-MIB (sum of cell-bound and dissolved phase) and the latter the dissolved 2-MIB.

The analytical procedure for 2-MIB followed that of Lin et al. (2003). In brief, a head-space solid phase micro-extraction (HS-SPME), using a PDMS fiber (No.57328-U, Supelco, USA), was used to concentrate the samples, and a GC/MSwas used to quantify 2-MIB. Detailed experimental procedures are provided in Lin et al. (2003).

2.8. Statistical analysis

The field data obtained in this study were analyzed for the correlation between 2-MIB concentrations and *mib*C gene copies of the samples. In addition, the 95% prediction intervals for the linear regressions were calculated with Eq. (1) (Montgomery et al., 2015),

$$\hat{y} \pm t^*_{n-2} s \sqrt{1+\frac{1}{n}+\frac{(x^*-\bar{x})^2}{(n-1)s_x^2}} \quad (1)$$

where $\hat{y}$ is predicted 2-MIB concentration from linear regression, t^*_{n-2} is the *t*-value for 95% (two-tailed), n-2 is the degree of freedom, s is the standard deviation of the 2-MIB concentration, n is the sample size, and $\bar{x}$ and s_x is the mean and the standard deviation of gene copy number per sample volume, respectively. The regression analysis was conducted with SPSS Statistics 17.0 software (IBM, New York, USA).

3. Results and discussion

3.1. Isolation and identification of 2-MIB producing strains

In February 2012, a high level of 2-MIB was detected in Tsair-Pu-Wo Reservoir (TPWR) and its water treatment plant (WTP) in Siju Island, one of the Matsu islands in Taiwan. Water samples from the reservoir and the processed water of the WTP were analyzed with the results shown in Fig. 1. As noted, the total 2-MIB (cell-bound + dissolved) concentration in TPWR was 21,500 ng/L, about 2200 times the odor threshold concentration (OTC, 10 ng/L) (Cook et al., 2001; Watson et al., 2008), while that of the WTP raw water was about 3300 ng/L. The sedimentation process removed about 50% of 2-MIB, reducing the level of 2-MIB to 1750 ng/L. As a fraction of cyanobacteria may be removed by sedimentation, the reduction of 2-MIB is probably due to the removal of cell-bound cyanobacteria. After sedimentation, the processes did not significantly remove the dissolved 2-MIB. Following filtration, 2-MIB rose to 3790 ng/L in finished water, due primarily to an increase of the cell-bound 2-MIB. As cyanobacteria were found in finished water (3220 cells/mL), it was suspected that cyanobacteria were present in the finished water tank.

To better understand the 2-MIB issue in the reservoir and WTP, isolation and identification of 2-MIB-producing microorganisms were conducted for TPWR. In the reservoir samples, the dominant cyanobacteria were filamentous in shape, and five strains were isolated and grown in laboratory cultures. Among the isolated strains, two were confirmed to be 2-MIB producers via a GC/MS analysis. The isolated 2-MIB-producing strains were then identified by their morphology using a microscope. Fig. S2 shows the photos for the 2 strains. The 2 strains are present in both planktonic phase and benthic phase in the culture receiving no agitation.

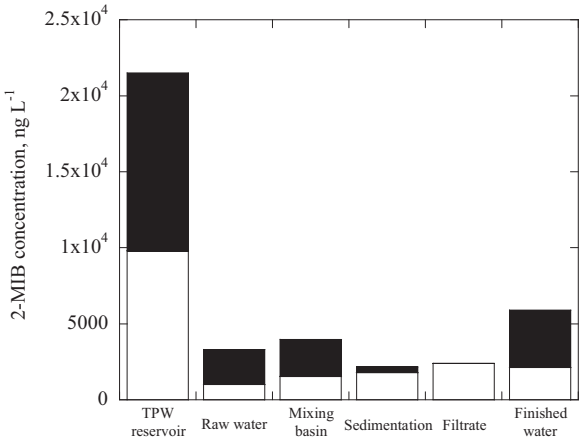

Fig. 1. The cell-bound and dissolved 2-MIB concentration in the processes of Siju water treatment plant in Feb. 2012. ■ represents the 2-MIB concentration in cell-bound phase; □ represents the 2-MIB concentration in dissolved phase.

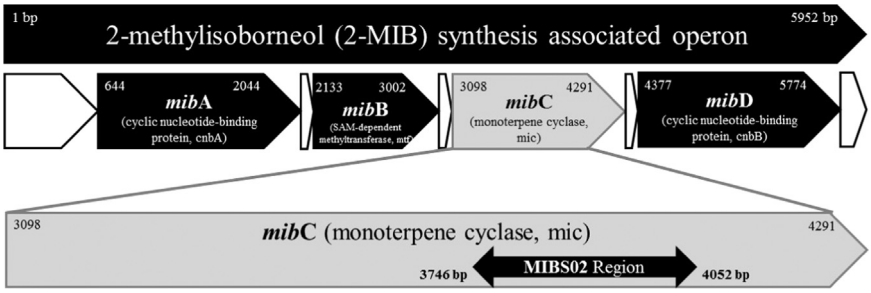

Fig. 2. Primer positions and alignment of the amplicon form standard strain on *Pseudanabaena* sp. dqp15 2-methylisoborneol (2-MIB) synthesis associated operon, complete sequence (HQ830028.1).

The isolated samples were analyzed for their genetic properties using the 16S rDNA sequence analysis. The 2 strains exhibited 99% similarity and *Pseudanabaena galeata* was identified based on the NCBI database. However, the genetic analysis using internal transcribed spacer (ITS) region showed that the two strains are different from the strains listed in the database. Therefore, the 2 isolated strains were named as *Pseudanabaena galeata* TWNCKU13 and *Pseudanabaena galeata* TWNCKU14.

3.2. Method development for quantification of the mibC genes

3.2.1. Primer selection

To quantify the *mib*C genes, a primer set (MIBS02) with TaqMan probe, detailed in Table 1, was designed based on the monoterpene cyclase of the 2-MIB synthesis-associated operon of the standard strain, *Pseudanabaena* sp. dqp15 (HQ830028.1) (Fig. 2). The primer set was further tested for specificity using other 2-MIB producers. As shown in Table S2, a total of 17 cyanobacterial strains from 9 genera, including *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Leptolyngbya*, *Microcystis*, *Oscillatoria*, *Planktothricoides*, *Planktothrix*, and *Pseudanabaena*, were first cultured in the laboratory and tested for 2-MIB-producing capability and the *mib*C genes. Among the tested strains, four of them were identified to be 2-MIB producers by GC/MS analysis of the culture samples. The PCR results show that the *mib*C genes present completely matched the 2-MIB detected in the same culture samples, indicating that the primer set was able to capture the *mib*C genes present in all the samples.

In the NCBI database, 13 cyanobacterial strains were reported to be 2-MIB producing cyanobacteria (accessed on March 27, 2016), including the genera of *Leptolyngbya*, *Oscillatoria*, *Planktothricoides*, *Planktothrix*, and *Pseudanabaena*, as shown in Table S2. The designed MIBS02 primer set was also compared with the *mib*C genes for these 2-MIB producers using BLAST analysis. The results demonstrate that for all the 13 2-MIB producing strains, their *mib*C genes were perfectly matched with the MIBS02 primer set. The results further confirm that the designed primer set may detect all the 2-MIB producers available in the literature.

The MIBS02F/R primer set was successfully amplified and the targeted DNA region sequenced from 2-MIB-producing strains. The obtained sequences were proven to the homologues of the monoterpene synthases. This result verified the specificity of the MIBS02F/R for 2-MIB-producing cyanobacterial species.

3.2.2. Standard curves for real-time PCR

To establish the real-time PCR-TaqMan system for *mib*C gene, the DNA extracted from a laboratory strain (*Pseudanabaena galeata* TWNCKU13) was used to prepare a standard. After DNA extraction, DNA from TWNCKU13 was amplified by conventional PCR and then the PCR products were cloned using TOPO® TA Cloning® Kit. After cloning, the plasmid, which cloned the monoterpene-synthases amplicon, was diluted using a tenfold serial dilution step

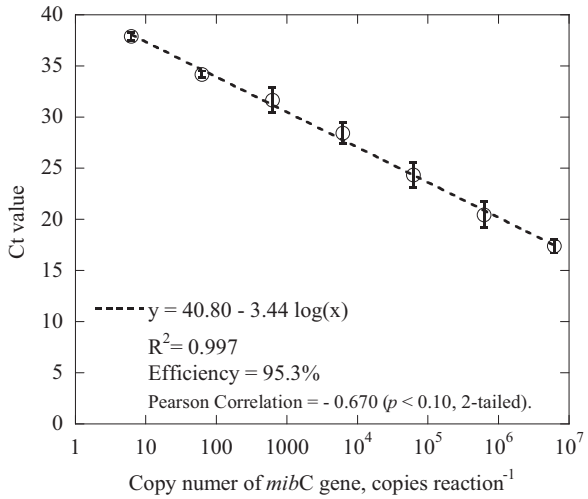

Fig. 3. Standard curve for real-time PCR using primer MIBS02F/R with TaqMan system.

(10^2 to 10^7 copies/μL) and then quantified by real-time PCR.

The standard curve shown in Fig. 3 demonstrates a high linearity from 10^2 to 10^7 copies/μL with a correlation coefficient R^2=0.998. The real-time PCR efficiency (E) calculated with $E=10^{-1/S}-1$ was 92.3%, where S is the slope of the standard curve. The high linearity and correlation coefficient of the calibration curve and the high PCR amplification efficiency suggest that the analytical protocol is reasonable.

3.3. Applications in monitoring mibC genes in 29 reservoirs

The real-time PCR approach was further applied to quantify 2-MIB producers from 29 reservoirs in Taiwan, including 10 drinking water reservoirs (DWRs) in Taiwan main island, 10 DWRs in Kinmen islands, and 9 DWRs in Matsu islands. Fig. 4 shows the time course of *mib*C genes and 2-MIB levels in water collected from three of the studied reservoirs, FSR in Taiwan main island, JHR in Kinmen islands, and LDWR in Matsu islands, from October 2012 to October 2015. In the figure, the *mib*C gene copies were compared with both total 2-MIB (cell-bound + dissolved) and cell-bound 2-MIB levels. As shown in Fig. 4a and b for FSR, the overall change of *mib*C gene copies follows more closely with that of the cell-bound 2-MIB, although the total 2-MIB levels in 6 samples collected before August 2014 show almost an identical trend with the gene copies. After August 2014, *mib*C gene was below the detection limit (3.1×10^1 copies/mL), although 10–120 ng/L of total 2-MIB were detected in water samples. Similar results were found for the samples from JHR (Fig. 4c and d), in which the trends for *mib*C genes copies and total 2-MIB concentrations were similar for samples collected before October 2014 and dissimilar thereafter. For samples from LDWR, the time courses of the *mib*C gene

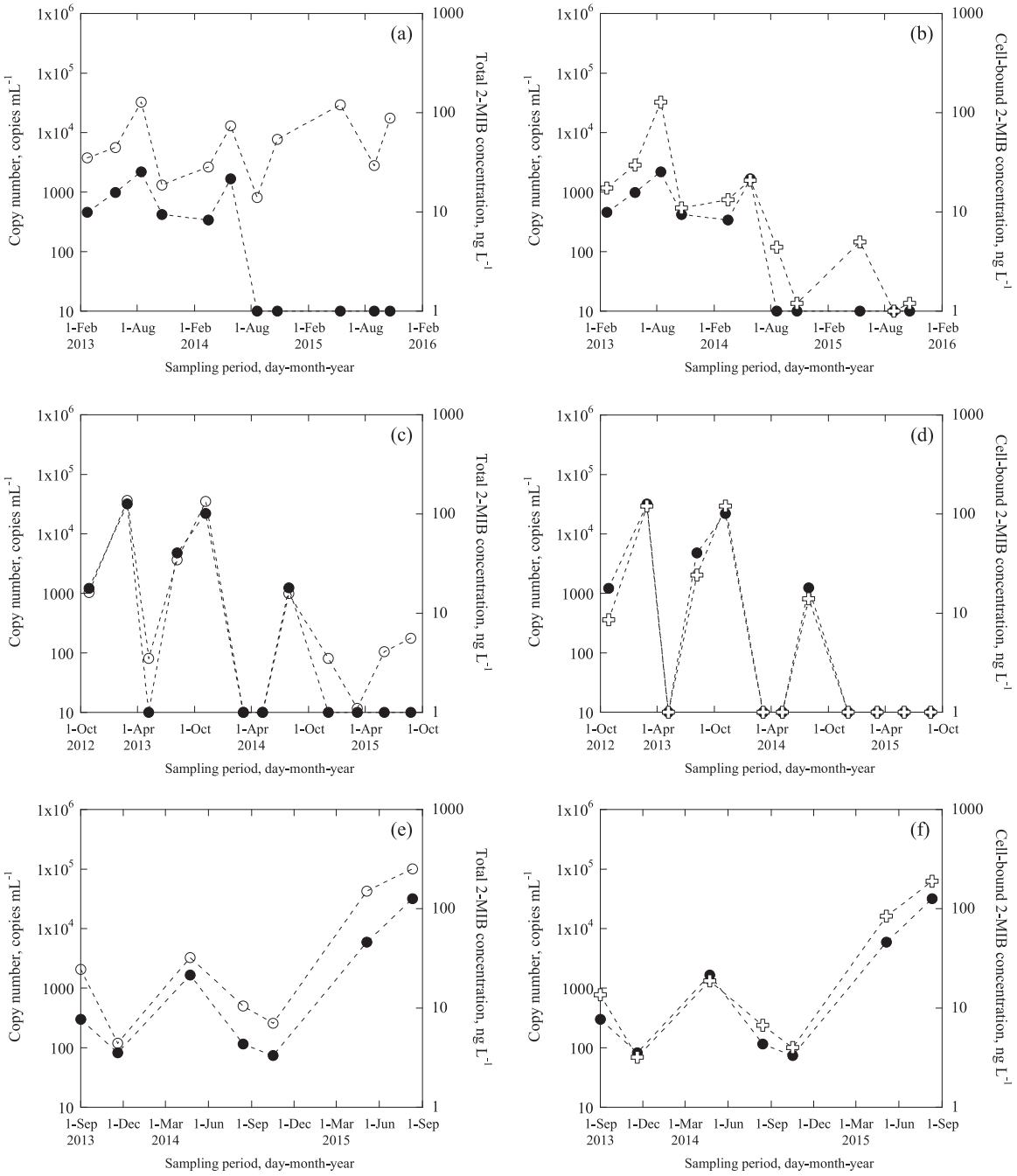

Fig. 4. Time course of *mib*C gene and 2-MIB concentrations for Taiwan's reservoirs, where (a) and (b) are for FSR in Taiwan main island, (c) and (d) are for JHR in Kinmen islands, and (e) and (f) are for LDWR in Matsu islands. ● is the copy number of *mib*C gene; ○ is the total 2-MIB (cell-bound + dissolved) concentration; ✚ is the cell-bound 2-MIB concentration.

followed the levels of both total and cell-bound of 2-MIB closely (Fig. 4e and f).

The three reservoir cases in Fig. 4 show that the trends of *mib*C gene abundance follow closely to that of both total 2-MIB and cell-bound concentrations for many of but not all the samples. In this study, 2-MIB was measured for those present within cells and in dissolved phase. However, for *mib*C genes, those bound with cells are believed to be the major one to be detected in the current study. The *mib*C gene is reported to be present in the genome of a 2-MIB operon (Giglio et al., 2011; Wang et al., 2011) and should be measurable with intact cells during sampling and analysis. For the DNA released from lysed cells, DNA fragmentation is expected (Dwyer et al., 2012; Kerr et al., 1972) to occur in short time (on the time scale of hours) (Gavrieli et al. (1992)), making amplication of targeted DNA difficult. In addition, in the current experimental protocol, only cyanobacteria cells were collected and dissolved DNA was expected not to be included in the cyanobacterial pellets after centrifugation. Therefore, only the cell-bound *mib*C genes are expected to be detected. It is believed that the cell-bound 2-MIB may be better correlated with the cell-bound *mib*C genes. Indeed, the data in Fig. 4b, d, and f show better fits between *mib*C genes and cell-bound 2-MIB as compared to the corresponding fits with total 2-MIB (Fig. 4a, c, and e), especially when the cell-bound 2-MIB concentrations were low.

The samples from FSR (Fig. 4a and b) before and after August 2014 were found to have 0–53% and 71–99% 2-MIB, respectively, in

the dissolved phase. Therefore, the detected *mib*C gene copies in the samples follow closely the levels of both total and cell-bound 2-MIB before August 2014 but only the level of cell-bound 2-MIB thereafter. Similarly, samples collected from JHR (Fig. 4c and d) show the same characteristics for *mib*C genes and 2-MIB concentrations before and after October 2014. The *mib*C gene copy exhibits a similar trend with either the total or the cell-bound 2-MIB level, even there was no 2-MIB present in water bodies around April 2014. After October 2014, there was no detected *mib*C gene due to a lower amount of cell-bound 2-MIB, a result similar to that shown in Fig. 4a and b. In the samples, 0–47% and 72–93% of 2-MIB were observed in the dissolved phase before and after

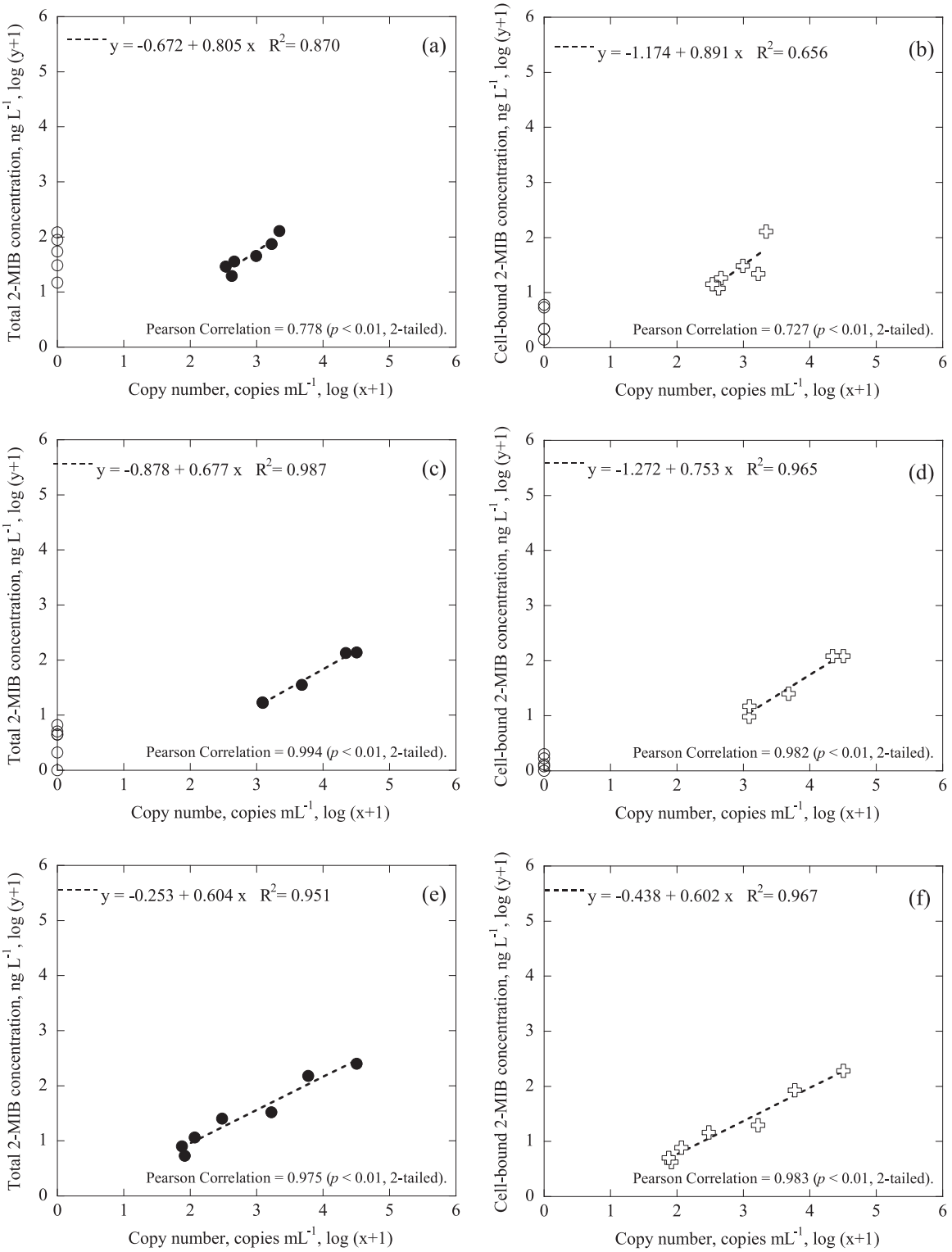

Fig. 5. Correlation between 2-MIB concentrations determined by HS-SPME/GC/MS and copy number measured by real-time PCR with MIBS02F/R. ● represents the total 2-MIB (cell-bound+dissolved) concentration; ✚ represents the cell-bound 2-MIB concentration; ○ represents the data with measurement by real-time PCR being < detection limit. (a) and (b) are for FSR in Taiwan main island (number of samples, n=6); (c) and (d) are for JHR in Kinmen islands (n=5); (e) and (f) are for LDWR in Matsu islands (n=7).

October 2014, respectively. For samples from LDWR (Fig. 4e and f), as the fractions of the cell-bound 2-MIB were between 56% and 75%, no significant difference was found between total and cell-bound 2-MIB concentrations. According to the monitored results of 2-MIB levels during culturing *Pseudanabaena galeata* TWNCKU13 (Fig. S3), 70–93% of 2-MIB concentration were present as cell-bound during logarithmic and stationary phases. A similar cell-bound 2-MIB ratio of 78% was also reported by Wu and Jüttner (1988) for *Oscillatoria tenuis*. The two laboratory observations both suggest that if 2-MIB producing cyanobacteria are healthy, most 2-MIB remains with the cells. However, the cell-bound 2-MIB started to release into the water during the decayed phase, thus increasing the dissolved phased concentration. It is likely that in samples having a large portion of dissolved 2-MIB, the 2-MIB producers were mainly in the decayed phase, resulting in a low cell-bound level in the water samples. Therefore, based on the observed data of this study, the abundance of *mib*C genes is a good indicator for the cell-bound 2-MIB level in the water. As the genetic method used in this study mainly detects cell-bound DNA, when applying this method in field monitoring, the measured gene abundance may represent the number of intact cells for the producers and the 2-MIB present within the cells, but is not necessary strongly linked to lysed cells and released 2-MIB in the water.

3.4. The mibC gene copies and 2-MIB concentrations

The data from the 29 DWRs were used to evaluate the correlation between the levels of *mib*C genes and 2-MIB. Fig. 5 shows correlations of the data from three reservoirs, FSR in Taiwan main island, JHR in Kinmen islands, and LDWR in Matsu islands. It is noted that in the analysis, both total (cell bound + dissolved) and cell-bound 2-MIB concentrations were correlated with the level of *mib*C genes. In addition, only the data with both detectable *mib*C genes and concentrations were used in the correlation analysis. Good correlations were found between the *mib*C gene copy and both the total and cell-bound 2-MIB levels, with $R^2=0.656–0.987$ ($p<0.01$). A similar correlating coefficient was observed for the data from each of the 12 other reservoirs, as shown in Table S3. It is interesting that the slopes in the correlation of the *mib*C gene copy with both total and cell-bound 2-MIB levels are fairly comparable to the data from each reservoir. However, for the different reservoirs, the slope of log[total (or cell-bound) 2-MIB concentration] to log[*mib*C gene copy] (unit in $\log(\text{ng L}^{-1})/\log(\text{copy mL}^{-1})$) varies from 0.33 to 1.04. Wang et al. (2015b), the only study to date reported the correlations between 2-MIB and *mib*C gene concentrations for a laboratory culture of *Pseudanabaena* sp. isolated from Lushui Reservoir, China. In the study, the total 2-MIB concentration (in log) to *mib*C gene copy (in log) slopes were 0.748 and 0.604 for the laboratory culture and reservoir samples, respectively, both being within the range of this current study.

Several parameters may affect the correlation slope of 2-MIB to *mib*C genes, such as 2-MIB production rate and cell quota (2-MIB mass per cell), 2-MIB producing species, and gene copy of the producing species. Cell quota of cyanobacterial metabolites has been reported to vary in a wide range in different growth phases in laboratory cultures and different sampling locations in a reservoir. Wang et al. (2015b) reported that the 2-mic gene quota (2-MIB mass per mic gene copy) was 10–45 (fg/mic gene copy) and 11–57 (fg/mic gene copy) for laboratory culture and reservoir samples, respectively. Similarly, the laboratory *Pseudanabaena* culture examined in this study was found to have cell quota of cell-bound 2-MIB varied from 5.96 to 51.1 (fg/cell) for different growth phases (Fig. S3). Another commonly observed cyanobacterial odorant, geosmin, its cell quota for *Anabaena circinalis* was reported to be 15–838 fg/cell in Myponga Reservoir, South Australia (Tsao et al., 2014), while the cell quota for a cyanotoxin, cylindrospermopsin, was 4.28–19.9 fg/cell for cylindrospermopsin-producing cyanobacteria in North Pine Dam, Queensland, Australia (Rasmussen et al., 2008). These studies all showed that the cell or gene quota of cyanobacterial odorant/toxin may vary in a wide range for the same cyanobacteria species and for the samples collected from the same reservoirs. For gene copies present in a producing species, the environmental monitoring results from Tsao et al. (2014) and Rasmussen et al. (2008) show that the geosmin-producing gene copy per *Anabaena circinalis* cell was in the range of 1.1–35.6 copies/cell, and the cylindrospermopsin-producing gene copy per cell was 1.8–6.0 copies/cell. The two studies both suggest that the gene copies per producing cell also vary in a relatively wide range. Although there are many factors that may affect the correlation slope of 2-MIB to *mib*C genes, based on the observation of this study and those reported in the literatures (Rasmussen et al., 2008; Tsao et al., 2014; Wang et al., 2015b), a good general correlation between the *mib*C gene copy and 2-MIB concentration for a given reservoir may be established, if long-term monitoring data is available.

For some data points in Fig. 5, the *mib*C gene level was below the detection limit although 2-MIB was detected, as indicated by the empty circles in the plot. This scenario may be a result of some possible causes. The first is that some 2-MIB were not produced indigenously, e.g., by benthic cyanobacteria (Berglind et al., 1983; Izaguirre, 1992; Izaguirre et al., 1982; Krasner et al., 1983; Wood et al., 2001), which migrated to the sampling site, and thus, no *mib*C genes were detected. This is in accordance with the observation that lower 2-MIB concentrations bind with cells compared with total 2-MIB. For example, for samples in which no *mib*C genes were detected, total 2-MIB concentrations were between 14.0 and 120.1 ng/L for FSR (Fig. 5a), while only $<1.0–4.4$ ng/L was observed as cell-bound (Fig. 5b). Another possible cause is due to the method detection limit. Based on the data collected in this study, it is observed that in most cases, when cell bound 2-MIB concentrations were below ~5 ng/L, the *mib*C genes in the same reservoir water samples were below detection limit.

The data collected from all reservoirs were further analyzed in terms of their geographic locations: 10 reservoirs in Taiwan main island (Fig. 6a and b), 10 reservoirs in Kinmen islands (Fig. 6c and d), and 9 reservoirs in Matsu islands (Fig. 6e and f). As noted, positive correlations were found between *mib*C genes and concentrations for the samples from each island ($R^2=0.302–0.796$), in which the slopes of the *mib*C copy versus total and cell-bound 2-MIB levels were also similar. However, the slopes were sufficiently different for the data from the three islands studied, being 0.317–0.369, 0.409–0.539, and 0.697–0.726, respectively, with Taiwan, Kinmen, and Matsu islands. The 95% prediction interval was shown in Fig. 6. As seen, the majority of data fall within the 95% prediction intervals. Or more exactly, 95.3% of the total and 100% of the cell-bound data from Taiwan island, 97.2% of the total and cell-bound data from Kinmen islands, and 93.1% (total) and 95.5% (cell-bound) data from Matsu islands. This suggests that if no long-term data are available for a reservoir, the correlation developed between 2-MIB species and genes in a region may be used as a preliminary correlation for that reservoir in the region.

Fig. 7 shows the overall correlation for all 195 data points with all reservoirs combined. It is noted that in Fig. 7(a) a few outlier data points with high *mib*C gene quota were found. These data were all from the samples collected from Matsu islands, which were shown to have larger slope of 2-MIB to *mib*C genes (*mib*C gene quota) than those collected elsewhere (Fig. 6(a), (c) and (e)). The *mib*C gene ranges from about 7.5×10^1 to 2.4×10^5 copies/mL, and the corresponding 2-MIB levels from 3.9 to 1715.5 ng/L for total 2-MIB and from 1.5 to 1479.2 ng/L for cell-bound 2-MIB. For

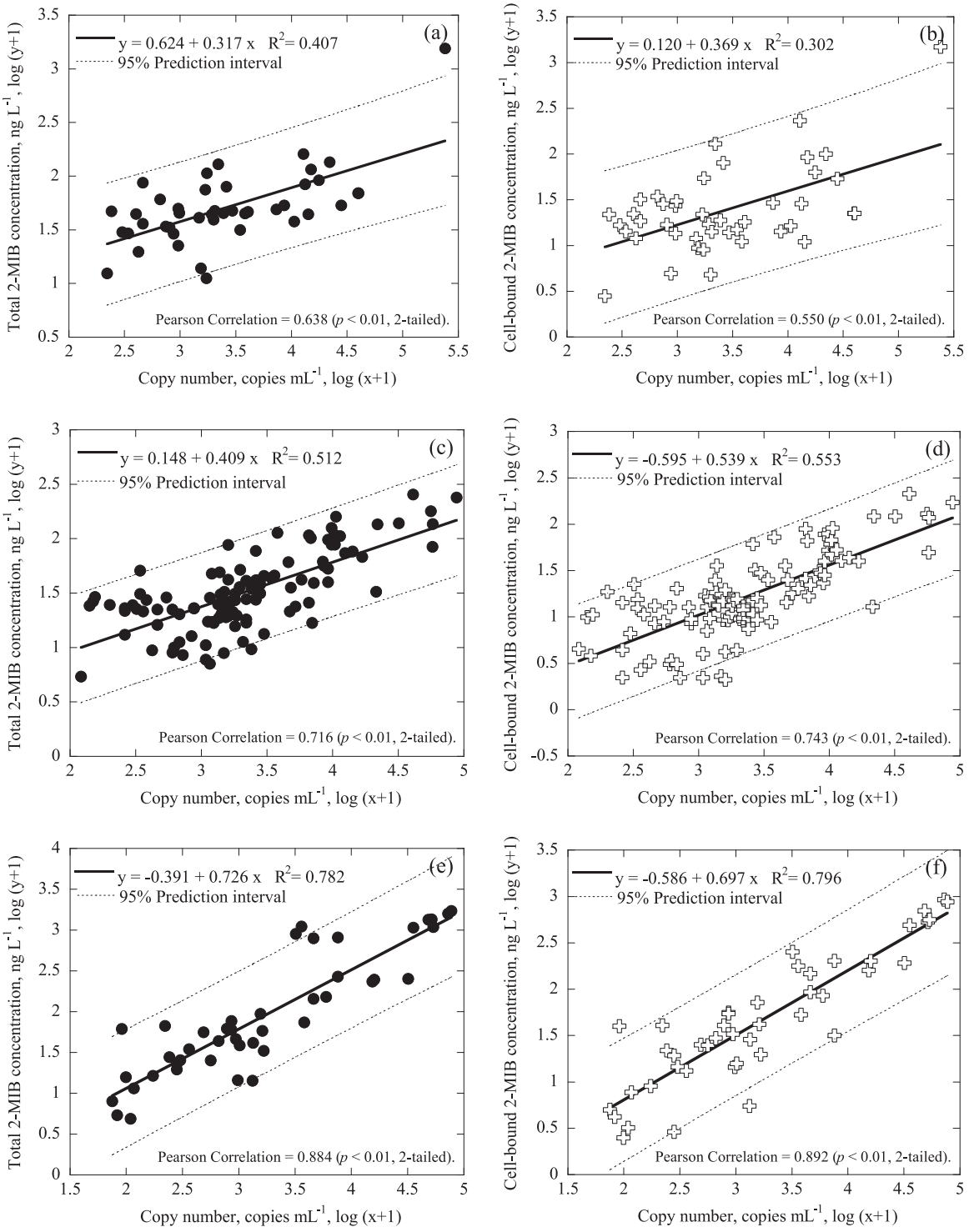

Fig. 6. Correlation between 2-MIB concentrations and copy number for three areas with 95% prediction interval (broken line). ● represents the total 2-MIB (cell-bound + dissolved) concentration; ✚ represents the cell-bound 2-MIB concentration; ○ represents the data with measurement by real-time PCR being < detection limit. (a) and (b) are for the all data in Taiwan main island (number of samples, n=43); (c) and (d) are for Kinmen islands (n=108); (e) and (f) are for Matsu islands (n=44).

such wide ranges of *mib*C genes and concentrations, the data show a reasonably good correlation coefficient of $R^2=0.473$ for total 2-MIB and $R^2=0.479$ for cell-bound 2-MIB. About 94.9% (total) and 97.4% (cell-bound) of the data are within the 95% prediction interval for total and cell-bound 2-MIB, respectively. The correlations thus provide a good link between 2-MIB producers and 2-MIB concentrations at a reservoir.

4. Conclusions

As discussed in this study, the developed real-time PCR was capable of well quantifying *mib*C genes in cyanobacteria in Taiwan's DWRs. The designed MIBS02F/R primer completely matched the 2-MIB producing capability for the 17 cultured strains and effectively amplified the targeted DNA for 13 2-MIB-producing strains listed in the literature. These results illustrate that the primer set was able to capture the *mib*C genes for 2-MIB-

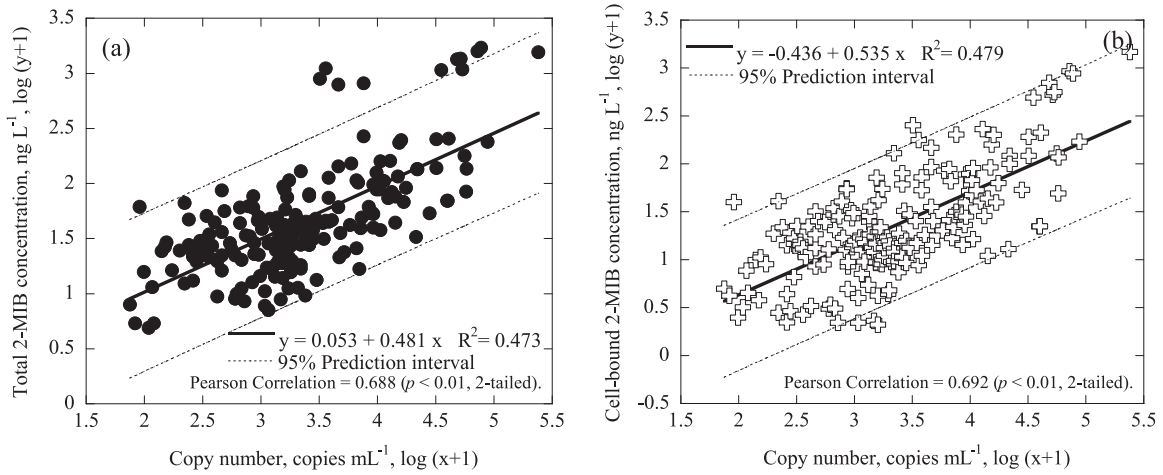

Fig. 7. Correlation between 2-MIB concentrations and copy number for the all data (n=195) with 95% prediction interval (broken line). (a) for the total 2-MIB (cell-bound+dissolved) concentration; (b) for the cell-bound 2-MIB concentration; ○ represents the data with measurement by real-time PCR being < detection limit.

producing cyanobacteria. The real-time PCR approach was further used to quantify 2-MIB producers in 29 reservoirs in Taiwan. High correlations were found between *mib*C genes and both total and cell-bound 2-MIB concentrations for samples from each reservoir, while reasonable correlations were also found for samples from all reservoirs of the three islands. The developed real-time PCR method may serve as an alternative method to develop useful reference data for water utilities confronted with 2-MIB problems in source water.

In particular, as the real-time PCR is capable of detecting multiple samples in a single run, a large quantity of samples may be analyzed in a short time. The estimation of 2-MIB in a whole reservoir is expected to be feasible using the real-time PCR analysis combined with the correlations established for 2-MIB and *mib*C genes. The scheme should provide timely information to managers of reservoirs and water utilities when facing 2-MIB-related incidents.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.08.034.

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