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RESEARCH PAPER

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# Development of a Quantification and Detection Method for 2-MIB-producing Cyanobacteria

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**Abstract**

Consumers often complain about taste and odor (T&O) in drinking water and freshwater fishery. One of the common T&O compounds, 2-methylisoborneol (2-MIB), can be detected by humans even when the concentration is below 10 ng/L. A forecast method of T&O occurrence is required to control drinking water plants and fishery farms to determine the timing of exchange of activated carbon or exchange the water in earthy pond. Traditional monitoring methods such as PCR, microscopy, and chemical analysis require a long time, are high in cost, and have a complex operation. We conducted this study to develop whole-cell PCR and whole-cell qPCR assays for rapid detection and quantification of 2-MIB-producing cyanobacteria without DNA extraction to detect 2-MIB cyclase gene (*mtc*). *Pseudanabaena foetida* strain 1705-12 (Lake Kasumigaura), strain 1803-12 (Lake Kasumigaura), and strain PTG (Lake Biwa) of 2-MIB-producing cyanobacteria were used in the study. The positive correlation between the results of whole-cell PCR, whole-cell qPCR and chlorophyll a (Chl.a), and gene abundances illustrated that whole-cell PCR and whole-cell qPCR assays could rapidly and conveniently detect and quantify 2-MIB-producing cyanobacteria. Thus, this study provides a valuable tool for prediction of T&O events in drinking water and freshwater fishery.

## Introduction

Taste and odors (T&O) are produced by some cyanobacteria and actinomycetes in water reservoirs and aquaculture earthen ponds (Robin *et al*., 2006, Ma *et al*., 2012). T&O can help an ordinary consumer to determine the safety and satisfaction of drinking water and cultured fish. In addition, T&O can lead to consumer complaints and significant economic losses on water sector and farmers. Examples of economic losses include lower market prices for cultured fish and the inability to collect fees for the use of tap water. (Engle *et al*., 1995; Whangchai *et al*., 2011; Burr *et al*.,2012). T&O events occur more in summer, but recently also occur in spring and winter. (Watson et al. 2001; Wang *et al*., 2005; Dzialowski *et al*., 2009; Wang Z *et al*., 2016). Geosmin and 2-methylisoborneol (2-MIB) are the main T&O compounds, they are not toxic to humans (Burgos et al., 2014), but they affect consumers' sense of smell from food and drinks.

Geosmin and 2-MIB are volatile microbial metabolite sesquiterpenes and monoterpenes, respectively (Suurnäkki *et al*., 2015). Human beings are sensitive to these two compounds, their threshold concentrations should be below 10 ng/L (Young *et al*., 1996). Previous studies have indicated that 2-MIB is mainly produced by filamentous cyanobacteria in aquatic environment (Izaguirre and Taylor, 2004; Jüttner and Watson, 2007; Smith *et al*., 2008; Watson *et al*., 2008), including *Planktothrix*, *Pseudanabaena* (Planktonic), *Planktothricoides*, *Oscillatoria*, and *Lyngbya* (Chiu *et al*., 2016, Izaguirre and Taylor, 1998, Izaguirre and Taylor, 2004, Zimba *et al*., 1999, Acinas *et al*., 2009, Zahang *et al*., 2009). Previous reports found that 2-MIB biosynthesis occurs via a two-step reaction: first, geranyl diphosphate (GPP) is converted to methyl GPP catalyzed by methyltransferase as *mtf* gene and then methyl GPP is cyclized to 2-MIB by 2-MIB synthase/monoterpene cyclase as *mtc* gene (Giglio et al., 2011a; Komatsu et al., 2008).

Musty and earthy odors affect the fishy of fish in the water environment, causing cyanobacteria commonly found in freshwater to produce musty and earthy odors that dissolve in the water and enter the bloodstream through the gills, skin via osmosis or by ingesting food, which can then accumulate in their flesh (Whangchai et al., 2011). One of the most promising food options is a sustainable source of protein and a nutritious and accessible fish diet. However, the foul odor of fish limits its production and consumption. Freshwater fish and marine fish have different fishy odor substances. The main compounds found in freshwater fish are geosmin and 2-MIB (Liu et al., 2021). Thus, it is important to monitor the industry for rapid quantification of T&O producers to control the quality of flesh.

T&O events not only affect fishery but also the quality of drinking water. Therefore, the removal of musty and earthy odors is a very curial process for drinking water treatment plants (DWTPs). Coagulation/flocculation and filtration processes are widely used to remove algae in drinking water treatment plants (DWTPs) (Teixeira *et al*., 2010; Pan *et al*., 2012). Additionally, peroxidation with chlorine, ozone, ferrate (VI), and permanganate has been implemented to remove algal cells (Daly *et al*., 2007; Pan *et al*., 2012; Fan *et al*., 2013, 2018; Zamyadi *et al*., 2019); however, peroxidation combined with traditional water treatment process has negative effects with the release of intracellular metabolites in water bodies. (Srinivasan and Sorial, 2011; Zamyadi *et al*., 2015). Strong oxidants such as ozone and chlorine and adsorption on activated carbon are generally more effective in 2-MIB removal. Ridal *et al*. (2001) found that the 2-MIB removal efficiency is directly related to $Cl_2$ residual. DWTPs can effectively remove 2-MIB and geosmin by increasing the residual amount of chlorine and contact time once odor occurs. Therefore, effective monitoring of 2-MIB and geosmin is important for DWTPs.

Microscopy and gas chromatography-mass spectrometry (GC-MS) are usually used for monitoring T&O. Microscopy is used to observe the cellular morphology of cyanobacteria and count the number of cyanobacteria. GC-MS is usually used for measuring musty and earthy odors volume. However, they have different defects, microscopic observation requires more time, and it cannot distinguish between T&O producers and non-producers (Jüttner 2007; Lee *et al*., 2017; Lu *et al*., 2019). GC-MS is time consuming. For many samples, the cleanup and separation step can reduce the speed of turnover, thus, increasing the sample analysis time. Complex operating procedures require technical expertise, and the large size and cost of instrument limit its usage for on-site application (Bristow *et al*., 2019; John *et al*., 2018; Lu *et al*., 2019). Additionally, the GC-MS results directly show the present and past situations. However, the users of waterworks and fishery require data that can be used for predicting T&O events.

Traditional quantitative PCR (qPCR) assay using total DNA extracted from samples taken from a monitored environment as a template is highly sensitive, low-cost, and requires a small sample size; additionally, it has been extensively used in the past to identify geosmin and/or 2-MIB-producing cyanobacterial population (Su *et al*., 2013; Tsao *et al*., 2014; Lindholm-Lento *et al*., 2019; Rong *et al*., 2018; Kim *et al*., 2020; Shen et al., 2021). However, the qPCR thermal cycle is costly and requires the extraction of DNA as a template. If the assay can monitor the population more simply and cheaply, it will be very useful for small-to-medium-sized businesses and institutes. Whole-Cell PCR and qPCR are rapid methods without DNA extraction to monitor T&O-producers. They can reduce the analysis time and cost of experimental equipment. However, no study has used the methods to monitor T&O-producers. This study aimed to develop a monitoring method for rapid detection and quantification of 2-MIB-producing cyanobacteria using whole-cell PCR and qPCR methods.

## Materials and Methods

### Strain and Culture Conditions

*Pseudanabaena foetida* strain 1705-12 and *Pseudanabaena foetida* strain 1803-12 were separated from Lake Kasumigaura (Japan), and *Pseudanabaena foetida* strain PTG (former name was *Phormidium tenue* PTG) was separated from Lake Biwa (Japan) (Niiyama *et al*., 2016). *P. foetida* strain 1705-12 and strain 1803-12 were cultured in CT liquid medium (pH 8.2) while *P. foetida* strain PTG was cultured in MA liquid medium (pH 8.6). All samples were cultured using 100 mL culture in 300 mL Erlenmeyer-flasks at 25°C under 12 h light-dark cycle (30 µmol/$m^2$/s) for 28 days. The initial chlorophyll a (Chl. a) concentration was set to 40 µg/L via inoculation from preculture. Samples for Chl.a

concentration and DNA extraction for qPCR, whole-cell PCR, and whole-cell qPCR (SYBR and TaqMan Probe) were obtained every 4 days in the 28 days experiment period.

**Analysis of Growth Using Chl. a Concentration**

The growth of *P. foetida* and *P. tenue* strains was determined by measuring the biomass in terms of Chl. a concentration as previously described (Shen *et al*., 2021). Briefly, 5 mL cultures were collected and filtered through a GF/C glass microfiber filter (Cytiba (Whatman), Tokyo, Japan). Chl. a was extracted from each sample with 10 mL of 90% methanol with 0.2% $MgCl_2$ at 70°C for 30 min in the dark. The absorbance of each sample was measured at 750 nm, 665 nm, 645 nm, and 630 nm using a spectrophotometer, and Chl.a concentration was determined using the following formula:

$$\text{Chl.a } (\mu g/L)=(11.6\times(A665 - A750) - 1.31\times(A645\text{-}A750) - 0.14\times(A630\text{-}A750))\times Vm\ (mL) / Vf\ (mL),$$

where Vm and Vf are the volumes of methanol and filtered cultures, respectively.

**Whole-cell PCR**

Cell culture (2 µL) was used as a template for whole-cell PCR using a Mighty Amp DNA Polymerase Ver.3 kit (Takara Bio Inc., Shiga, Japan) according to manufacturer's protocol. The primer set used in this study is shown in Table 1. The amplification of 2-MIB cyclase gene (*mtc* gene) using PCR was used the Mtcf primer (0.2 µL, 10 µM) as forward primer and Mtcr primer (0.2 µL, 10 µM) as reverse primer (Wang *et al*., 2011). The product size of the amplified target of PCR was 152 bp. We developed semi-quantitative PCR applied to whole-cell PCR to accomplish rapid detection and quantification at a low cost. The thermal cycle condition was set as initial denaturation at 98°C for 2 min, and this was followed by X cycles of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and extension at 68°C for 20 s. The whole-cell PCR amplified products were analyzed via 2% agarose gel electrophoresis. The relative brightness of the amplified target was analyzed using ImageJ (NIH, USA). A 25 bp marker (Thermo Fisher SCIENTIFIC (Invitrogen), Waltham Massachusetts, USA) was used as a reference after subtracting the background noise. The same amount of DNA in the marker was applied to all agarose gel electrophoresis experiments. Relative brightness was calculated as the ratio of specific band brightness to the marker brightness. To develop semi-quantitative PCR, we performed a preliminary experiment to determine the optimal number of cycles of whole-cell PCR. We tested every 5 cycles from 10 to 35 cycles for preliminary optimization, and we narrowed the cycle number range where the bands and their brightness differed. There was no obvious amplified PCR band within 20 cycles; however, the PCR amplified bands were observed at 25, 30, and 35 cycles. Based on the result of the relative coefficient of every 1 cycle from 20 to 25 cycles between brightness and Chl. a concentration shown in Table 2, the optimal cycle number was decided as 25 cycles in the thermal condition mentioned above.

**Table 1.** List of primers used in the study

| Gene | Primer TaqMan Probe | Sequence (5'–3') | Reference |
|---|---|---|---|
| *mtc* gene | Mtcf | CGCTCGCTTTGTGAGTGAGATAG | Wang *et al*., 2011 |
| | Mtcr | GGCAGTAGAGTGGTGAGGCAGTT | |
| | MIBcyclaseF | GTGAGTGAGATAGCCAAG | This study |
| | MIBcyclaseR | ATAGGCAGGATCACAATC | |
| | MIBcyclaseProbe | ACCTAGATTCAGGACTACTCCAGACA | |

**Table 2.** Correlation between Chl.a and relative brightness using whole-cell PCR at different cycles

| Strains | Cycles | Correlation($R^2$) |
|---|---|---|
| *Pseudanabaena foetida* strain 1705-12 | 21 | N |
| | 22 | * |
| | 23 | 0.9023 |
| | 24 | 0.8825 |
| | 25 | 0.9296 |
| *Pseudanabaena foetida* strain 1803-12 | 21 | * |
| | 22 | 0.5711 |
| | 23 | 0.4189 |
| | 24 | 0.8272 |
| | 25 | 0.8420 |
| *Pseudanabaena foetida* strain PTG | 21 | * |
| | 22 | 0.6214 |
| | 23 | 0.7639 |
| | 24 | 0.7336 |
| | 25 | 0.8921 |

* Shows no band on target product size at cycles.

**Whole-cell qPCR Assay**

SYBR Green assay and TaqMan Probe assay were conducted for Whole-Cell qPCR and qPCR. All qPCR operations were performed using an ABI 7500 system (Thermo Fisher SCIENTIFIC (Applied Biosystems), Waltham Massachusetts, USA).

**Whole-cell qPCR Assay Using SYBR Green**

The primer set Mtcf and Mtcr shown in Table 1 was used to amplify the *mtc* gene (Wang *et al*. 2011). The 20 µL SYBR Green qPCR Master Mix contained 2 µL cell culture, 1 µL primer (10 µM, forward and reverse, each), 10 µL TB Green *Premix Ex Taq* II (2 X) (Takara Bio Inc., Shiga, Japan), 0.4 µL of ROX Reference Dye II (50 X), and 5.6 µL of $ddH_2O$. The thermal cycle condition for the PCR set was as follows: preheating at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 34 s, and finally one cycle of dissociation stage at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 sec. All samples were amplified in triplicate.

**Whole-cell qPCR Assay Using TaqMan Probe**

The primer set of MIBcyclaseF, MIBcyclaseR, and MIBcyclaseProbe as TaqMan Probe shown in Table 1 were used for TaqMan Whole-Cell qPCR assay. Cell culture was used as the template for DNA amplification using Premix *Ex Taq*™ (Probe qPCR) kit (Takara Bio Inc., Shiga, Japan) according to manufacturer's protocol. The 20 µL qPCR reaction mixture comprised 0.2 µL of each primer (10 µM), 10 µL of Premix *Ex Taq* (Probe qPCR), 0.8 µL of TaqMan probe, 0.2 µL of ROX Reference Dye II (50X), 6.2 µL of $ddH_2O$, and 2 µL cell culture as a template. The PCR thermal cycle condition was set as follows: preheating at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. All samples were tested in triplicate.

**Total DNA Extraction**

Cyanobacterial cells (2 mL) were collected from cultures, and they were treated via centrifugation at 10,400 *g* for 5 min, after which the supernatant was discarded to collect the cell pellets for DNA extraction. The TE buffer contained 200 µL of 50 ng RNase and 400 µL Solution Ⅰ (1% sarkoysl, 0.5 M NaCl, 1% SDS) in tubes containing cell pellets, and the tubes were then incubated at 35℃ for 10 min. The cells were disrupted by bead beating at 4600 rpm for 40 s using a bead beader (Mini-BEAD BEATER, BioSpec Products, Inc. Bartlesville, Oklahoma, USA). Chloroform and isoamyl alcohol solution (25:24, 600 µL) was added into the sample tubes, and the mixture was treated via centrifugation at 12,000 *g* for 5 min. The supernatant (500 µL) was transferred to a new 2 mL tube, and 50 µL of sodium acetate buffer (3 M, pH 5.2) as 1/10 of the supernatant volume and 500 µL of isopropanol at the same volume as that of the supernatant were added into new tubes. The precipitate was harvested via centrifugation at 10,400 *g* and 4℃ for 15 min. The precipitate was then washed using 500 µL of 70% ethanol via centrifugation at 10,400 g and 4℃ for 15 min twice, and the extracted DNA was dried and dissolved in 25 µL of 1/10 TE buffer (pH 8.5). After manipulation for DNA extraction, the extracted total DNA was checked via agarose gel (0.8%) electrophoresis assay. After confirmation, the extracted total DNA samples were stocked in a freezer at –30℃.

**The qPCR Assay**

The qPCR using SYBR Green and TaqMan probe were used as references for the developed method. The qPCR using SYBR Green and TaqMan probe was performed using the same protocol as that of whole-cell qPCR using SYBR Green and TaqMan probe. DNA amplification used TB Green Premix *Ex Taq* II (2 X) (TaKaRa bio, Shiga, Japan) according to manufacturer's protocol using 50 ng of extracted DNA. The qPCR thermal cycle condition was the same as the Whole-cell qPCR assay. All samples were amplified in triplicate.

**Standard DNA Preparation for Whole-cell qPCR and qPCR Analysis**

The standard DNA using plasmid for quantification of *mtc* gene was prepared as previously reported (Shen *et al*., 2021). The concentrations of plasmid DNA were determined using a spectrophotometer (NanoVue Plus, Biochrom Ltd., Cambridge, UK) according to manufacturer's protocol. The gene copies were calculated using the following formula (Wang *et al*., 2016):

$$N\ (copies/mL) = cDNA\ (ng/mL) \times 10^{-9} \times 6.022 \times 10^{23} / (L\text{-}plasmid\ (bp) \times 660),$$

where cDNA and L-plasmid are the concentration and length of the linear plasmid, respectively.

A tenfold serial dilution of linear plasmid ($10^1$ to $10^7$ copies/mL) and the corresponding CT values was used to construct standard curves.

**Statistical Analysis**

Correlation analysis was performed using SPSS 26.0 (SPSS Inc., Chicago, Illinois, USA) statistical software.

## Results

**Growth Curve of Cyanobacteria Strains**

Chl.a concentration of *P. foetida* strain 1705-12 gradually increased, with a maximum value of 2493.4 µg/L observed on Day 20, which decreased slightly and

was finally relatively stable (Figure 1). *P. foetida* strain 1803-12 and strain PTG reached their maximum Chl. a concentration on Day 12 with 2156.9 µg/L and 2461.4 µg/L, respectively. *P. foetida* strain 1803 was stable for 12 to 16 days, it quickly decayed, and finally gradually deceased. *P. foetida* strain PTG slowly declined the growth activity after reaching its maximum value at 12 days, and it remained stable from 24 to 28 days (Figure 1).

**Applicability Analysis of Whole-cell PCR**

The *mtc* gene abundance using qPCR with SYBR Green assays was shown in Figure 2A. The *mtc* gene abundance of *P. foetida* strain 1705-12 reached a peak with $10.8\times10^5$ copies/mL at 20 days. The *mtc* gene abundance of *P. foetida* strain 1803-12 reached a peak with $1.94\times10^6$ copies/mL at 12 and 16 days. *P. foetida* strain PTG reached the maximum *mtc* gene abundance

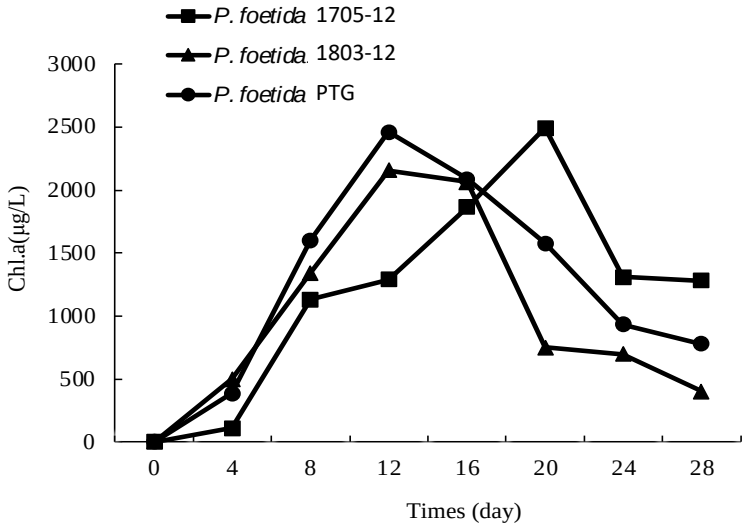


**Figure 1**. Chl.a of *Pseudanabaena foetida* strain 1705-12, strain 1803-12, and strain PTG.

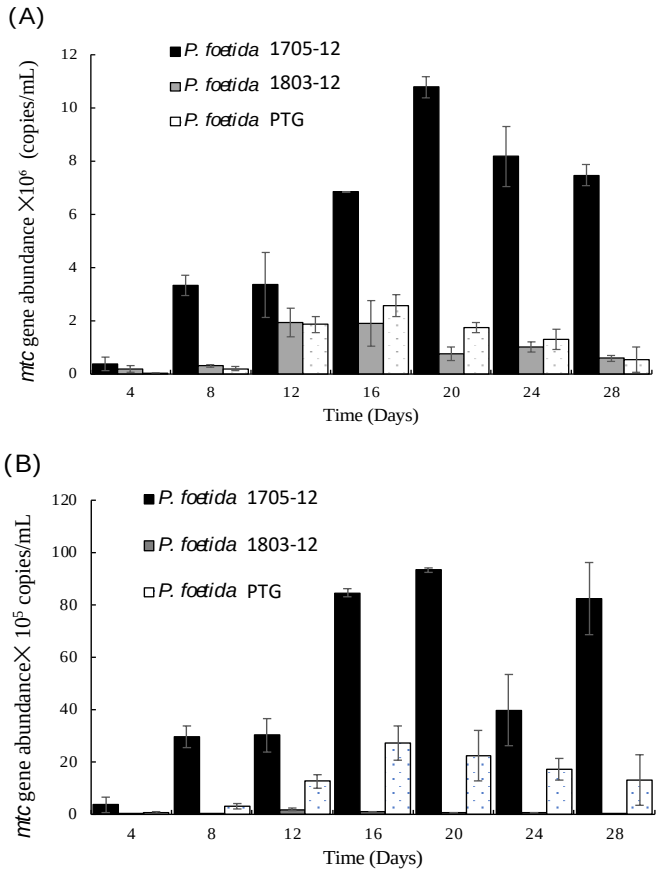


**Figure 2.** Abundance of *mtc* gene using qPCR. SYBR Green qPCR (A), TaqMan qPCR (B).

was $2.58\times10^6$ copies/mL at 16 days. Figure 2B shows the *mtc* gene abundance using qPCR with TaqMan probe assays. The *mtc* gene abundance of *P. foetida* strain 1705-12 reached maximum with $93.4\times10^5$ copies/mL at 20 days. *P. foetida* strain 1803-12 and *P. foetida* strain PTG reached their maximum *mtc* gene abundance with $15.2\times10^5$ copies/mL at 12 days and $25.8\times10^5$ copies/mL at 16 days, respectively.

Figure 3 shows a correlation between mtc gene abundance via qPCR using both SYBR Green and TaqMan probe assays and relative brightness of whole-cell PCR. The correlation of *P. foetida* strain 1705-12, P. *foetida* strain 1803-12, and *P. foetida* strain PTG was 0.95, 0.88, and 0.89, respectively, as compared to qPCR using SYBR Green assay (Figure 3A1, B1, ad C1). The correlation of *P. foetida* strain 1705-12, *P. foetida* strain 1803-12, and *P. foetida* PTG was 0.94, 0.94, and 0.76, respectively, as compared to qPCR using TaqMan probe assay (Figure 3A2, B2, ad C2). The positive correlation between *mtc* gene abundance via qPCR and relative brightness of whole-cell PCR suggested that the whole-cell PCR assay could detect and quantify *mtc* gene to estimate the population of 2-MIB-producing cyanobacteria.

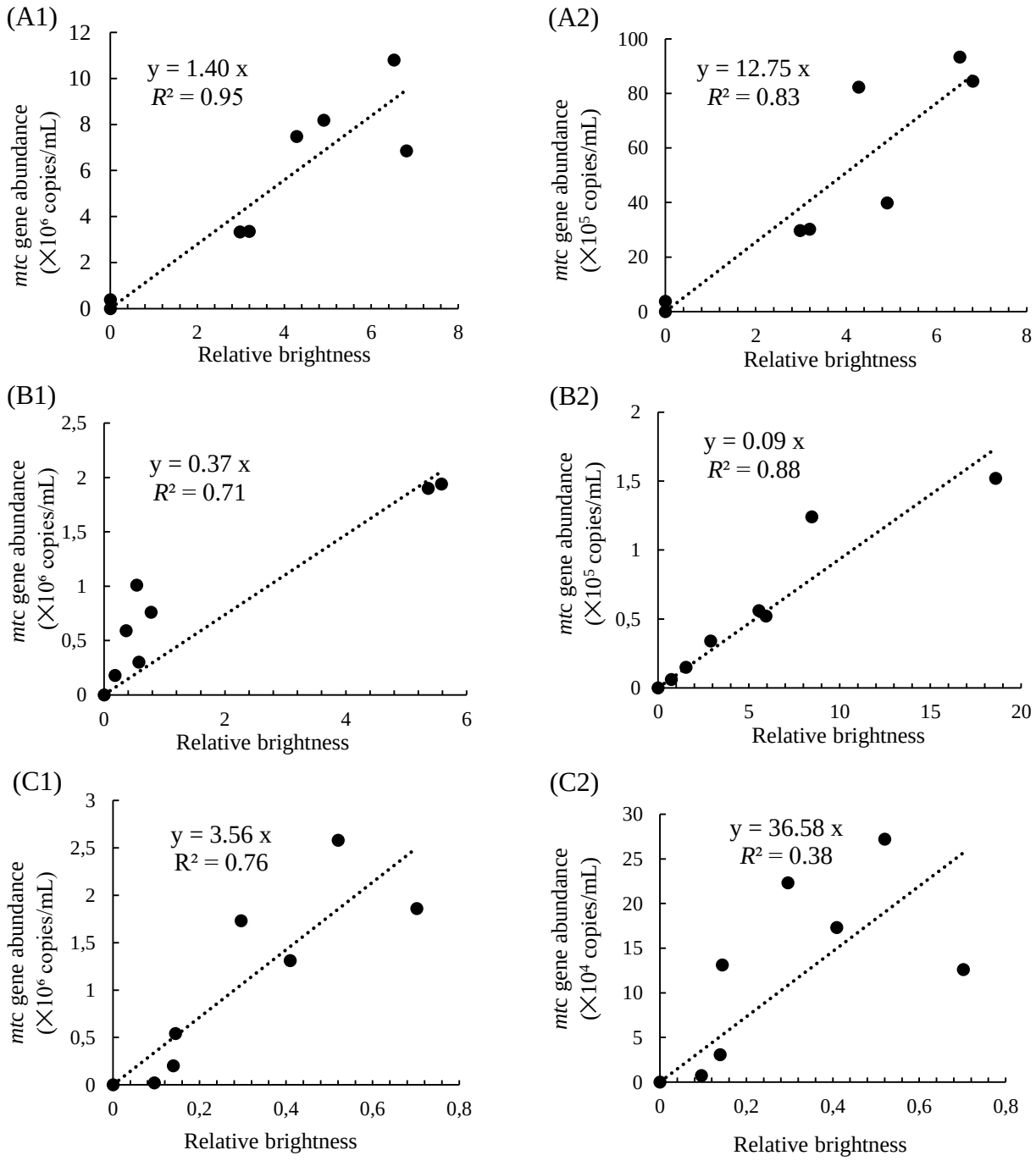


**Figure 3**. Correlation between *mtc* gene abundance and relative brightness of Whole-Cell PCR of *P. foetida* 1705-12 via qPCR using both SYBR Green and TaqMan Probe assay (A), *P. foetida* 1803-12 (B), *P. foetida* PTG (C). SYBR Green qPCR (A1, B1, C1), and TaqMan probe qPCR (A2, B2, C2).

**Applicability Analysis of Whole-cell qPCR**

The culture of *P. foetida* strain 1705-12, *P. foetida* strain 1803-12, and *P. foetida* strain PTG was tested to quantify *mtc* gene abundance, leading to the quantification of population to assess whole-cell qPCR assays. The amplification efficiencies of *P. foetida* strain 1705-12, strain 1803-12, and strain PTG were 102.2%, 100.0%, and 108.0% via whole-cell qPCR using SYBR Green assays, respectively, with a linear range from $4.5\times10^1$ to $4.5\times10^7$ *mtc* gene copies ($R^2$=0.99, 0.97, and 0.99, respectively). The *mtc* gene copy number of *P. foetida* strain 1705-12 was $1.51\times10^8$ to $1.33\times10^9$ copies/mL, and the strain reached the maximum value at 20 days. The *mtc* gene copies of *P. foetida* strain 1803-12 varied from approximately $8.89\times10^6$ to $6.20\times10^9$ copies/mL. The *mtc* gene copies of *P. foetida* strain PTG varied from approximately $3.86\times10^7$ to $2.05\times10^{10}$ copies/mL. *P. foetida* strain 1803-12 and *P. foetida* strain PTG reached the maximum value at 12 days. Correlation of *mtc* copy number via whole-cell qPCR using SYBR Green assay and the corresponding Chl. a concentration as the biomass from *P. foetida* strain 1705-12, strain 1803-12, and strain PTG showed positive relationship as $R^2$=0.95, 0.70, and 0.71, respectively (Figure 4). Correlation of *mtc* gene copy number via whole-cell qPCR using SYBR Green assay and the *mtc* copy number via qPCR from *P. foetida* strain 1705-12, strain 1803-12, and strain PTG showed positive relationship as $R^2$=0.83, 0.46, and 0.65, respectively (Figure 5). The strong positive correlation revealed that the whole-cell qPCR using SYBR Green assay is a valuable method to quantify 2-MIB-producing cyanobacteria.

For whole-cell qPCR TaqMan Probe assay, the amplification efficiencies of *P. foetida* strain 1705-12, strain 1803-12, and strain PTG were 110.1%, 110.7%, and 104.7% via whole-cell qPCR using TaqMan probe assay, respectively, with a linear range from $44.4\times10^1$ to $44.4\times10^7$ *mtc* gene copies ($R^2$=0.99, 0.98, and 1.00, respectively). The *mtc* gene copy numbers of *P. foetida* strain 1705-12, strain 1803-12, and strain PTG cultures varied from approximately $1.14\times10^8$ to $1.85\times10^9$ copies/mL, from $5.56\times10^4$ to $1.48\times10^7$ copies/mL, and from $2.01\times10^6$ to $2.51\times10^9$ copies/mL, respectively. *P. foetida* strain 1705-12 reached the maximum value at 20 days, and *P. foetida* strain 1803-12 and *P. foetida* strain PTG reached the maximum value at 12 days. Correlation of *mtc* copy number via whole-cell qPCR using TaqMan probe assay, and the corresponding Chl. a concentration as the biomass from *P. foetida* strain

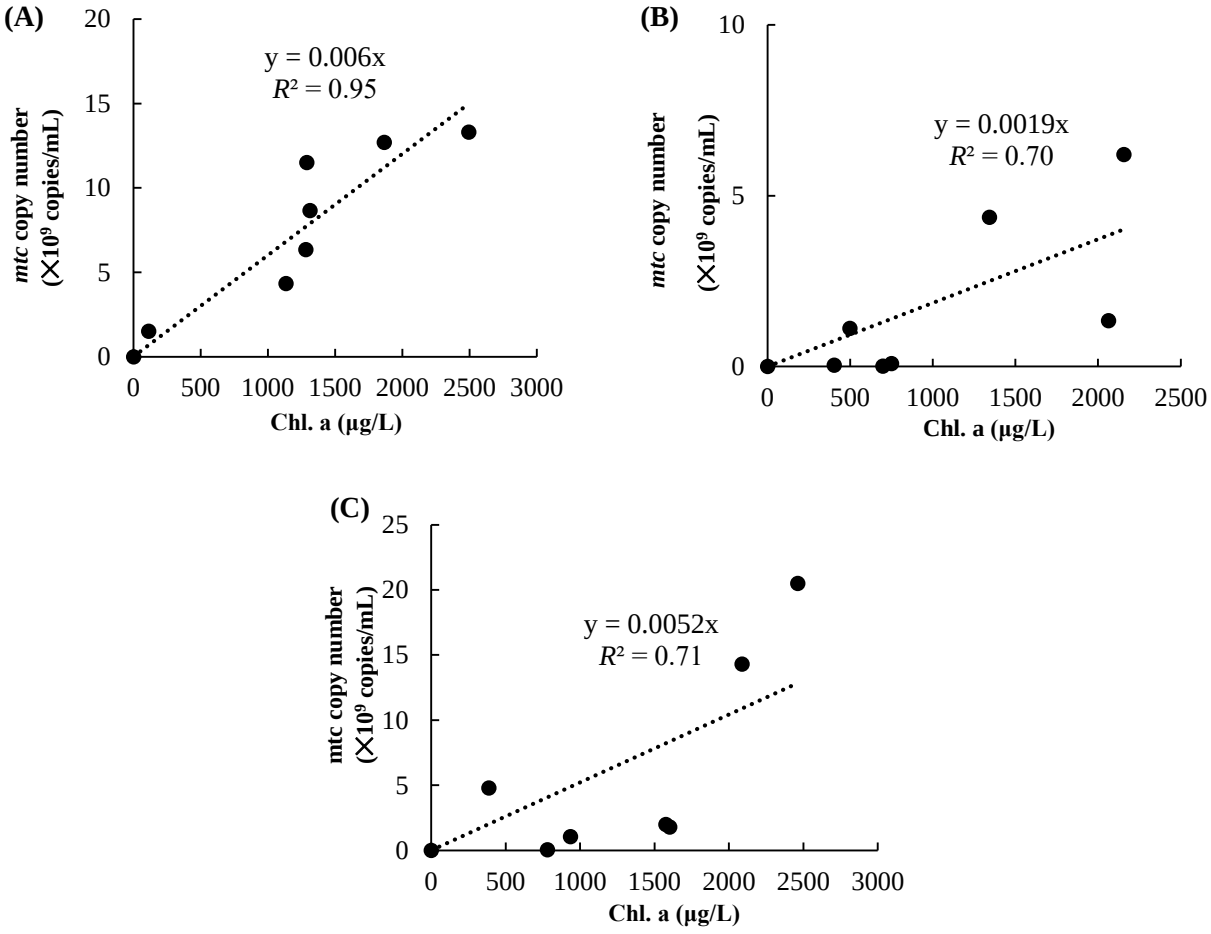


**Figure 4.** Correlation between Chl. a and *mtc* gene copy number using whole-cell SYBR Green qPCR of *P. foetida* 1705-12 (A), *P. foetida* 1803-12 (B), *P. foetida* PTG (C).

1705-12, strain 1803-12, and strain PTG showed positive relationship as $R^2$=0.94, 0.71, and 0.71, respectively (Figure 6). The correlation between *mtc* copy number via qPCR and whole-cell qPCR using TaqMan probe assay from three strains, *P. foetida* strain 1705-12, strain 1803-12, and strain PTG, showed as $R^2$=0.83, 0.52, and 0.25, respectively (Figure 7). The strong positive correlation revealed that whole-cell qPCR using the TaqMan probe assay could rapidly detect and quantify 2-MIB-producing cyanobacteria.

## Discussion

Due to the limiting factors of chemical and traditional biological monitoring analyses such as the inability to estimate the populations of musty and earthy odor producers, requirement of analytical techniques, high cost of installation of analytical instruments, and long analysis times, there is no warning system to forecast T&O occurrence in fishery farms and drinking water plants. PCR/qPCR is a method to determine the population of T&O-producing cyanobacteria. qPCR was first reported for monitoring geosmin-producing *Anabaena* populations in freshwater (Ming Su *et al*., 2013). After some years, qPCR was found to monitor 2-MIB-producing cyanobacteria (Wang *et al*., 2016). However, PCR and qPCR require a long time because DNA needs to be extracted from the sample before PCR/qPCR operation (Giglio *et al*., 2011; Wang *et al*., 2016). The manipulation of DNA extraction requires a long time, causing a heavy burden on the staff in Waterworks and fishery farms. The rapid detection and quantification methods of 2-MIB-producing cyanobacteria developed in this study is based on the PCR/qPCR detection method, and it is more rapid and easy to operate.

The gene-based method for 2-MIB event forecast systems is more accurate and specific than the traditional time-consuming cell count method (Lu *et al*., 2019). Numerous studies have shown that cell count is positively correlated with the *mtc* gene number (Devi *et al.*, 2021), thus, this study attempted to quantify *mtc* gene number using cells directly as a template for PCR and qPCR. PCR has more experimental errors, such as errors in sampling, DNA extraction, and different DNA extraction methods lead to variation extraction efficiency. However, whole-cell PCR and whole-cell qPCR can reduce the errors caused by DNA extraction.

Whole-cell PCR and whole-cell qPCR assays are not necessary to extract DNA, and the cell can be directly

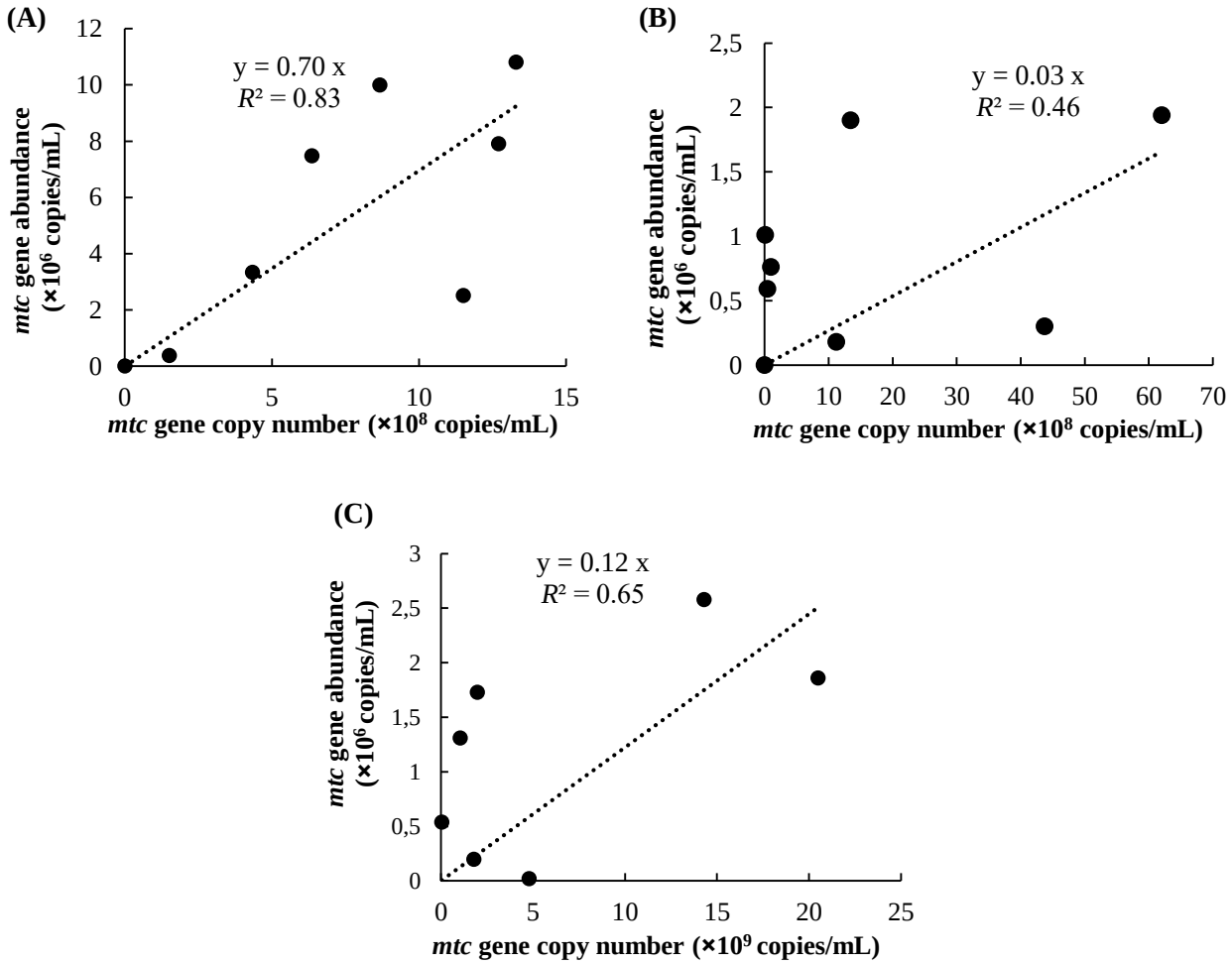


**Figure 5.** Correlation between *mtc* gene abundances using qPCR and *mtc* gene copy number via whole-cell SYBR Green qPCR (intercalate) of *P. foetida* 1705-12 (A), *P. foetida* 1803-12 (B), *P. foetida* PTG (C).

used as a template, thus, saving up to 2 to 5 h per sample. Additionally, whole-cell PCR uses a low price thermal cycler at small waterworks and fishery farms as compared to the thermal cycler for qPCR. Whole-cell PCR and whole-cell qPCR can be used as rapid and reliable methods to detect microcystin producers (Li *et al*., 2011; Al-Tebrineh J *et al.*, 2011; Ban *et al*., 2006). Additionally, microcystin is another compound produced by cyanobacteria. Li *et al*. (2011) found high positive correlations ($R^2$=0.98) of Microcystis colonies with *mcyA*-containing cells using microscopic count quantified via whole-cell qPCR.

The positive relationship ($R^2$=0.46 to 0.95) between *mtc* gene abundance determined via qPCR and the results of whole-cell qPCR and whole-cell qPCR assays revealed that whole-cell PCR and whole-cell qPCR could determine the population of 2-MIB producers to forecast 2-MIB outbreak in water reservoirs and freshwater fishery farms. Although qPCR using extracted DNA is reliable for monitoring, Whole-cell PCR/qPCR detects 2-MIB-producing cyanobacteria and is accurate and sensitive enough to monitoring. In addition, the manipulation time for the Whole-cell PCR/qPCR is less than qPCR using extracted DNA because we do not need to extract DNA from the sample. Further studies should focus on the accuracy assessment of whole-cell PCR and whole-cell qPCR and improving the actual environmental water or freshwater fishery. Extending studies on whole-cell PCR/whole-cell qPCR assays for 2-MIB-producing cyanobacteria will make the monitoring of water quality more feasible, rapid, and sensitive, allowing an early forecast of T&O events.

## Ethical Statement

This study has been conducted in an ethical and responsible manner, and s n full compliance with all relevant codes of experimentation and legislations.

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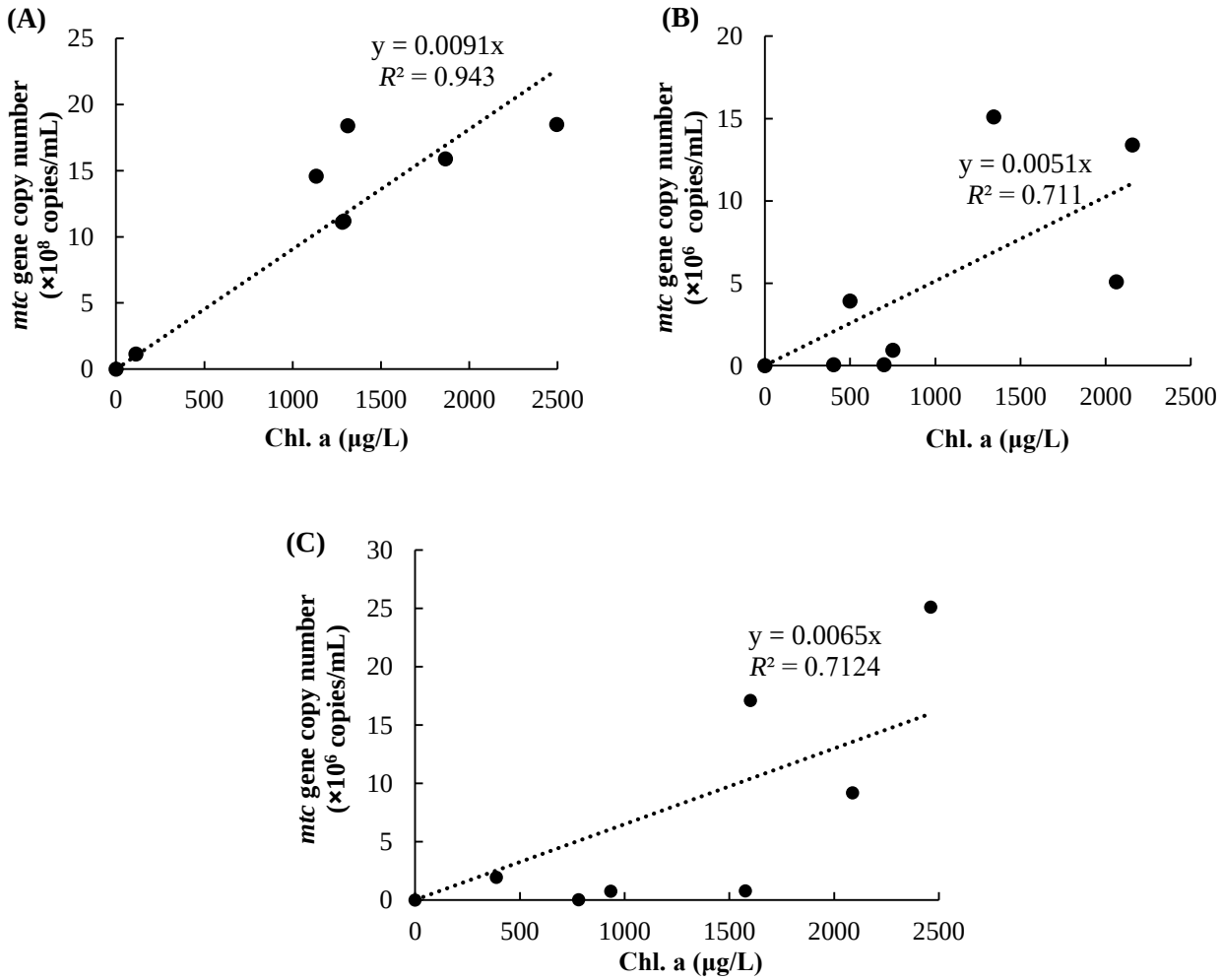


**Figure 6.** Correlation between Chl. a and *mtc* gene copy number using whole-cell TaqMan probe qPCR of *P. foetida* 1705-12 (A), *P. foetida* 1803-12 (B), *P. foetida* PTG (C).

## Author Contribution

Ji Zhang: the data curation, formal analysis, conceptualization, writing—original draft, methodology, investigation, and validation; Qingyue Shen: the writing—original draft, data curation, and validation; Hanchen Miao: the investigation and methodology; Qintong Li: the data curation and writing—review and editing; Marie Shimada: the resources, data curation, and validation; Yuan Tian: the writing—review and editing; Motoo Utsumi: the writing—review and editing, resources, and supervision; Zhongfang Lei: the writing—review and editing and supervision; Zhenya Zhang: the writing—review and editing and supervision; Hirokazu Takanashi: the writing—review and editing and conceptualization; Naoshi Fujimoto: the resource and writing—review and editing; Satoshi Ichise: the resources and writing—review and editing; Yasuhiro Asada: the resource and writing—review and editing; Osamu Nishimura: the methodology and writing—review and editing; Michihiro Akiba: the conceptualization, funding acquisition, project administration, and writing—review and editing; Kazuya Shimizu: the conceptualization, writing—original draft, writing—review and editing, supervision, and project administration. All of the authors read and approved the final manuscript.

## Conflict of Interest

The authors declare that we have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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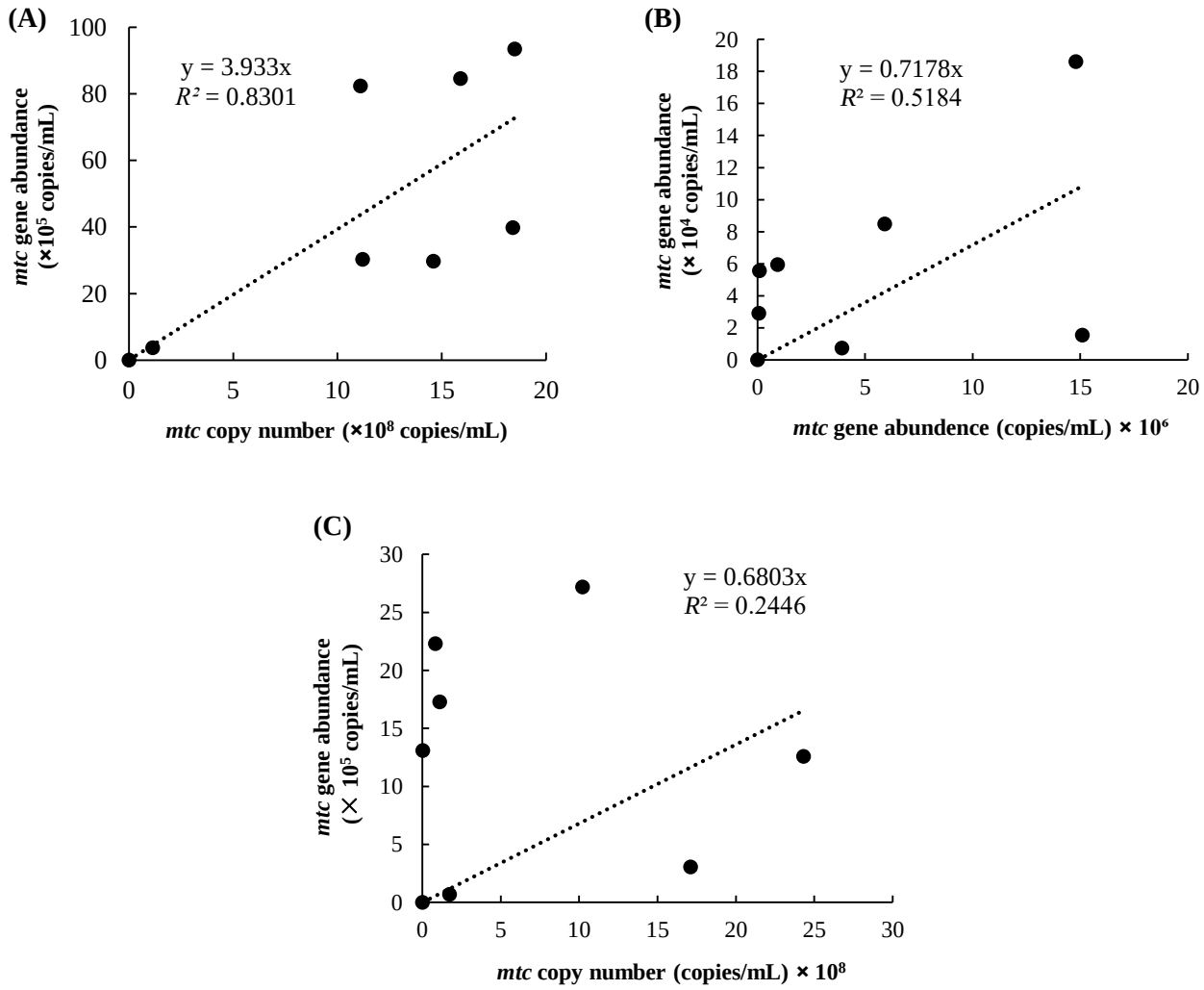


**Figure 7.** Correlation between *mtc* gene abundances using qPCR and *mtc* gene copy number via whole-cell TaqMan probe qPCR of *P. foetida* 1705-12 (A), *P. foetida* 1803-12 (B), *P. foetida* PTG (C).

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