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Real-Time PCR Detection of Host-Mediated Cyanophage Gene Transcripts during Infection of a Natural *Microcystis aeruginosa* Population

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The aim of this study was to develop a quantitative real-time reverse transcription-PCR (real-time RT-PCR) assay to detect and quantify mRNA of cyanophages within infected *Microcystis aeruginosa* cells in a freshwater pond. Laboratory-based data showed that the relative abundance of the cyanophage *g91* mRNA within host cells increased before cyanophage numbers increased in culture. This transcriptional pattern indicated the kinetics of the viral infection suggesting the real-time RT-PCR method to be a potential tool for environmental monitoring of cyanophage infections. In this field survey, the numbers of infected *M. aeruginosa* cell populations estimated from cyanophage numbers were low at 0.01–2.9 cells mL⁻¹. The highest relative abundance of phage *g91* RNA (10⁻² per *rnpB* transcript) was at about the same levels of expression as laboratory-based growth data for Ma-LMM01 (estimated density of infected host cells: 10⁵ cells mL⁻¹); and was observed when cyanophage numbers rapidly increased (as well as a decrease in host cell numbers). Quantification of cyanophage numbers is important to understand ecological relationships between the phage and its hosts. Our data suggest the quantification of phage gene transcripts within a natural host cell population to be a strong tool for investigating the quantitative effects of phage lysis during infection of the host population.

Key words: cyanophage, *Microcystis aeruginosa*, real-time RT-PCR, succession, toxic cyanobacteria

Microcystis aeruginosa is a well-known toxic cyanobacterial species that commonly develops blooms in eutrophic freshwater throughout the world. This species includes strains that can produce potent hepatotoxins called microcystins (2). There are several reports of deaths in wild and domestic animals as well as humans due to acute poisoning which causes massive hepatic hemorrhage (1, 6, 16).

Cyanophages are considered to be a significant factor regulating the abundance, clonal diversity, and composition of their cyanobacterial host populations (9, 10, 12, 14, 15, 19, 20, 25). The phages also play a major role in nutrient cycling and genetic transfer (21, 22, 26). In contrast to the vast majority of research having focused on marine cyanophages (12, 14, 15, 19, 20, 25), there are few studies concerning freshwater cyanophages (9, 10). Reports suggest phages play an important role in regulating the bloom dynamics of *M. aeruginosa* blooms. Manage *et al.* (9, 10) observed an increase in cyanophage titers (the numbers of particles forming plaques on an *M. aeruginosa* lawn) accompanied by a large decrease in the abundance of *M. aeruginosa* in a natural freshwater environment. Recently, during a field survey of a Japanese freshwater lake, real-time monitoring of *M. aeruginosa*-cyanophage abundance with quantitative PCR assays showed the seasonal dynamics of the cyanophage community in freshwater that may affect

shifts in the clonal composition of diverse *M. aeruginosa* populations (*e.g.*, microcystin-producing and non-producing populations); rather than having a quantitative impact on the total *M. aeruginosa* abundance (31). Despite the various molecular approaches used to study the diversity and population dynamics of both marine cyanophages (3, 8, 13, 24, 27, 28, 35) and freshwater cyanophages (4), there have been no studies concerning environmental monitoring and the seasonal patterns of cyanophage gene expression during infection in a natural host population.

The aim of this study was to develop a quantitative real-time reverse transcription-PCR (real-time RT-PCR) assay that could detect and quantify the RNA of cyanophages within naturally infected *M. aeruginosa* cells in a freshwater pond. For the real-time RT-PCR assay, we used primers targeting the viral sheath protein-coding gene (*g91*) of *M. aeruginosa* cyanophages (23). Because *g91* is a highly conserved region among isolated *M. aeruginosa* cyanophages, real-time PCR amplification of this gene has been used to quantify *M. aeruginosa* cyanophages in environmental samples (23, 31). In laboratory experiments, we show using real-time RT-PCR that cyanophage strain Ma-LMM01 *g91* mRNA from infected *M. aeruginosa* NIES298 can be detected; and *g91* expression and the temporal dynamics during the infection can be monitored. This approach may be useful for *in situ* monitoring of cyanophage infections of host cells. We therefore examined whether this monitoring system can detect the dynamics of potentially *M. aeruginosa*-

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infectious cyanophage transcripts in host cell samples from Hirosawanoike Pond.

Materials and Methods

Laboratory host infection experiments and sample collection

M. aeruginosa strain NIES298 was obtained from the National Institute for Environmental Studies (NIES), Environmental Agency, Japan. It was grown at 30°C in CB medium (7) at a light intensity of *ca.* 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under a 12 h light-12 h dark cycle under cool fluorescent lamps (32) with 0.5% (v/v) CO₂ aeration. An exponentially growing culture of *M. aeruginosa* NIES298 was inoculated at an initial cell density of 1×10^5 cells mL⁻¹ into 2 L of CB medium and incubated. After 4 d, exponentially growing host cells (about 1×10^6 cells mL⁻¹) were filtered through a 3.0- μm polycarbonate membrane (Millipore, Bedford, MA, USA). The host cells collected on the filter were infected with cyanophage strain Ma-LMM01 at a multiplicity of infection (MOI) of 0.01. After Ma-LMM01's addition (0 time), 310 mL of the sample was collected from each culture at 0, 1, 3, 6, 9, and 12 h. Each sample was fractionated and used for analyses. Subsamples were used for a transcriptional analysis of cyanophage mRNA and quantitative analysis of phage DNA.

For the transcriptional analysis of the cyanophage mRNA, 300 mL of the culture with infected cells was collected on a 3- μm PTFE membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) and transferred into a 50-mL tube containing 4 mL of stop solution (TE-saturated phenol:ethanol=5:95 [v/v]) to kill the host cells rapidly and to stop any degradation reactions (17). After resuspending the cells from the filter mixing using vortexing, the filter was removed and the suspension was stored at -20°C. For the DNA analysis, 10 mL of the sample collected at 24 h was filtered through a 0.2- μm polycarbonate filter (Toyo Roshi Kaisha) and concentrated using ultracentrifugation at $111,000 \times g$ for 1.5 h at 4°C. The phage DNA was extracted as described previously (23). Each extracted DNA was used as the PCR template for real-time PCR.

Environmental sampling

A 410-mL water sample was collected from the surface (0.5 m) of Hirosawanoike Pond (Japan) once every month from May 2006 to November 2008 at a fixed point (35°02' N, 135°41' E). For bacterial DNA extraction, particles including cyanobacterial cells from 100 mL of the pond water were sonicated gently and harvested using centrifugation at $14,400 \times g$ for 10 min. DNA was extracted and purified using the previously described xanthogenate method (29). For viral nucleic acid extraction, 10 mL and 300 mL of the samples were used for DNA and RNA, respectively.

RNA extraction, purification, and cDNA synthesis

Total RNA was extracted from 1 mL of the stored cell suspension (33). Cells collected by centrifugation were treated with 5% (w/v) SDS, and the RNA was extracted using 1 mL of Sepazol RNA I (Nacalai Tesque, Kyoto, Japan) followed by 200 μL of

chloroform. Two additional extractions were made with TE-saturated phenol and chloroform/isoamylalcohol (49:1 [v/v]). The RNA was precipitated by adding one volume of isopropanol; and the precipitate was washed with 70% (v/v) ethanol. The pellet was re-suspended in dimethyl-dicarbonate (DMDC)-treated water and the DNA was removed using a Turbo DNA-free kit (Applied Biosystems, Foster City, CA, USA). For cDNA synthesis, 1 to 2 μg of RNA was reverse transcribed with random hexamers using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for reverse transcription-PCR; and the cDNA was treated with 2 U of RNase H for 20 min at 37°C according to the manufacturer's instructions.

Real-time PCR and real-time RT-PCR

The primers for real-time PCR and real-time RT-PCR are listed in Table 1. The real-time PCR primer pairs, 188F-254R, M1rF-M1rR, and SheathRTF-SheathRTR (Table 1), were used to amplify the phycocyanin intergenic spacer (PC-IGS) gene (66 bp), the microcystin synthetase gene, *mcyA* (107 bp), and the *g91* gene (132 bp) fragments, respectively. The PC-IGS and *mcyA* real-time PCR were used to quantify numbers of the total *M. aeruginosa* and the potentially microcystin-producing *M. aeruginosa* subpopulation, respectively, in natural water samples, as previously described by Yoshida *et al.* (30). This method could distinguish between microcystin-producing and non-producing *M. aeruginosa* in natural mixed populations. *g91* real-time PCR was conducted as described by Takashima *et al.* (23). For the real-time RT-PCR to detect the cyanophage RNA and the host *M. aeruginosa* RNA as an internal control, the primer sets SheathRTF-SheathRTR and *rnpB*RTF-*rnpB*RTR (Table 1) targeting the cyanophage *g91* and RNase P RNA (*rnpB*) genes of *M. aeruginosa* (33), respectively, were used.

External standards used to determine the PC-IGS, *mcyA*, and *rnpB* and *g91* copy numbers were prepared using the genomic DNA from *M. aeruginosa* strain NIES298 and the cyanophage strain Ma-LMM01, respectively. Copy numbers of these genes were enumerated using the size of each DNA fragment. A 10-fold dilution series of the DNAs was prepared and amplified with the PC-IGS, *mcyA*, *rnpB*, and *g91* real-time PCR assays. For these genes, linear regression equations to obtain cycle threshold (*C_t*) values were calculated as a function of the known DNA copy numbers.

Each real-time RT-PCR mixture contained 25 μL of PCR Master Mix (Toyobo, Osaka, Japan) and 200 nM of each primer. After initial denaturing for 1 min at 95°C, 35 cycles were performed: 15 s at 95°C, 15 s annealing, and 30 s extension (Table 1) using an iCycler real-time PCR machine (Bio-Rad, Hercules, CA, USA). The iCycler software analysis program (Bio-Rad) was used to calculate the *C_t* values to determine the sample concentrations as compared to a standard count. A standard curve of the *C_t* values obtained from serially diluted PCR products for the *g91* gene was constructed first; then the relative transcription amounts were calculated using the *rnpB* gene transcripts of the host *M. aeruginosa* to normalize the data. All tests were performed using three samples that were taken at the same time and location; and each was processed separately.

Table 1. Primers used in this study

Primer	Target gene	Sequence (5' to 3')	Temperature (°C)		Reference
			Annealing	Extension	
188F	PC-IGS	GCT ACT TCG ACC GCG CC	60	84	(30)
254R	PC-IGS	TCC TAC GGT TTA ATT GAG ACT AGC C			(30)
M1rF	<i>mcyA</i>	AGC GGT AGT CAT TGC ATC GG	65	83	(30)
M1rR	<i>mcyA</i>	GCC CTT TTT CTG AAG TCG CC			(30)
SheathRTF	<i>g91</i>	ACA TCA GCG TTC GTT TCG G	58	84	(23)
SheathRTR	<i>g91</i>	CAA TCT GGT TAG GTA GGT CG			(23)
<i>rnpB</i> RTF	<i>rnpB</i>	GTG GGG AGC AAG GTG G	56	80	This study
<i>rnpB</i> RTR	<i>rnpB</i>	CTT TTA CCT TTG TTG GAA TAG AG			This study

Results and Discussion

Transcriptional pattern of the g91 M. aeruginosa-cyanophage gene during infection in laboratory experiments

The *g91* gene expression of the cyanophage Ma-LMM01 was examined *in vitro* to determine whether the real-time RT-PCR assay could be used to monitor the changes in the abundance of the phage transcripts during the infection cycles of the host *M. aeruginosa* NIES298 culture (Fig. 1). To normalize the raw expression levels of the phage *g91* mRNA, the relative abundance of *g91* compared to the control gene *rnpB* was obtained. No phage *g91* mRNA was detected from infected host cells at the onset of infection. At 1 h post infection (PI), the relative abundance of *g91* in Ma-LMM01 was 2.1×10^{-4} . This value increased rapidly, reaching a maximum at 6 h PI of 10^{-1} and remained high for the rest of the sampling period. The period (0–6 h PI) when the *g91* relative abundance increased significantly fit well within the Ma-LMM01 latent period (6–12 h PI) as previously reported for the infection kinetics of this phage (34).

To examine the abundance and fluctuation of potentially

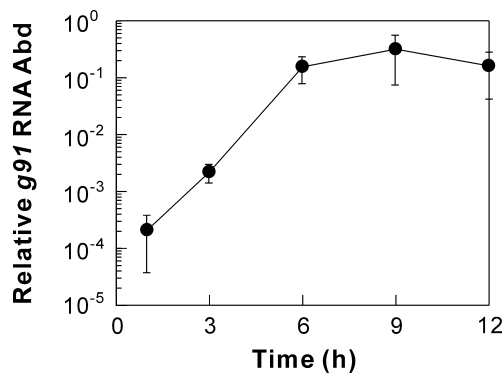


Fig. 1. Expression profiles of the relative amounts of Ma-LMM01 *g91* RNA within infected *Microcystis aeruginosa* NIES298 cells. *g91* relative abundance was monitored using real-time RT-PCR and determined by dividing the numbers of RNA copies in the cyanophages by the numbers of RNA copies of the host *M. aeruginosa* determined using the *rnpB* primer set. No phage *g91* mRNA was detected in host cells at the onset of infection (the detection limit, $<1 \times 10^{-5}$). Abd: abundance.

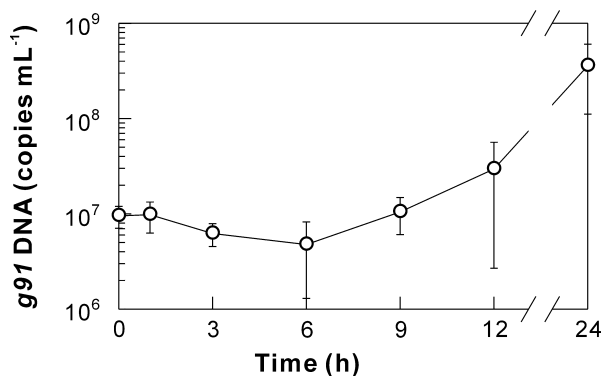


Fig. 2. Changes in Ma-LMM01 abundance determined using real-time PCR with the *g91* primers. The error bars indicate the SD from three samples taken at the same time and location; and each sample was processed separately.

M. aeruginosa-infectious cyanophages during a cyanophage infection, phage *g91* DNA in the viral filtrate samples from the host culture was monitored using the real-time PCR assay (Fig. 2). The numbers of Ma-LMM01 *g91* DNA copies did not change between 0 and 9 h PI (approximately 10^7 copies mL^{-1}); and thereafter at 24 h PI *g91* sharply increased to above 10^8 copies mL^{-1} . The increase in copy numbers of Ma-LMM01 *g91* DNA from the culture filtrates indicated the continuous replication of cyanophage DNA and eventual release of the phage particles harboring the *g91* DNA during the infection cycle.

Seasonal patterns of M. aeruginosa-cyanophage transcripts during infection in M. aeruginosa populations

Using the real-time PCR assays, the abundance of *M. aeruginosa* and the cyanophages in Hirosawanoike Pond were monitored during the sampling period of 2006–2008 (Fig. 3A). The PC gene copy numbers of *M. aeruginosa* were between 1.3×10^3 and 5.8×10^8 copies mL^{-1} during the field survey. During this period, the cyanophage *g91* DNA copy numbers ranged from 1.5×10^0 to 1.7×10^3 copies mL^{-1} . However, there was no clear relationship between the phage abundance and the host *M. aeruginosa* numbers. The numbers of infected *M. aeruginosa* cells as estimated from the cyanophage abundance in Hirosawanoike Pond were low at 0.01–2.9 cells mL^{-1} . The infected host cell numbers (*i*) per milliliter at each sampling date was calculated using $i=C/b$ where *C* is the cyanophage abundance per milliliter at each sampling date and *b* is the burst size of the phage Ma-LMM01 (*ca.* 100 cells⁻¹) (34). Previously, we reported that Lake Mikata (Japan) cyanophage abundance was inversely correlated with the numbers of *M. aeruginosa*; whereas the cyanophage assemblage may infect only in a small percentage of the *M. aeruginosa* population (31). Similarly, the data shown here also suggested the influence of the cyanophages only resulted in replacement of phage-sensitive populations by phage-insensitive populations; rather than a quantitative impact on the entire *M. aeruginosa* population (31).

To evaluate the effect of the cyanophages on the internal dynamics of the total *M. aeruginosa* community, we examined the fluctuation in the abundance of potentially microcystin-producing *M. aeruginosa* populations using real-time PCR targeting the *mcyA* gene; and monitored the relative size of the microcystin-producing subpopulation compared to the total population in relation to cyanophage numbers in the field. The *mcyA* copy numbers of the potentially microcystin-producing populations were found to be between 2.5×10^2 and 2.1×10^7 copies mL^{-1} (Fig. 3B). The ratio of the *mcyA*-containing subpopulation to the total *M. aeruginosa* population was 3.6 to 47.5%. The relative abundance of the microcystin-producing subpopulation of *M. aeruginosa* fluctuated between bloom stages especially in 2006. In the early summer of 2006, a distinct shift in the relative abundance of the microcystin-producing subpopulation was observed when the cyanophage abundance increased and the *M. aeruginosa* abundance declined notably (Fig. 3A). Thus, our data show viral infection to be a factor, at times, and related to shifts in the composition of the different *M. aeruginosa* populations present during the bloom season as previously reported (31).

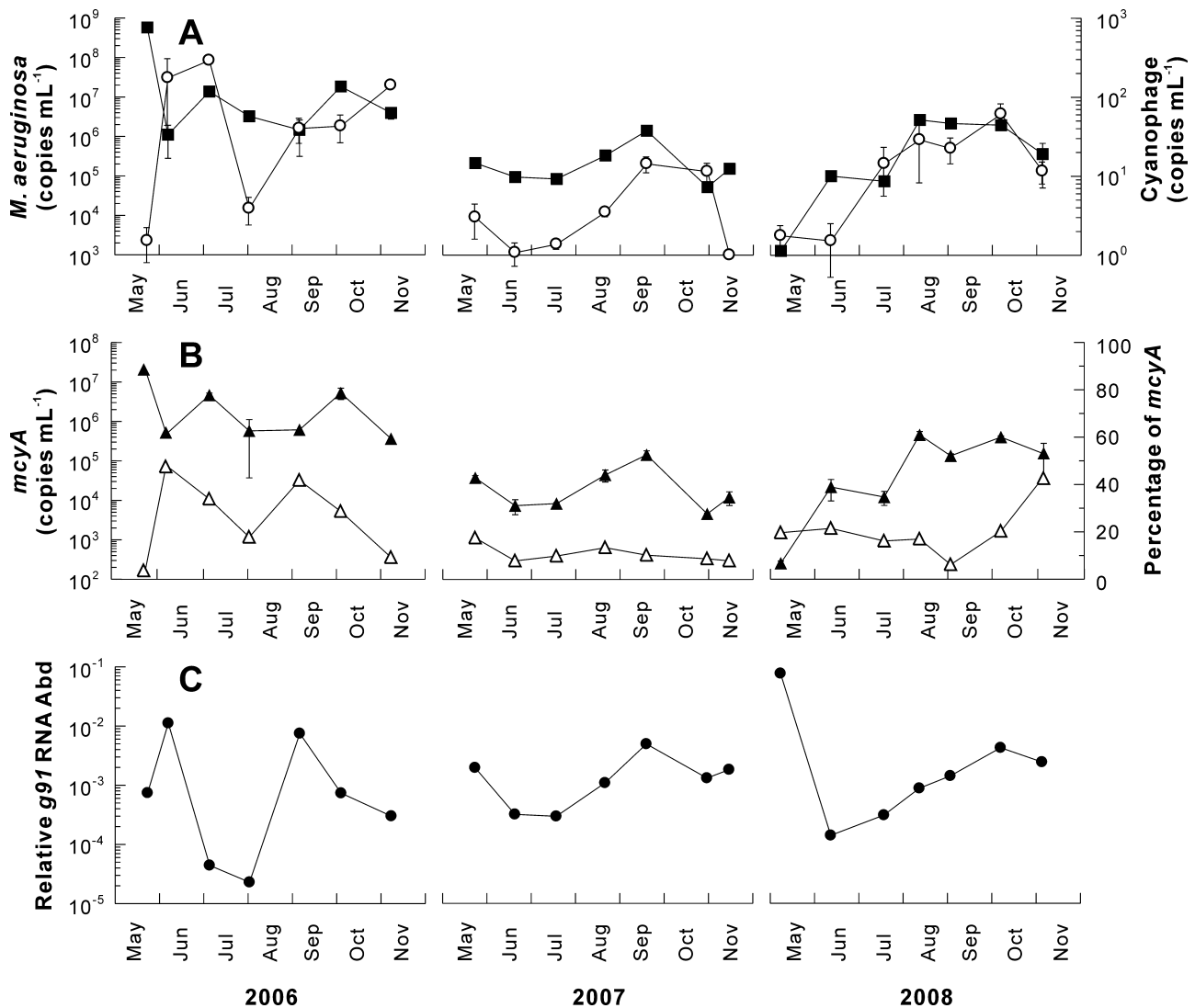


Fig. 3. (A) *Microcystis aeruginosa* (PC-IGS, closed square) and cyanophage (*g91*, opened circle) abundance in Hirosawanoike Pond from May 2006 to November 2008. The abundance of *M. aeruginosa* and the cyanophage was determined using the PC-IGS and *g91* real-time PCR, respectively. The error bars indicate the SD ($n=3$). (B) *mcyA* subpopulation abundance (closed triangle) and percentage of *mcyA* (opened triangle) in Hirosawanoike Pond from May 2006 to November 2008. The numbers of *mcyA* gene copies per milliliter were determined using real-time PCR. The percentage of *mcyA* was determined by dividing the numbers of DNA copies in the *mcyA* subpopulation by the total number of copies in *M. aeruginosa* determined using the PC-IGS primer set. (C) Expression of the cyanophage *g91* RNA within the infected host cells in Hirosawanoike Pond from May 2006 to November 2008. *g91* relative expression was monitored using real-time RT-PCR and determined by dividing the numbers of RNA copies from the cyanophages by the number of copies in the host *M. aeruginosa* determined using the *rnpB* primer set. Data are means of measurements ($n=3$). Abd: abundance.

g91 mRNA was observed throughout the field survey; and its relative abundance compared to the host *rnpB* mRNA was 2.3×10^{-5} to 7.6×10^{-2} (Fig. 3C). In June and September of 2006, the relative abundance of phage *g91* RNA was high (10^{-2} per *rnpB* transcript) in comparison to the Ma-LMM01 growth data (Fig. 1) showing the possible proliferation of cyanophages in the host cells. In addition, a rapid increase in the numbers of cyanophages and a clear decline in the host *M. aeruginosa* numbers were also found during May to June and August to September of 2006. Therefore, the possible proliferation of cyanophages in the host cells may account for the lysis of the *M. aeruginosa* associated with the phage in the pond water. In contrast to the data from 2006, the changes in the relative amounts of the phage RNA transcripts during 2007 and 2008 showed a similar temporal pattern to

the *M. aeruginosa* numbers. This trend was also found in the relationship between the cyanophage and *M. aeruginosa*. This data implies that the viral infection did not contribute to the control of *M. aeruginosa* abundance during the 3-year study period. However, the relative abundance of the cyanophage *g91* mRNA in this pond was at the same levels of expression as those from laboratory-based growth data on Ma-LMM01 (Fig. 1) where the estimated number of infected host cells was at least 10^5 cells mL⁻¹. This data suggests that viral infection occurs in a larger portion of the natural *M. aeruginosa* cell population (e.g. here, 0.01–2.9 cells mL⁻¹). Further, the results suggest that the cyanophage numbers in the pond determined by real-time PCR underestimate the actual numbers of phage particles released from the host population; and underestimate the quantitative effect of

phage lysis on overall *M. aeruginosa* abundance. This underestimation may be due to the rapid destruction of viruses in the environment (e.g., by solar radiation and increased water temperature) (5). Alternatively, grazing of the cyanobacteria by protozoa (e.g., heterotrophic nanoflagellates) may be involved in the decrease in the number of phages released into the water (11, 18).

In conclusion, we developed a real-time RT-PCR method using the *g91* gene of the *M. aeruginosa* phage Ma-LMM01; and used it to monitor the expression of the gene within infected host cells in laboratory experiments and a field survey. Phage infection is suggested to be an ordinary event occurring during the bloom season suggesting that the fresh-water cyanophage infection may not contribute to the control of *M. aeruginosa* abundance.

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