

# Effects of arsenate on the growth and microcystin production of *Microcystis aeruginosa* isolated from Taiwan as influenced by extracellular phosphate

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**Abstract** Arsenic pollution and eutrophication are both prominent issues in the aquaculture ponds of Taiwan. It is important to study the effects of arsenic on algal growth and toxin production in order to assess the ecological risk of arsenic pollution, or at least to understand naturally occurring ponds. The sensitivity of algae to arsenate has often been linked to the structural similarities between arsenate and phosphate. Thus, in this study we examined the effects of arsenate ( $10^{-8}$  to  $10^{-4}$  M) on *Microcystis aeruginosa* TY-1 isolated from Taiwan, under two phosphate regimes. The present study showed that *M. aeruginosa* TY-1 was arsenate tolerant up to  $10^{-4}$  M, and that this tolerance was not affected by extracellular phosphate. However, it seems that extracellular phosphate contributed to microcystin production and leakage by *M. aeruginosa* in response to arsenate.

Under normal phosphate conditions, total toxin yields after arsenate treatment followed a typical inverted U-shape hormesis, with a peak value of  $2.25 \pm 0.06$  mg L<sup>-1</sup> in the presence of  $10^{-7}$  M arsenate, whereas  $10^{-8}$  to  $10^{-6}$  M arsenate increased leakage of ~75% microcystin. Under phosphate starvation, total toxin yields were not affected by arsenate, while  $10^{-6}$  and  $10^{-5}$  M arsenate stimulated microcystin leakage. It is suggested that arsenate may play a role in the process of microcystin biosynthesis and excretion. Given the arsenic concentrations in aquaculture ponds in Taiwan, arsenate favors survival of toxic *M. aeruginosa* in such ponds, and arsenate-stimulated microcystin production and leakage may have an impact on the food chain.

**Keywords** Arsenate · Phosphate · Cyanobacterium · *Microcystis aeruginosa* · Microcystins

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## Introduction

Arsenic is ubiquitous in the environment and potentially toxic to humans (Smith et al. 2002). Its threat to the Western Pacific region has been noted (Suk et al. 2003). Of the various sources of arsenic in the environment, water-borne arsenic probably poses the greatest threat to human health. The spectrum of arsenic concentrations in natural waters ranges from 0.5 to 5,000 µg L<sup>-1</sup> (about  $6.7 \times 10^{-9}$  to  $6.7 \times 10^{-5}$  M; Smedley and Kinniburgh 2002). Arsenic species comprise arsenate, arsenite, monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), and other complex arseno-compounds such as arsenobetaine, arsenocholine and arsenosugars (Yukiko et al. 1996; Argese et al. 2005). Inorganic arsenic species can have a significant effect on the structure or physiology of phytoplankton communities (Knauer et al. 1999). Arsenate, the thermodynamically dominant

species of arsenic in marine and estuarine surface waters, is taken up by the phosphate transport systems of phytoplankton and plants due to its structural similarity to phosphate (Smedley and Kinniburgh 2002; Manomita et al. 2004). Arsenate has been demonstrated in many cases to significantly reduce phosphate uptake in a competitive fashion (Wångberg and Blanck 1990; Bun-ya et al. 1996; Bleeker et al. 2003). Studies have indicated that the sensitivity of phytoplankton to arsenate may be species-specific (Planas and Healey 1978; Knauer et al. 1999); however, algal sensitivity to arsenate is related not only to the phosphate concentration in the culture medium (Planas and Healey 1978; Thiel 1988), but also to nutritional history (Creed et al. 1990).

Cyanobacterial blooms in freshwaters, especially cyanotoxins from some of the blooms, pose a major challenge for the supply of safe drinking water. Globally, the most common cyanotoxins are the cyclic peptide hepatotoxins of the microcystin family, which are mainly from *Microcystis* blooms and can cause liver damage (Sivonen and Jones 1999). Like many other areas in the world, *Microcystis* blooms occur frequently in the eutrophic waters of Taiwan, especially in the aquaculture ponds used for eel, tilapia and carp (Lee et al. 1998). The groundwater used for aquaculture on the southwestern coast of Taiwan contains high concentrations of arsenic (Lin et al. 2004). It is not known whether arsenic has any relationship with cyanobacterial growth and toxin production in these aquaculture ponds. The water of culture ponds is dominated by arsenate (Huang et al. 2003). Therefore, our research, which investigated the effects of arsenate on *Microcystis aeruginosa* TY-1 isolated in Taiwan, under two phosphate regimes, was designed to answer the questions of whether extracellular phosphate protects cells from arsenate; and whether arsenate affects microcystin production and leakage under different phosphate regimes.

## Materials and methods

Axenic cultures of unicellular *M. aeruginosa* TY-1 were isolated from Tauryuan, Taiwan (Lee et al. 1998). The strain was cultivated under a constant cool-fluorescent light intensity of 35  $\mu\text{mol photons PAR m}^{-2} \text{ s}^{-1}$ , 16:8 h light: day cycle and a temperature of  $28 \pm 1^\circ\text{C}$  in BG-11 medium. The cells in stationary phase were harvested by centrifugation and washed three times with sterile Milli-Q water ( $18.0 \text{ m}\Omega \text{ cm}^{-1}$ ). Parts of the pellets were inoculated into 250 mL Erlenmeyer flasks in modified BG-11 medium, to which  $\text{PO}_4^{3-}$  was added at  $1.0 \mu\text{M}$  for the phosphate-normal medium or omitted for the phosphate-starved medium. Arsenate, in the form of  $\text{Na}_2\text{HAsO}_4$  was added to the culture medium at concentrations ranging from  $10^{-8}$  to  $10^{-4}$  M. The reason for the high concentrations of arsenate, such as  $10^{-4}$  M, was to explore the inhibitory threshold dose of

arsenate for growth of *M. aeruginosa* TY-1. The phosphate concentration used in this study ( $1.0 \mu\text{M}$ ) is similar to that under natural conditions according to United States Environmental Protection Agency standards (US EPA 1988). A culture in the same growth medium without addition of any arsenate was used as a control. Culture conditions for each treatment and the control were as described above, and all cultures were shaken at 100 rpm to maintain cells in suspension. All samples were run in triplicate.

The stock bottles and culture flasks were soaked in 10% (v/v)  $\text{HNO}_3$  for 12 h, rinsed in Milli-Q water, dried overnight and sterilized prior to use. Culture media were autoclaved for a minimum of 30 min at  $121^\circ\text{C}$ . Analytical reagent grade chemicals and Milli-Q water were used for preparation of all stock solutions and media.

Culture density was monitored by direct cell counts using a hemocytometer. Subsamples of  $20 \mu\text{L}$  were taken for the enumeration of cell density. All cultures were continued over the complete growth period. In the late exponential growth phase, samples of 1 mL were mixed with 9 mL acetone to achieve a final concentration of 90% acetone, extracted at  $4^\circ\text{C}$  for 24 h, and chlorophyll-*a* was determined spectrophotometrically using specific absorption coefficients (Jeffrey and Humphrey 1975).

For analysis of microcystins using HPLC, a 30 mL subsample was harvested in the late exponential growth phase and filtered through a CF/C glass fiber filter under low vacuum pressure (0.1 MPa). Cells on the filters were preserved at  $-20^\circ\text{C}$  prior to microcystin analysis. For cell toxin analysis, samples were prepared and measured according to Ramanan et al. (2000). Toxins dissolved in the growth medium were concentrated and analyzed from 50 mL filtered supernatant according to Rivasseau et al. (1998).

For microcystins, each test solution was analyzed directly by HPLC with photodiode array ultraviolet detection (HPLC-PDA-UV). Separation was accomplished under reverse-phase isocratic conditions with an octadecyl silica (ODS) column (Cosmosil 5C18-AR,  $4.6 \times 150 \text{ mm}$ ; Nacalai, Kyoto, Japan) and a mobile phase of 0.01 M ammonium acetate: acetonitrile (73:27). The flow rate was  $1 \text{ mL min}^{-1}$ . Microcystins were identified from their characteristic spectra. Quantification was carried out using peak areas of the test samples and comparing them with those of the standards available at 238 nm. The microcystin (MC) standards (MC-LR, MC-RR and  $[\text{Dha}^7]\text{MC-LR}$ ) were obtained from Kanto Reagents (Tokyo, Japan). The liquid chromatography component consisted of a Hitachi (Tokyo, Japan) L-6200 pump and a Hitachi L-4200 UV-vis detector.

Statistical analysis was carried out with Microcal Origin (version 6.1, Microcal Software, Northampton, USA). Standard errors (SE) were calculated and Student's *t* test was used to compare results obtained from the control and treatment cultures. The normality and homogeneity of variances were

previously checked. In all statistical tests, a significance level of 5% was adopted.

**Results**

Figure 1 shows the effects of arsenate on the growth of *Microcystis aeruginosa* TY-1 at different phosphate concentrations. The growth patterns of *M. aeruginosa* at arsenate concentrations ranging from  $10^{-8}$  to  $10^{-4}$  M were very similar to the control under phosphate-normal and phosphate-starved conditions (Fig. 1,  $P>0.05$ , two-sample paired *t* test). The chlorophyll *a* content of *M. aeruginosa* exposed to arsenate ranging from  $10^{-8}$  to  $10^{-4}$  M also showed no difference from that of the control under both phosphate regimes (data not shown).

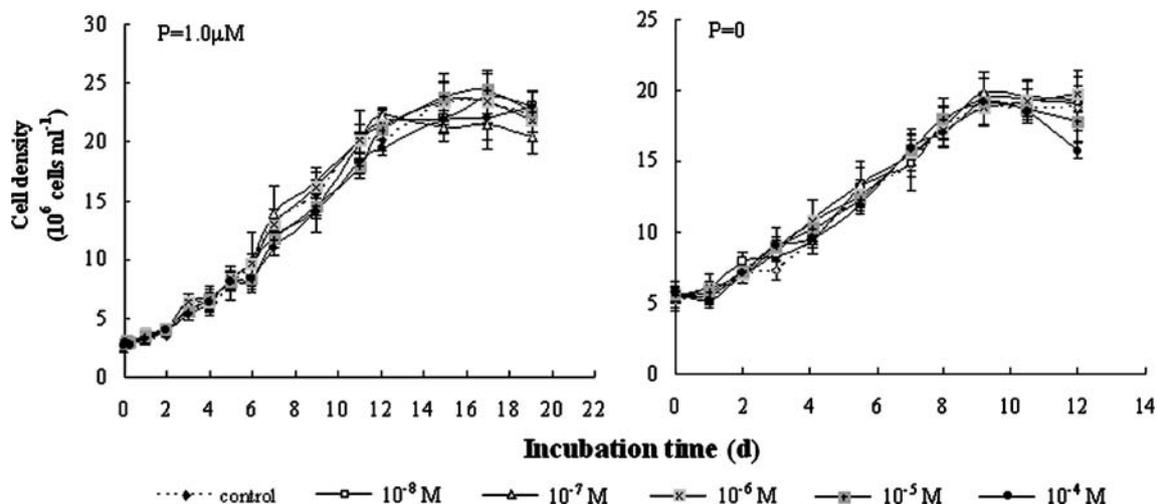
*Microcystis aeruginosa* TY-1 produced predominantly MC-LR and [Dha<sup>7</sup>]MC-LR. Compared with the control, intracellular MC-LR quotas of the phosphate-normal cells were inhibited by 32.2% and 44.8% in the presence of  $10^{-5}$  and  $10^{-4}$  M arsenate, respectively (Fig. 2a,  $P<0.05$ , two-sample *t* test). During phosphate-normal conditions, the extracellular MC-LR content was enhanced ~45% with arsenate from  $10^{-8}$  to  $10^{-6}$  M as compared to the control, whereas the extracellular [Dha<sup>7</sup>]MC-LR content was stimulated by exposure to arsenate at concentrations from  $10^{-8}$  to  $10^{-5}$  M (Fig. 2b,  $P<0.05$ , two-sample *t* test). The phosphate-starved cells had an intracellular MC-LR quota approximately 1.4 times higher than that of the control when treated with  $10^{-4}$  M arsenate, while intracellular [Dha<sup>7</sup>]MC-LR content was not affected by arsenate (Fig. 2c,  $P<0.04$ , two-sample *t* test). The highest extracellular MC-LR content of the toxic TY-1 at  $10^{-5}$  M arsenate was an average of 61% higher than that of the control under phosphate starvation, but the

extracellular [Dha<sup>7</sup>]MC-LR content changed about two-fold when exposed to  $10^{-5}$  M arsenate (Fig. 2d,  $P<0.05$ , two-sample *t* test). In particular, the extracellular MC-LR content was only ~14% of that of the control with  $10^{-4}$  M arsenate under both phosphate regimes (Fig. 2b,d).

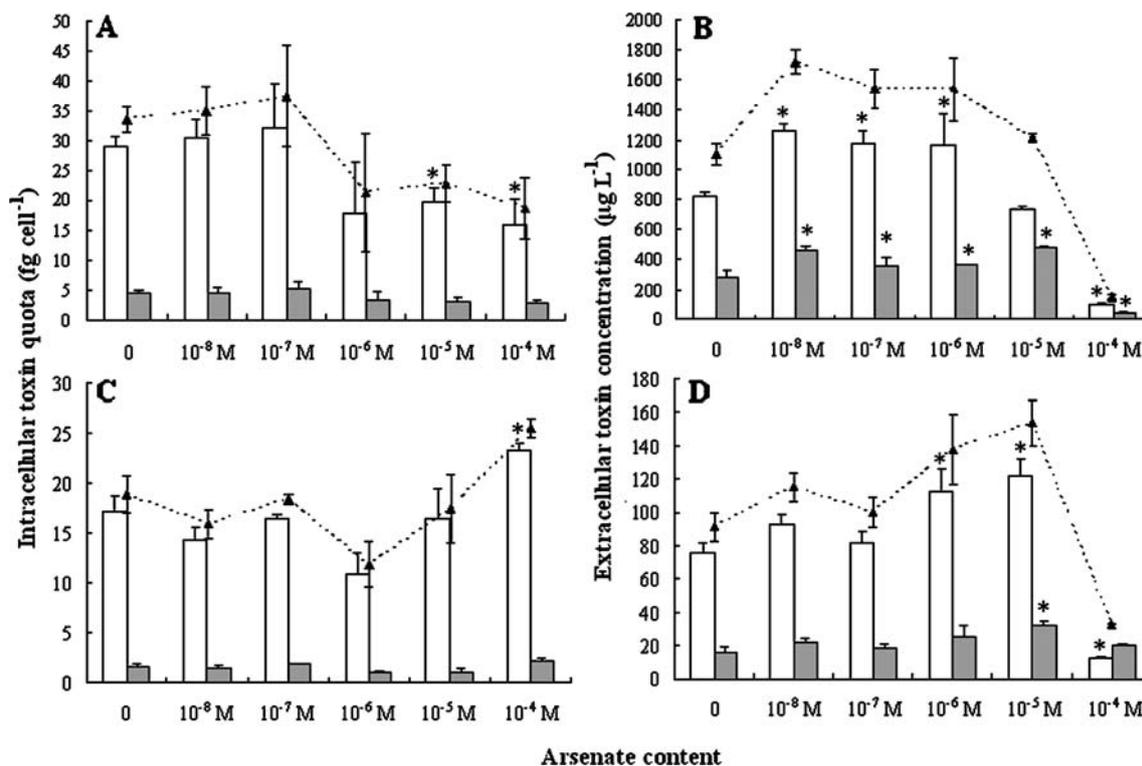
Both the extra- and intra-cellular MC-LR contents were expressed as microgram per liter of cyanobacterial culture medium, and then the total toxin yield and ratio of extracellular microcystins to total toxins were calculated (Fig. 3). Under phosphate-normal conditions, total toxin yields after arsenate treatment followed an inverted U-shape pattern, with a peak value of  $2.25\pm0.06$  mg L<sup>-1</sup> in the presence of  $10^{-7}$  M arsenate (Fig. 3a). The highest percentage in the growth medium was an average of 76.6% of total toxins in the presence of  $10^{-6}$  M arsenate under phosphate-normal conditions (Fig. 3b). Both the total toxin yield and the ratio of extracellular microcystins to total toxins increased with the concentration of arsenate in the range of  $10^{-8}$ – $10^{-6}$  M, and decreased at an arsenate concentration higher than  $10^{-5}$  M. However, such a phenomenon was not observed during phosphate starvation. Total toxin yields were not affected by arsenate under phosphate starvation, but the ratio of extracellular microcystins to total toxins was stimulated by  $10^{-6}$  and  $10^{-5}$  M arsenate as compared to the control ( $P<0.05$ , two-sample *t* test).

**Discussion**

Hardly any reports found in recent literature on the toxicity of arsenate to *Microcystis* consider the phosphate status of this cyanobacterium, although arsenate competition with phosphate has been reported for other phytoplankton species and organisms (Thiel 1988; Creed et al. 1990; Wängberg and



**Fig. 1** Growth curves of *Microcystis aeruginosa* TY-1 exposed to various concentrations of arsenate under two phosphate regimes. Error bars Standard deviation ( $n=3$ )



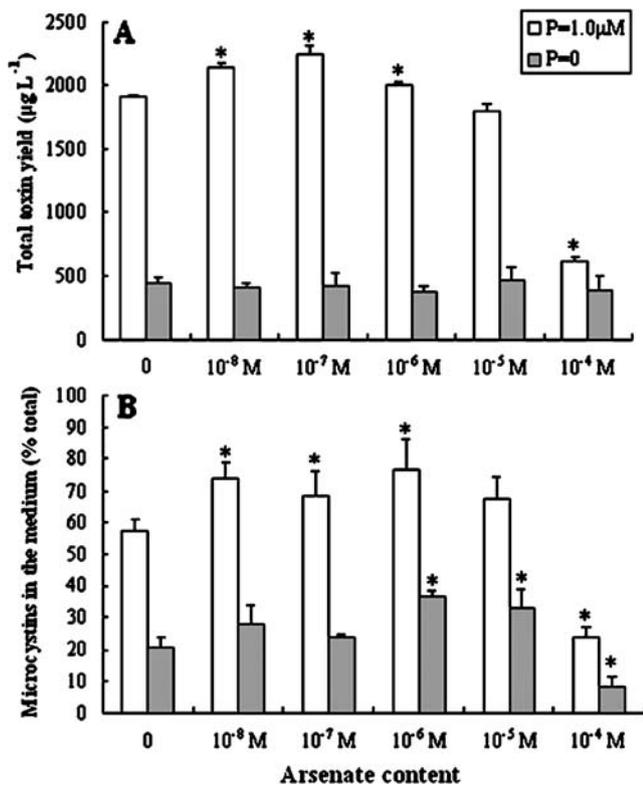
**Fig. 2 a–d** Intra- and extra-cellular microcystin (MC)-LR levels (*open bars*), [Dha<sup>7</sup>]MC-LR levels (*solid bars*) and total MC levels (*dotted line*) of *M. aeruginosa* TY-1 exposed to various concentrations of arsenate under two phosphate regimes. **a** Intracellular MC quotas after 15 days exposure to phosphate-normal conditions; **b** extracellular MC content

after 15 days exposure to phosphate-normal conditions; **c** intracellular MC quotas after 9 days exposure to phosphate-starved conditions; **d** extracellular MC content after 9 days exposure under phosphate-starved conditions. *Error bars* Standard deviation ( $n=3$ ), \*significant difference from the control ( $P<0.05$ )

Blanck 1990; Knauer and Hemond 2000). Growth is a good indicator of the action of noxious compounds in susceptible microorganisms, and reflects the metabolism of the cell (Blankley 1973). It was demonstrated that the arsenate/phosphate ratio outside the cells did not directly govern the toxicity of arsenate, as shown by the growth curves of *M. aeruginosa* TY-1 (Fig. 1), which does not support the traditional view that phosphate-starved cells are more sensitive to arsenate (Thiel 1988; Wängberg and Blanck 1990). The tolerance of this *Microcystis* strain to arsenate was much greater than that of many freshwater microalgae, such as *Chlamydocapsa* cf. *peterfii* (Wängberg and Blanck 1990), *Stichococcus bacillaris* (Pawlik-Skowronska et al. 2004), and *Monoraphidium arcuatum* (Levy et al. 2005). The higher tolerance of *M. aeruginosa* TY-1 to arsenate may be the result of one (or a combination) of three alternatives. The first is that the high-affinity phosphate/arsenate uptake system may be suppressed by arsenate and was insensitive to cyanobacterial phosphorous status, as is the case in some arsenate-resistant plants (Meharg and Macnair 1992). The second explanation is that *M. aeruginosa* can detoxify arsenate via transfer to arsenite, and/or further to methylated arsenic species (Sanders 1979), and an arsenate reductase, pI258 ArsC, has been found in the cyanobacterium

*Synechocystis* sp. PCC6803 (Li et al. 2003). A mathematical model has been constructed to simulate arsenate transformation in algae under P-limited and P-non-limited conditions (Hellweger et al. 2003). The third explanation is that high intracellular concentrations of phosphorus reduce the possibility of arsenate successfully competing with phosphate for enzymatic sites (Meharg 1994), since *M. aeruginosa* is capable of luxury uptake and accumulation of phosphate in several-fold excess of demand when exposed to considerable amounts of phosphate in BG-11 medium prior to arsenate exposure (Jacobson and Halmann 1982; Baldia et al. 2007).

*Microcystis* with hepatotoxicity, which can cause liver damage or even liver haemorrhage, is a common bloom-forming cyanobacterium in eutrophic freshwaters world wide (Sivonen and Jones 1999). At least 76 different microcystin analogs have been found in natural blooms and laboratory cultures of cyanobacteria (Acero et al. 2005). In particular, MC-RR, LR and [Dha<sup>7</sup>]MC-LR are commonly found in freshwater cyanobacterial blooms in China (Chen et al. 2006). *Microcystis aeruginosa* TY-1 isolated from Taiwan produces two main microcystins, MC-LR and [Dha<sup>7</sup>]MC-LR. MC-LR is the most common variant of all the microcystins, and also the most toxic, with an LD<sub>50</sub> of 50 μg kg<sup>-1</sup> (Dawson 1998), whereas the toxicity of [Dha<sup>7</sup>]MC-LR is



**Fig. 3** Total toxin content (a) and the ratio of extracellular microcystins to total toxin content (b) for *M. aeruginosa* TY-1 exposed to various concentrations of arsenate under two phosphate regimes. Error bars Standard deviation ( $n=3$ ), \* significant difference from the control ( $P<0.05$ )

about one-fifth of that of MC-LR (Sivonen and Jones 1999). Thus, it was important to analyze the microcystin composition resulting from exposure to arsenate. Our results showed that toxicity was related mainly to the intracellular level of MC-LR, which was the only microcystin type altered by arsenate under both phosphate regimes. However, variation in the toxicity of the culture medium was directly correlated with changes in both extracellular microcystin levels when the cyanobacterium was exposed to arsenate.

Microcystins are considered as being primary intracellular, and microcystin leakage is generally considered to be linked to a decrease in cell integrity (White et al. 2005). However, in *M. aeruginosa* TY-1, microcystins are released during the log phase of growth and accumulate continuously in the medium, and extracellular microcystins contribute up to ~60% of total toxin yields in the late exponential growth phase when this strain is cultivated in medium containing 0.3 mM  $\text{PO}_4^{3-}$  (Lin 2007). It was also observed in this study that  $57.6\pm 3.7\%$  of total microcystins of *Microcystis* TY-1 without arsenate exposure leaked into the culture medium under phosphate-normal conditions ( $1.0 \mu\text{M PO}_4^{3-}$ ). However, only  $20.7\pm 3.0\%$  of the total microcystin yield was released under phosphate starvation without arsenate expo-

sure. On the other hand, extracellular phosphate did not affect microcystin leakage in *Microcystis* PCC7806 under phosphate starvation (Gong et al. 2008). It is worth mentioning that *Microcystis* PCC7806 stores most microcystins in cells, and only 4% of the total toxin amount is found outside the cells in late exponential growth phase (Rivasseau et al. 1998). A single amino acid difference in the *mcyH* sequence has been found in *Microcystis* TY-1 in comparison with *Microcystis* PCC7806 (Lin 2007). The sequence of *mcyH* is known to be homologous to the bacterial ABC transporter, but it is also known as a key protein that maintains the integrity of microcystin synthetase clusters (Pearson et al. 2004). *Microcystis* TY-1 is suggested to have the inherent ability of microcystin excretion. Phosphate has been shown to support the necessary enzymes, precursors and energy charge for microcystin synthesis (Dai et al. 2008). Therefore, it is hypothesized that phosphate can inhibit microcystin production and leakage when it is the limiting factor for cells (Fig. 3). Meanwhile, as *Microcystis* PCC7806 does not possess the ability to excrete microcystin, extracellular phosphate would not affect the leakage of microcystins, but could have a similar effect on total microcystin yields as for *Microcystis* TY-1 (Gong et al. 2008).

The present study has shown that arsenate has some effect on the dissolved microcystin level under both phosphate regimes. The cyanobacterial cells were expected to produce more extracellular microcystins due to induction of the transcription of *mcy* genes when exposed to low doses of arsenate, since both total microcystin yields and microcystin excretion increased with  $10^{-8}$ – $10^{-6}$  M arsenate under phosphate-normal conditions. The most likely reason for microcystin production in cyanobacteria is defence against grazers (Rohrlack et al. 1999). It is presumed that the defence capability of *Microcystis* cells is enhanced by exposure to arsenate provided as a competitor of phosphate. However, it is not clear that total microcystin yields were invariable with arsenate exposure under phosphate starvation, while microcystin excretion was variable. Intracellular phosphate/arsenate perhaps does not play a role in microcystin production but rather affects the release of microcystin from cells under phosphate starvation. Further experiments are needed to study the expression levels of the toxin and transporter genes to clarify the above problems.

The response to arsenate of total microcystin production in this *M. aeruginosa* strain under phosphate-normal conditions seemed to be an inverted U-shaped hormesis, as described by Stebbing (1982), which varied from being enhanced at lower doses to being decreased (due to toxicity) at higher doses. The peak values measured here were ~120% of control values when treated with  $10^{-7}$  M arsenate, which is within the range of typical hormetic responses proposed by Calabrese and Baldwin (1997). Based on a recent report on the range of arsenic levels of aquaculture ponds in Taiwan ( $1.92\times 10^{-7}$ –

$2.95 \times 10^{-6}$  M) and the predominance of arsenate in the water (Huang et al. 2003), it is likely that *M. aeruginosa* TY-1 could grow well, in addition to producing and excreting more microcystins in arsenic-polluted ponds with the coexistence of phosphate. The bioaccumulation of microcystins by fish has been reviewed by Ibelings and Chorus (2007). This implies that risks to human health might be increased through consumption of fish harvested from culture ponds containing arsenate and toxic *Microcystis*.

In summary, this is the first report on the effects of arsenate on the growth and microcystin production of *M. aeruginosa* isolated from Taiwan under different phosphate regimes. The traditional view that phosphate-starved cells were necessarily more sensitive to arsenate toxicity may not be correct. Our study suggests that *M. aeruginosa* TY-1 is tolerant to arsenate, and this tolerance is not affected by extracellular phosphate content. The stimulation of microcystin production in *M. aeruginosa* TY-1 at low concentration of arsenate ( $10^{-8}$ – $10^{-6}$  M) under normal phosphate conditions might be an effect of hormesis. It is speculated that arsenate may play a role in the process of microcystin biosynthesis and excretion, yet this remains to be verified. Given the known arsenic concentrations in aquaculture ponds of Taiwan, it is likely that arsenate favors survival of toxic *M. aeruginosa* in the ponds; it is evident that low doses of arsenate stimulate microcystin production and leakage, which may increase the risk of human exposure to hepatotoxins through the food chain.

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