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Contributed Paper

Effects of Toluene and Sodium Azide on Microcystins Releasing from *Microcystis* Cells

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ABSTRACT

Analysis of microcystins in Thailand is of interest due to *Microcystis aeruginosa* bloom in some freshwater reservoirs. Toxin analysis has been done and included in water quality monitoring. During water sampling, toluene as preservative was added to cover surface of water sample. It acts as a barrier between the air and the surface of the specimen. In this work, the effects of preservative chemicals including toluene and sodium azide were tested with permeabilised *Microcystis*. Toluene was found to act as accelerator of toxins release from cells to their environment. Samples without toluene contained almost 100% microcystins in their pellets. A release of microcystins from the cells was found in all samples with spiked toluene. Microcystins detected in supernatants of the samples with toluene were much higher than in those without toluene. Percentage of the toluene added was found to be related with a rate of toxin release as well as storage time. Long storage period presented low amount of microcystins detected in the pellets, whereas their high amount were found in the aqueous part. In comparison with added sodium azide samples, no major change had been observed on concentrations of microcystins both presented in pellet and supernatant in relation with time. Lyophilised *Microcystis* bloom materials from Sakon Nakhon Province, Thailand was also tested with toluene in order to see its effect on natural sample. We found a possible biotransformation peak that increases while the others are decreasing. It is possible that the biotransformation remains stable and at low concentrations when sterile, but increases when no sterile. In conclusion, sodium azide seemed to be more effective for preservation of microcystins in cells because no major change had been observed on concentrations of microcystins presented in pellet and supernatant in relation with time.

Keywords : cyanotoxins, microcystins, *Microcystis*, toluene, sodium azide.

1. INTRODUCTION

The contamination of water resources and drinking water supplies by human activities remains a major human health concern. Cyanobacterial toxins become widely recognised as a human health problem arising as a consequence of eutrophication, the biological response to the excess input of nutrients into a water body [1]. The frequent dominance by cyanobacteria in eutrophic waters is of additional concern to water quality considerations because of the common ability of these organisms to produce toxins [2].

The most frequently found cyanobacterial toxins in blooms from fresh water are the cyclic peptide toxins of the microcystin and nodularin family. They pose a major challenge for the production of safe drinking waters

from surface waters containing cyanobacteria with these toxins. Microcystins have been characterised from *Microcystis* species, commonly *Microcystis aeruginosa*. These species are linked most frequently to hepatotoxic blooms world-wide [1]. The microcystins are tumor-promoters. Disruption of liver structure and function occurs with hemorrhage into the liver and death by respiratory arrest [3]. Animal toxications and human illnesses have been associated with, or attributed to cyanobacterial toxins [4]. The deaths of more than 50 haemodialysis patients in Caruaru, Brazil, have been attributed to exposure to microcystins in dialysis water [5, 6].

The World Health Organization (WHO) is currently addressing health hazards presented by cyanobacterial toxins as part of a rolling revision of the WHO Guidelines for Drinking Water Quality. With the main aim of protecting public health, the WHO Guideline Value (GV) for total microcystin-LR in drinking water has been derived to 1.0 μg/l [7].

In Thailand, analysis of microcystins has been of interest due to algal, *Microcystis aeruginosa*, bloom in some freshwater reservoirs around the country in 1998 [8]. After that event, toxin analysis has being done and included in monitoring of water quality [8, 9]. Microcystins in water sample has been analysed after water sampling was done under preservation by adding an appropriate amount of toluene to cover the sample surface. Toluene is the only organic solvent that is still used as a preservative when presents in a large enough amounts, it acts as a barrier between the air and the surface of the specimens. Toluene, however, will not prevent the growth of anaerobic microorganisms, and because of its flammable nature it is a safety hazard [10]. In order to minimise a use of toluene, this research was conducted to prove whether toluene is really needed as a preservative of microcystins in water sample. How much is an appropriate amount of toluene to be added, if it was found out to be necessary. How long will microcystins be released from the cell after toluene was added? These questions are needed to be answers as a baseline data for water sampling in a purpose of microcystin analysis.

Sodium azide is another common preservative of samples and stock solutions in laboratories [11]. In this research work, toluene and sodium azide have been tested for their effects on toxin releasing from *Microcystis* cells in order to compare their capacities on sample preservation.

2. Sample Preparation and Extraction

2.1 Toluene Experiment

Approximately 35 ml permeabilised *Microcystis* sample was taken and centrifuged. Its pellet was re-suspended with 35 ml of Phosphate Buffered Saline (PBS) and centrifuged for 4 times. Their supernatants were collected and mixed well. Approximately 8 ml solution was taken into each of 18 glass bottle. A 1.5 ml of solution from each bottle was sampled as a time 0 sample. Three bottles were separated as control samples without toluene. Another 15 bottles left were divided into five groups and spiked with toluene in a percentage of 0.1, 0.25, 0.5, 1.0 and 2.0%, respectively. All samples were shaked well and only 1.5 ml triplicate samples from each toluene-concentration were collected at 5 mins, 1 hr and 24 hrs. After sample collecting, they were centrifuged. Their pellet and supernatant were then separated. The supernatants were left in a freeze-drier until dried. Both of pellet and dried supernatant parts were re-suspended in 150 ml of 70% methanol and centrifuged. Then 50 ml of the solution was injected onto HPLC-DAD [12].

2.2 Toluene and Sodium Azide Experiment

In order to compare the effect of toluene and sodium azide on toxins released from cells, 30 ml of permeabilised *Microcystis* cells were 3 times centrifuged and washed with PBS solution. Each of 10 ml centrifuged sample was transferred to a universal glass bottle and centrifuged again. Pellet and supernatant were separated and recorded as control. Toluene

(0.1% and 1.0%) was added to the aqueous solution of the first and the second bottles, whereas the last bottle was added with 0.002% sodium azide and shaken. Samples were immediately collected and recorded as time 0 sample. The rest were kept and collected after 1 hr and 24 hrs for centrifugation. Their pellet and supernatant were separated and left in a freeze-drier. Both of pellet and dried supernatant parts were re-suspended in 150 µl of 70% methanol and centrifuged. Then 50 µl of the solution was injected onto HPLC-DAD [12].

2.3 Experiment on Lyophilised *Microcystis* Bloom Materials from Thailand

Approximately 20 mg of lyophilised *Microcystis* bloom materials from Sakon Nakhon Province, Thailand were extracted with 10 ml milli-Q water. Samples were divided into four groups as shown in Table 1. Samples No. 2 and 4 were added with a 100 ml of toluene (1.0%) and shaken well. Samples No. 1 and 2 were left at room temperature, whereas the No. 3 and 4 were put in an autoclave for 10 mins. They were all left under dark condition during an experiment. One ml of each extracted sample was taken into Eppendorf vial and centrifuged. They were recorded as day 1 samples. Their pellet and supernatant were separated and dried in a freeze drier. After getting dry, they were re-suspended with 200 ml of 70% methanol and mixed well. After that 50 ml samples were injected onto HPLC-DAD [12]. All steps were then repeated again for day 3, day 9 and day 19.

Table 1. Groups of lyophilised samples according to preservation methods.

Sample Number	Preservation Methods	1% toluene added
1	Control	-
2	Control + Toluene	100 µl
3	Autoclave	-
4	Autoclave + Toluene	100 µl

3. Effects of Preservatives on Permeabilised *Microcystis* Samples

3.1 Effects of Toluene

A variation of toluene concentrations, 0.1, 0.25, 0.5, 1.0 and 2.0% of the samples were added to the extracted permeabilised *Microcystis* samples to find out its effect to toxin and its appropriate amount to preserve toxin in water samples. The samples without toluene in triplicate were also analysed as a control under the same conditions as other samples with toluene. All samples were analysed at time 0, 5 mins, 1 hr and 24 hrs, respectively. Result is shown in Table 2. It can be seen clearly that the samples without toluene contain almost 100% microcystins in their pellets except in time 0 and 24 hrs samples, which had about 15% and 30% of microcystin in their supernatants, respectively. A release of microcystins from the cell was found in all samples with spiked toluene. Microcystins detected in supernatants of the samples with toluene were much higher than in those without toluene. Moreover, the amount of microcystins detected in the supernatants of toluene spiked samples was direct proportional to times, whereas those in pellets were found in reverse order.

The concentrations of microcystin equivalent were plotted again against percentage of toluene added into the supernatant of the extracted permeabilised *Microcystis* samples as shown in Figures 1A and 1B. In order to compare effect of amount of toluene on toxin

Table 2. Average of microcystin concentration equivalence (MCE) (mg/ml) found in pellets and supernatants of extracted permeabilised *Microcystis* samples.

Toluene (%)	MCE in Pellets x 10^{-3} (µg/µl)				MCE in Supernatants x 10^{-3} (µg/µl)			
	Time 0	5 min	1 hr	24 hr	Time 0	5 min	1 hr	24 hr
0.00	7.19	8.08	4.91	6.39	1.15	0.28	0.40	2.75
0.10	7.16	5.60	2.81	1.20	1.12	1.61	2.64	5.40
0.25	7.46	4.01	1.32	0.54	0.84	1.78	3.79	4.66
0.50	7.71	6.07	2.01	0.79	0.42	1.50	3.74	4.62
1.00	8.04	5.68	1.77	0.93	0.37	1.64	4.11	5.84
2.00	8.48	3.62	1.87	1.26	0.22	2.48	3.88	3.32

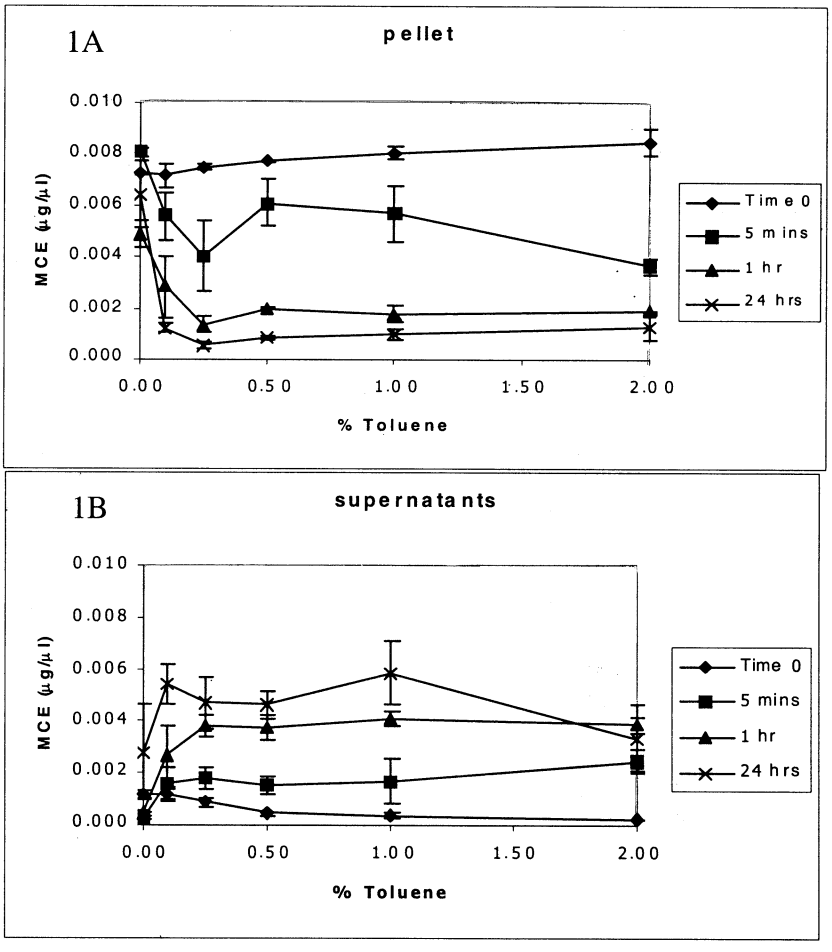

Figure 1. Concentration of microcystins found in pellets (1A) and supernatants (1B) of permeabilised *Microcystis* samples with variation of toluene added.

release, the MCE detected were continuously plotted with times. It can be seen clearly that the time 0 samples had a relatively constant microcystin concentrations, whereas a decline of microcystins was found in other samples. The variation of the added toluene samples related with a rate of toxin release, although only up to 0.5% toluene. Increased amount of toluene was resulted by toxin release from the pellets to the supernatants. However, samples in which toluene more than 0.5% was added showed no difference in microcystins detected.

The storage times play also an important role in toxin release. Long storage period presented low amount of microcystins detected in the pellets, whereas more were found in the supernatants. Moreover, a long storage time gave a high rate of toxin release, which can be seen from a slope of each line. The amount of toxin detected in a range of 0 to 0.1% added toluene presented a steep slope.

Up to this point it can be concluded that the amount of toluene added into water samples plays no important roll with an event of toxin release from the cells. The results show clearly that samples without toluene had relatively constant microcystin concentrations in their pellets, even though the concentrations in the 1-hr samples were relatively lower among the others. It can be concluded that the added toluene accelerated a toxin release from cells to their environment. Therefore the toxin concentrations found in the pellets were decreasing with times, whereas those in the supernatants were increasing.

How long will the toxin take to disperse from the pellets to supernatants after toluene was added? As can be seen in Figure 1B, when each line was compared with one another, it was found out that the toxin would be released immediately after toluene was added. Even in this experiment, the second sampling was done after 5 mins, it was obviously seen decreasing of microcystin in the pellets.

3.2 Effects of Toluene and Sodium Azide

Effect of toluene on microcystins releasing from cells can be seen from Figure 2. Immediately after adding toluene to the cell (time 0), no toxin was found in aqueous solution but in the cells. Amount of toluene added played no roll in toxin release at this stage. After 1 hr, nearly 95% of toxin released from cells to the aqueous solution in 0.1% toluene added sample (only 0.11×10^{-3} μg/μl was left in pellet), whereas none of toxins was found in the pellet of 1.0% toluene added sample (Figure 2B). After 24 hrs, none of toxin was left in the pellet of toluene added samples. Moreover, no major change was observed in control one, except only about 3% toxin was released from cell to aqueous solution after 24 hrs (Figure 2C).

In comparison with sodium azide effect, no major change had been observed on concentration of microcystins presented in pellet and supernatant in relation with time as shown in Figure 3. It can be concluded that sodium azide acted different from toluene in term of sample preservation. It has no effect on toxin release from cells to their environment.

4. Effects of Preservatives on Lyophilised *Microcystis* Bloom Materials from Thailand

Lyophilised *Microcystis* bloom materials from Sakon Nakhon Province, Thailand was tested with toluene in order to see its effect on real sample. Figure 4 shows decreasing of microcystins in control sample within 9 days, whereas the one added with toluene presented increasing of toxins within 3 days. Increasing of MCE related with effect of toluene on toxin releasing from cell to its environment, whereas the sterile samples presented not much change in both with and without toluene.

The main microcystin like-peaks for the control and the autoclave samples (both with and without toluene) were put onto charts as shown in Figure 5. We have found a possible biotransformation peak that increases while the others are decreasing as shown in Figure 5A. It is the possible biotransformation that remains stable and at low concentrations when sterile, but increases when no sterile.

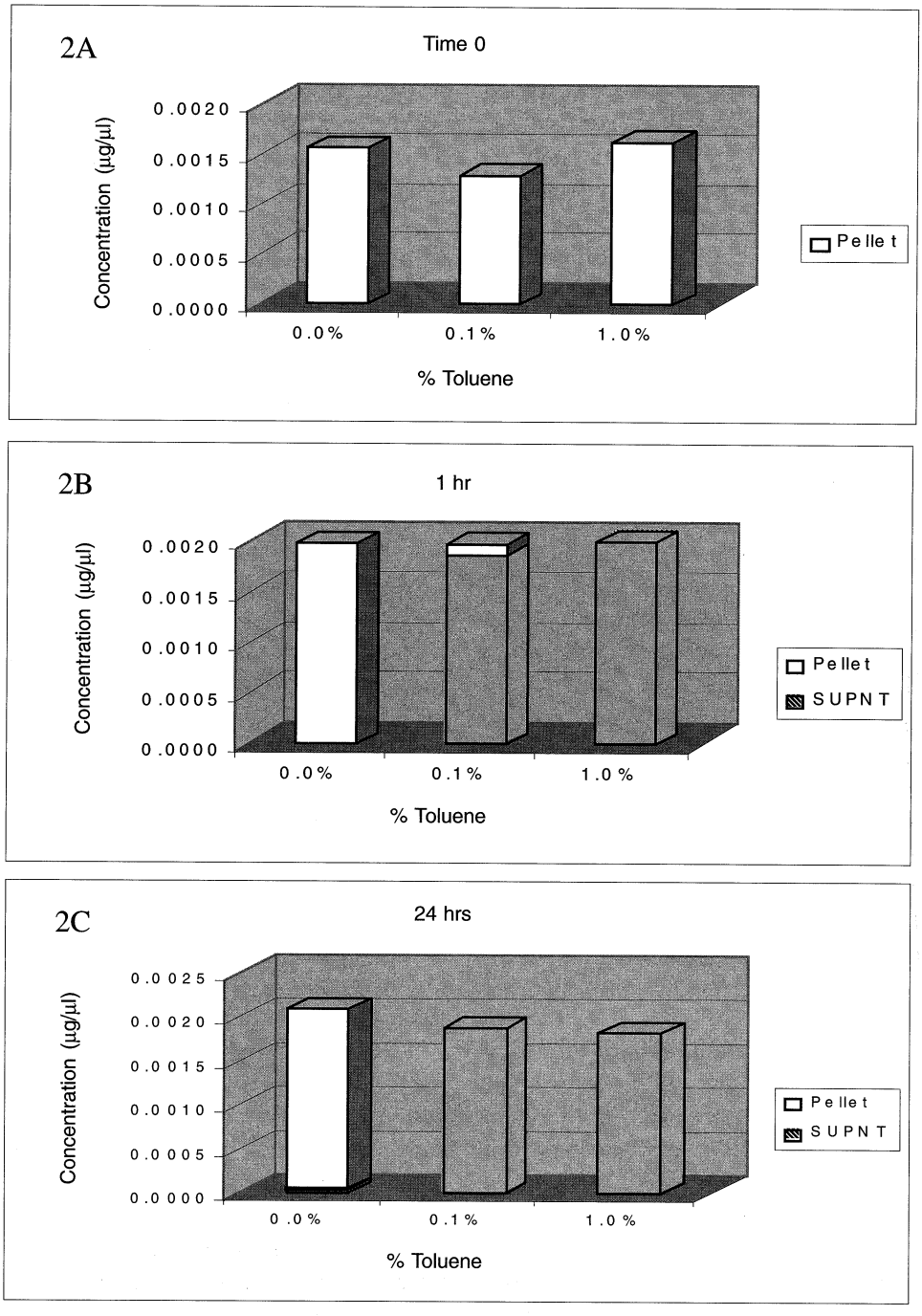

Figure 2. Concentrations of microcystins in pellet and supernatant (SUPNT) showing effect of toluene on toxin released from cells at different periods of time ranking from time 0 (2A), 1 hr (2B) and 24 hrs (2C).

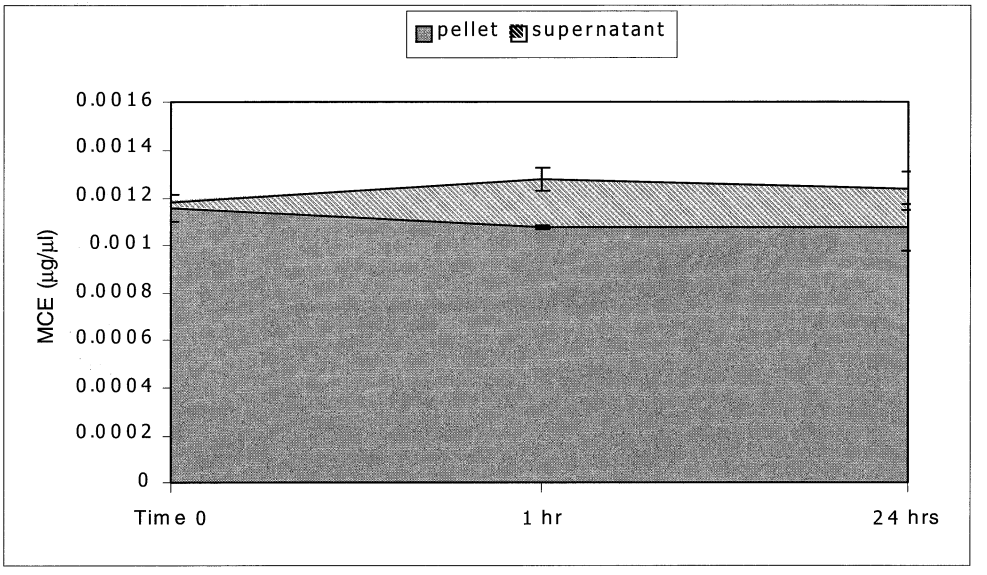

Figure 3. Effect of 0.002% sodium azide on microcystins releasing from cells.

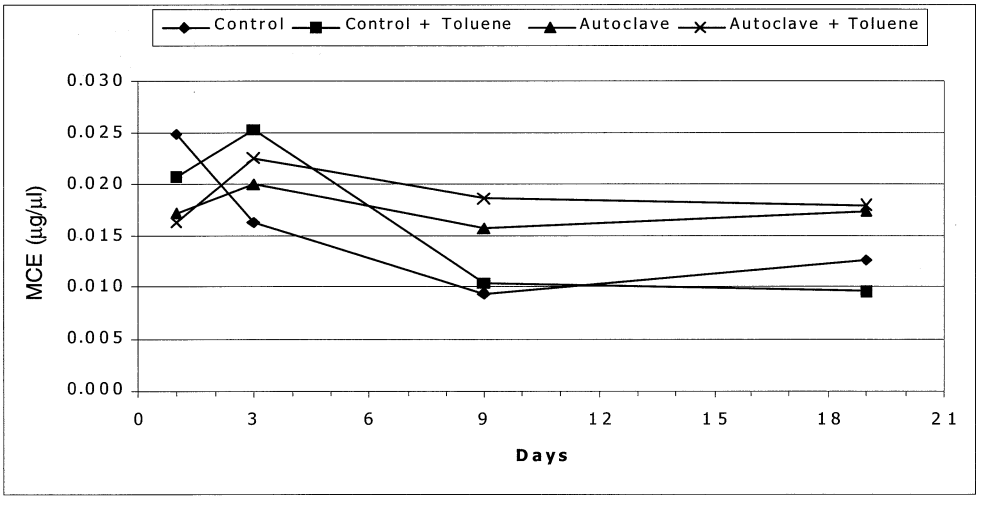

Figure 4. Total concentrations of microcystin equivalent detected in aqueous solution of four different conditions.

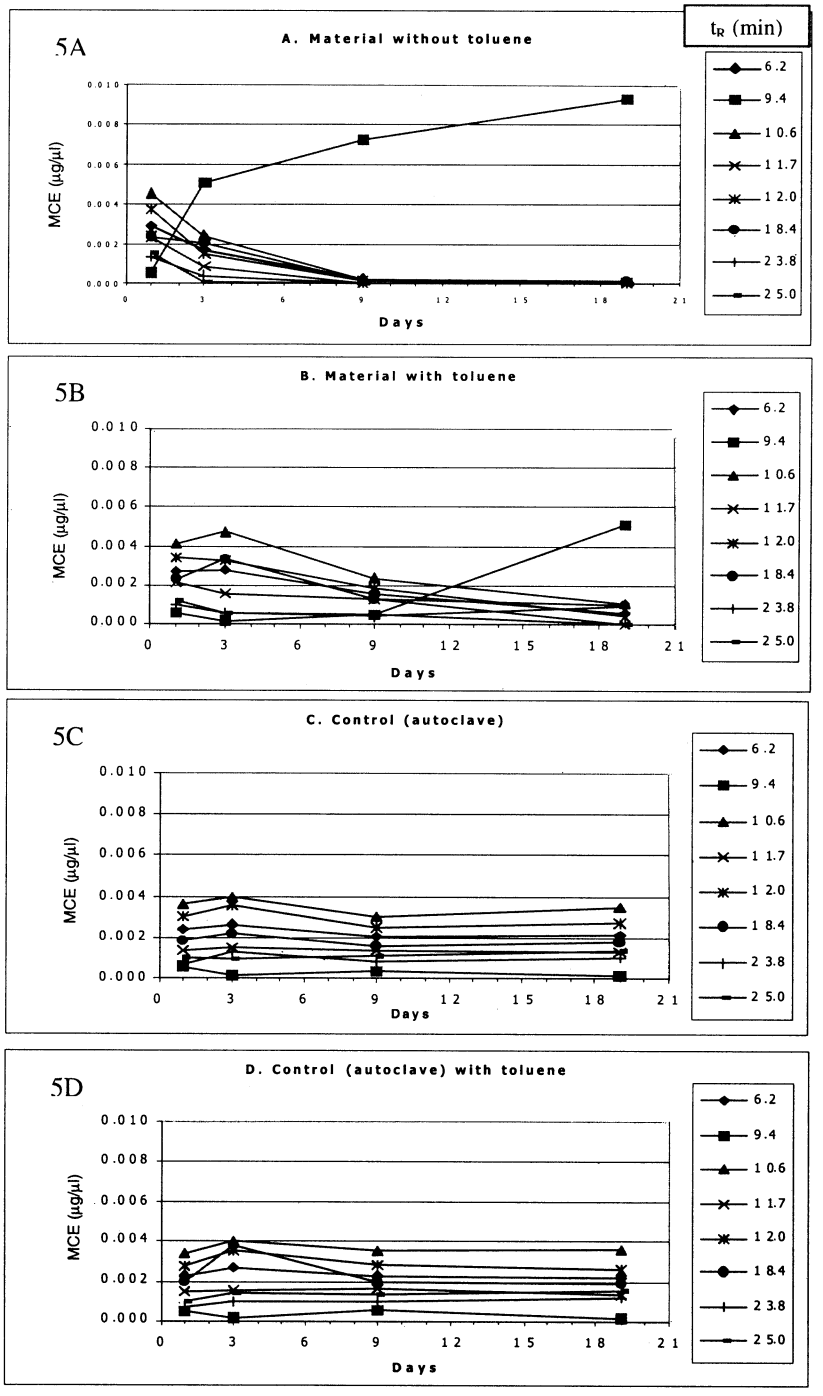

Figure 5. Biodegradation of microcystin-like peaks in aqueous extracts of lyophilised *Microcystis* bloom materials from Thailand. 5A : Material without toluene, 5B : Material with toluene, 5C : Control (autoclave) and 5D : Control (autoclave) with toluene.

5. CONCLUSION

Toluene is not necessary for using as a preservative of microcystins in water analysis. It only accelerates toxin release from cells to their environment. The variation of the toluene was found to be related with a rate of toxin release, although only up to 0.5% toluene. The storage time is also important in terms of toxin release. Long storage period presented low amount of microcystins detected in the pellets, whereas higher amount was found in the supernatants.

Sodium azide seemed to be more effective for preservation of microcystins in cells because no major change had been observed on concentrations of microcystin presented in pellet and supernatant in relation with time. Moreover, it maintained toxins in cells rather than accelerated and released them to environment as toluene acted. However, cool or frozen storage and protecting from direct sunlight are sufficient to preserve samples for toxin analysis. Using hazardous chemicals such as toluene and sodium azide as preservatives in water and environmental samples can be avoided.

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