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TOXICITY PROFILES BETWEEN STRAINSOF*ALEXANDRIUMTAMIYAVANICHII*

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Abstract- Phycotoxins are byproducts of toxic microalga that capable of causing severity on humans upon consuming contaminated seafood. The amount of toxins released by microalgae varies according to species as some of the microalgae synthesize toxins within small number of cells while others required a blooming event for the toxins to be detected. It is also possible for the toxicity of one species being gradually decreases throughout years in culture. *Alexandriumtamiyavanichii* has been reported to cause toxicity in seafood products that subject to hospitalized cases in Malaysia. Genus *Alexandrium*has been known for its stability in producing toxins yet no total loss of toxicity has been recorded. Two cultures of *A. tamiyavanichii* have been tested for its toxicity using high-performance liquid chromatography (HPLC) with GTX standards as reference. Strains isolated from Kuantan Port during recent cases of paralytic shellfish poisoning (PSP) outbreak showed extremely high in total toxin content (3.07 nmol.cell-1) while the clonal culture of *A. tamiyavanichii* established from Sebatu Malacca exhibited very low level of toxicity (1.167 fmol.cell-1). There are many factors influencing toxicity properties of *A. tamiyavanichii* with highlights in nutrients deprivation and adaptation as well as bacterial influences for the aged culture.

Keywords- Alexandrium spp., toxin profiling, post-column HPLC, gonyautoxins

I. INTRODUCTION

Harmful microalgae can be calamitous in many ways inclusive of economic loss in aquaculture industry and also human intoxication. The major function of microalgae as important producer within the ecosystem has deviated into causing harm to the co-occurring organisms (Lim *et al.,* 2012).The presence of microalgae is not only favorable to the ecosystem but also importance for the transfer of energy between populations via food web (Vasconcelos*et al*.,2010). However, the occurrence turns out to be nuisance as they begin to outnumber the normal population. This phenomena cause disturbance to fish and other sea creatures that depend on good circulation of oxygen apart from food supplements for growth continuity. Corals and other types of macroalgae like seaweed acquire light for photosynthesis under natural conditions. Excessive number of cells on the water surface could block the light penetration and oxygen diffusion through the water column.

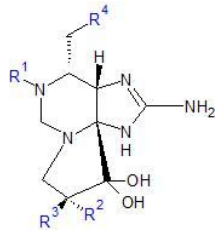
Massive assemblage of microalgae may pose great risks to the environmental issues as some of the microalgae are natural toxin producers. In certain cases the toxins may spread through aerosol but most incidents reported were due to eating contaminated shellfish (CetinkayaandMus, 2012). Shellfish is the modest vector of toxins as they are filter feeders that do not get affected by the toxins at all. The ability of synthesizing toxins is species-specific and varied in terms of toxins produced and symptoms developed in human. Dinoflagellates and diatoms are the source of toxins in aquatic life where there is no restriction with regards to cell numbers. Some species may induce toxins under certain circumstances such as nutrients limitation, water salinity and temperature (Laabir*et al*.,2013; Kodama, 2010).

Toxins from genus of Alexandrium

Dinoflagellates from genus Alexandrium contribute to most of toxicity cases reported in South China Sea. It is the only genus with diversity in toxin groups that comprise of saxitoxin, spirolides and goniodomins with the former as the most potent (Anderson *et al.*, 2012). Human intoxication due to saxitoxin is the worst effect inaugurated by species from the genus. Shellfish poisoning resulted from accumulation of toxins are the best route for toxin transfer. There are different types of shellfish poisoning but the one that is associated by *Alexandrium spp*. is widely known as paralytic shellfish poisoning (PSP). PSPcause a neurological malfunction of human immune system which develops right after consuming contaminated shellfish. Saxitoxins (STX) is the prototype of congeners mainly from *Alexandrium* sp. and responsible of inducing PSP to human (Etheridge, 2009;Hackett *et al.,* 2013). It has many varieties which basically having the structural bone as in Figure 1.

The congeners are developed from the skeletal structure of 3,4,6-trialkyltetrahydropurine compound into carbamate, decarbamoyl, N-sulfocarbamoyl and hydroxybenzoate with each varied in their toxic potential (Cetinkya and Mus, 2012; Rossini and Hess, 2010; Wiese *et al.*, 2010; Alonso *et al.*, 2016). These potent toxins act by blocking the voltage-gated of sodium channel thus stimulating paralysis to the body parts and functions (Murray *et al.,* 2011). Depolarization of membrane potential is suppressed due to the binding of STX at the receptor of membrane protein (Faber, 2012; Wiese *et al.*, 2010). The resting state of the membrane potential signals the onset of paralysis. Blocking of sodium

conductance eventually generates symptoms of numbness, tingling sensation of the oral parts and nausea to the patient (Gerssen*et al.*, 2010; Baier, 2000). The after affects took minutes to be expressed depending on the toxins concentrated within the shellfish (Etheridge, 2009).



STX	R^1	R^2	R^3	R^4
STX	H	H	H	$OCONH_2$
NeoSTX	OH	H	H	$OCONH_2$
GTX1	OH	OSO_3^-	H	$OCONH_2$
GTX2	H	OSO_3^-	H	$OCONH_2$
GTX3	H	H	OSO_3^-	$OCONH_2$
GTX4	OH	H	OSO_3^-	$OCONH_2$
GTX5 (B1)	H	H	H	$OCONHSO_3^-$
GTX6 (B2)	OH	H	H	$OCONHSO_3^-$

Figure 1
Structure of STX with relatives functional groups (Cusickand Sayler, 2013)

As the name infers, the acute toxicity brought by these species may even lead to death if no early detoxification was carried out. So far, the only remedy for PSP is prolong water insertion until the symptoms shown started to fade out (Baier, 2000; Wiese *et al.*, 2010). The deadliness of this attack is due to the fact that targeted receptor of these toxins mainly linked to the central nervous system which eventually caused paralysis to the whole body function.

Distributions of *Alexandriumtamiyavanichii*

However, not all species of Alexandrium are capable of synthesizing toxins yet more than half are natural toxin producers. *Alexandriumtamiyavanichii*is one of the known toxicants capable of inducing paralytic shellfish toxins (PST)at low cell counts. Propagation ofA. *tamiyavanichii* is considered worldwide as the species was detected both in tropical and temperate water around the globe. However, most discoveries were focusing on Asia coastal waters. The species was once misunderstood to be A. *cohorticula*however after further revision on its morphology, a new nomenclature known as A. *tamiyavanichii* was proposed. Strains of A. *tamiyavanichii*were discovered in Northern Brazil, Japan, Gulf of Thailand, Philippine and Malaysia (Menezes *et al.*, 2010; Oh *et al.*,2009; Fukuyo, Pholpunthinand Yoshida, 1988; Kon*et al.*, 2015; Usup*et al.*, 2006).

Pyrodinium, Alexandrium and Gymnodinium are the three genus of dinoflagellates affiliated to cause PSP worldwide (Etheridge, 2009). After that onwards, different species of microalgae were discovered.The worst scenario of harmful algae blooming had been recorded in Malaysia waters in involving *Pyrodiniumbahamense*1976 where large number of poisonings and casualties were reported (Roy, 1977). However, the prominent harmful species mostly recorded in Malaysia was dinoflagellate from the genus Alexandrium (Fukuyo*et al.*, 2011). The outbreaks were mostly occured alongside of Peninsular Malaysia coastal water where there is less water turbulence (Figure 2).

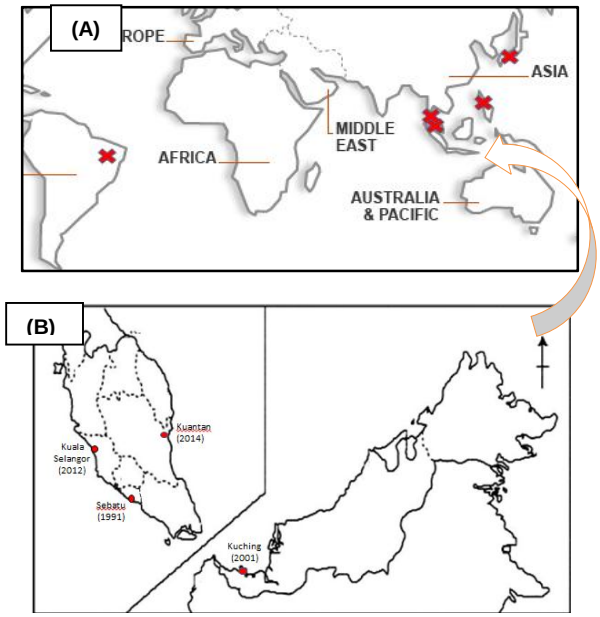


Figure 2
World map showing A. *tamiyavanichii* occurrences (A) with focus on its distribution along Malaysian coastal waters (B) (Menezes *et al.*, 2010; Oh *et al.*, 2009; Fukuyo, Pholpunthin and Yoshida, 1988; Kon*et al.*, 2015; Usup*et al.*, 2006)

The first case involving *Alexandriumtamiyavanichii* was discovered in Sebatu Malacca where farmed mussels were established (Usup*et al.*, 2002; Razali*et al.*, 2015; Lim *et al.*, 2012). It caused three people to be hospitalized after consuming the fouled mussels. Subsequent analysis on water sample taken from Straits of Malacca had successfully identified the presence of A. *tamiyavanichii*in Kuala Selangor yet no alarming effects was reported due to the low cell counts (Su-myat*et al.*, 2012). However, the presenceof this species inSemariang Sarawak showed similar toxins profile with the previous analysis on Sebatu samples (Hii, Tan and Lim, 2012). Recently poisoning outbreaks were reported in Kuantan Port. This study aimed to analyze toxin levels of A. *tamiyavanichii* isolated from different origins. Changes in toxicity level were to be expected but not in toxin compositions.

II. MATERIALS AND METHODOLOGY

Late exponential phase cultures of two clonal cultures of A. *tamiyavanichii*(1.0×10^5 cells mL-1) were

harvested by centrifugation at 3000 ×g for 5 min. Toxin was extracted with 0.05 M acetic acid, followed by liquid nitrogen freeze-thawing method for three consecutive times. The samples were then immersed in ultrasonic bath for 5 min for further cells disruption. The extracts were centrifuged again and supernatant containing toxins were collected. Impurities were removed prior to injection with filtration through a 0.45 µm syringe filter.

Toxin extracts were subjected to isocratic separation in a Waters 2475 HPLC system (Alliance, Singapore) followed by post-column derivatization(Oshima, 1989; Rodriguez *et al.*, 2010). The use of post-column has an advantage in which the peaks for each standard will be separated accordingly. Ten microliter of extracts were injected into a reversed-phase C8 column (150×3 mm, 5µm bead size) with a flow rate of 0.8 ml/min. Mobile phase was prepared deliberately for GTX toxins detection containing 0.1 M n-heptanesulfonic acid and 0.5 M phosphoric acid (pH 7).

The mobile phase for STX detection system has an additional of 5 % acetonitrile. Toxin standards used in this study were obtained from Food Safety Solution Division Group. A reservoir containing 0.35 M periodic acid and 0.25 M dipotassium phosphate was connected to the post-column effluent line at a flow rate of 0.4 mL/min. Acidification of the reaction mixture took place at 0.4 ml/min with

0.5 M acetic acid. The fluorescent eluted derivatives were detected using a Waters Multiwavelength Fluorescence Detector (Waters, USA) with excitation and emission wavelengths at 330 nm and 390 nm, respectively hence produced the toxin profiles for each strain.

III. RESULTS AND DISCUSSIONS

In this experiment, five standards were used to calibrate the instrument starting from GTX 1 to GTX 5. The chromatogram of standards was displayed in Figure 3 and the toxin profiles between the two strains were observed (Figure 4).

The amount deduced from the profiles showed that high toxin contents in strains were isolated from Kuantan Port in 2015 (3.07 nmole.cell^{-1}) while the strain obtained from the clonal culture of Sebatu sample in 1991 case was expressed in very low toxicity (1.167 fmole.cell^{-1}).

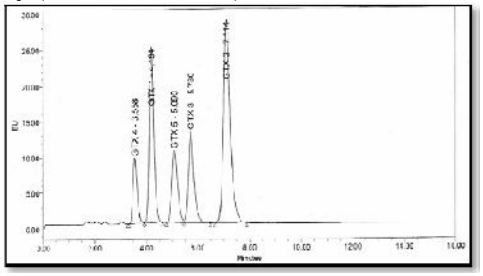
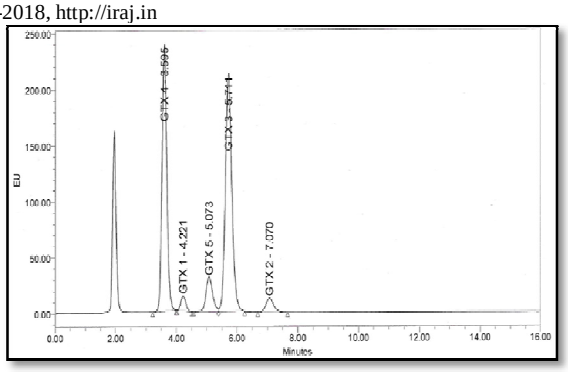
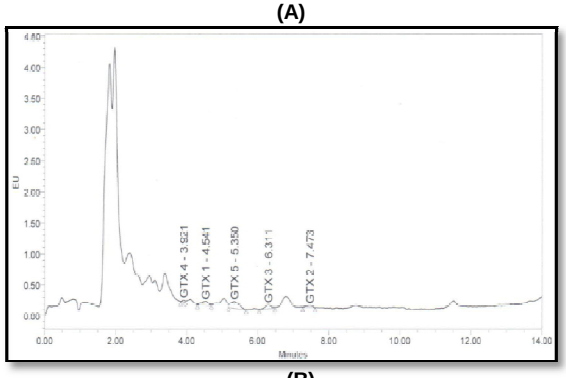


Figure 3
Chromatogram profile of gonyautoxins standards (GTX 1-GTX 5)



(A)



(B)

Figure 4
Toxin profiles of two strains of A. *tamiyavanichii*from Kuantan Port (A) and Sebatu (B) respectively

Table 1
Total toxins content in two strains of A. *tamiyavanichii*

Isolates	Toxins
Kuantan Port	3.07 nmol/cell
Sebatu Malacca	1.167 fmol/ cell

The highest peak was recorded at GTX4 and GTX3 for Kuantan Port isolates. The peaks eluted by the isolate were concise as compared to the other strain. It was unexpected to find that the strain from Sebatu Malacca expressed very low toxins.This is because toxicity attributes for A. *tamiyavanichii* to date were recorded high and stable throughout the time (Lim *et al.*, 2006). Table 2 summarized the toxin contents of A. *tamiyavanichii of* different originsbased on previous toxin analysis conducted. The fluctuation in toxin potential of A. *tamiyavanichii* is discussed in this paper.

Table 2
Total toxins content of A. *tamiyavanichii* worldwide

Isolates	Toxins	Year
Seto Island. Japan	112.5 fmol/cell	Hashimoto. T. *et al.* (2002)
Sebatu Malacca	**54.0 fmol/cell < 180 fmol/cell**	**Usup, G. *et al.* (2006) Lim *et al.* (2006)**
Northeastern Brazil	16.85 fmol/ cell	Menezes *et al.* (2010)

For many years, toxicity studies on harmful microalgae were mostly focused on the stimulating factors of toxic algae. Some of the researches had

discovered the reduction and even total loss of toxin contents from similar strains of microalgae thus eliminating the idea of stability in toxicity attributes (Hansen *et al.*, 2003; Martins *et al.*, 2004).Hence, the focus has shifted into identifying the potentials of losing toxins among HAB species. Previous studies conducted on several toxic species agreed on few reasons which include bacterial influences, altered growth conditions and presence of other microalgae under its original conditions.

Bacteria assemblage is believed to influence the toxicity among HAB species. Dantzer and Levin (1997) carried out an experiment on toxic A. *tamarense* by eliminating the extracellular bacteria using antibiotics and yet the algae remained toxic. The result is supported study by Silva (1989) whofound that bacterial extracts isolated from toxic microalgae showed negative for toxins but later introduced toxicity to previously non-toxic microalgae. Axenic and nonaxeniccultures of A. *catenella* were established for toxicity analysis in which the study justified the negative influence of associated bacteria in promoting toxicity of microalgae (Uribe andEspejo, 2002).However, the above phenomena were totally opposite tothe findings reported by Martins *et al.*, (2004). The teamhighlighted on the influence of natural bacteria occurrence towardstoxicity content of *Alexandriumlusitanicum.* The culture of A. *lusitanicum* which previously reported as toxic showed a complete loss of toxins when supplied with penicillin for a long term. The findings suggested thatbacteria presencehad great influence on the toxic properties of microalgae. After all, it can be concluded here that bacteria brought no toxicity but presumably induced toxicity by altering the metabolism within microalgae cells in whichthe mechanism remains unknown. The use of antibiotics may slightly alter or completely removed bacteria assemblage within the population of toxic microalgae.

There is also a suggestion of Other research suggested the possibility of genetic recombination as discovered in A. *tamarense* isolates from Japan (Ichimi*et al.*, 2002).In that particular analysis, different isolates showed variations in terms of toxin compositions. Similarly, two strains of A. *minutum* isolates from Irish coastal waters, which were collected from different regions, were found to betoxic as well as non-toxic (Touzet, Franco andRaine (2007). Genetic polymorphism is one of the plausible explanations for such difference apart from its environmental parameters. Media formulation is another factor that can cause toxicity deprivation among toxic microalgae. There are few reports suggested the limiting nutrients which favor toxin release among toxic algae but the results are conclusive. This is because toxin induction is species-specific. For instance some algae preferred high nitrogen to phosphorus ratio in order to outgrow and release toxins while others are vice versa (Laabir*et al.*,2013).

In this study, the divergence in toxin profiles between the two strains of A. *tamiyavanichii* was expected as geographical difference is taken into consideration to be the inducing factors for toxin analysis (Hansen *et al.*, 2003; Touzet*et al.*, 2007). The high toxin levels in Kuantan Port isolates revealed the strong toxic characteristic of A. *tamiyavanichii* even under low cell counts. However, the low toxin content of A. *tamiyavanichii*recorded from Sebatu clonal cultures may conform to the reasons mentioned earlier. Eventhough there is no antibiotic introduced throughout the culturing process; the toxicity withdrawal attribute may be due to the changes in genetic recombination within the cells. Besides, addition of soil culture in which the original purpose was to increase the cell numbers might change the growth condition of the clonal cultures. The soil culture may contain other type of bacteria that could modify the toxin metabolism within A. *tamiyavanichii* cells.

CONCLUSION

Difference strains isolated of *A. tamiyavanichii*from two locations in Malaysia revealed significant value. Kuantan Port during recent cases of paralytic shellfish poisoning (PSP) outbreak showed extremely high in total toxin content (3.07 nmol.cell-1) while the clonal culture of *A. tamiyavanichii* established from Sebatu Malacca exhibited very low level of toxicity (1.167 fmol.cell-1).

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