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Depuration Rate of Neurotoxin Domoic Acid in the Tissue of Blood Cockles *Anadara granosa*

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Abstract: *Nitzschia navis-varingica* is known as the major Amnesic Shellfish Poisoning toxin producer other than *Pseudo-nitzschia* species. Amnesic Shellfish Poisoning has effect on humans such as nausea, diarrhea and permanent short-term memory loss once the toxin exceeds the regulatory limit. The purpose of this study is to find out the growth rate of toxigenic N. *navis-varingica* and to determine the cell uptake and depuration rate of domoic acid in commercial blood cockles, *Anadara granosa*. The growth rate of N. *navis-varingica* cells grown in modified SWII media reached exponential phase from Day 3 to Day 11 where the growth rate of cell reaches 657 cell every two days. The graph reaches stationary phase whereby the cell density fluctuates within 3080 to 9182 x cells/L and the average growth rate is 287 cell every two days during stationary phase. Uptake rate in Tank B decreases drastically at the 6th hour for 12 cell every three hours and fluctuates at 1 cell every three hours during 9th hour to 18th hour. For toxin accumulation profile, *A.granosa* fed with cultured cells of toxic N. *navis-varingica* was harvested and the accumulated domoic acid was extracted in methanol by sonication and analyzed using LC-MS-MS instrument with Multiple Reaction Monitoring (MRM). The LC-MS-MS chromatogram of cockles' extract did not show detectable DA at retention peak 312.1 m/z. This research had met two objectives and is able to provide useful data in the fisheries industry.

Keywords: *Amnesic Shellfish Poisoning; blood cockles; growth; uptake; depuration*

INTRODUCTION

Suspension-feeding bivalves are important vectors of domoic acid (DA); a neurotoxic amino acid produced by diatoms mainly of the genus *Pseudo-nitzschia* and *Nitzschia* [1][2], hence the causative organisms of amnesic shellfish poisoning (ASP). Although *Pseudo-nitzschia* cells can release relatively large amounts of DA in the water column, especially under macronutrient- [3] or iron-limitation [4], only minor toxin incorporation (0.3–0.6% of available DA in 5–24 h) has been reported from the dissolved phase by mussels [5]. Recently, *N. navis-varingica* was reported to produce a significant amount of DA [6], [7]. Also reported as well is its wide distribution in Southeast Asian waters [1][8]. Some strains of *N. navis-varingica* isolated from Bulacan Estuary in northern Manila Bay produced a significant amount of isodomoic acids A and B instead of DA, and same species from several estuaries in southern parts of Manila Bay produced isodomoic acid B together with DA [1]. In Malaysia, *N. navis-varingica* was also found along the waters of Tebrau Straits, Johor [9].Other than that, *N. navis-varingica* was also found for the first time in the Mediterranean Sea on the year of 2018 [10][11].

It has been reported that that *N. navis-varingica* is distributed widely in brackish water from temperate to tropical areas. Although there is no report on domoic acid in brackish water animals, it could be possible for these animals to accumulate domoic acid from *N. navis-varingica* via food webs [1].

During toxic blooms, accumulation of DA to levels exceeding the regulatory limit (RL) of [$\mu g/g^{-1}$ in bivalve tissues thus depends on the density and toxicity of cells in suspension, which can be highly variable even for a single *Pseudo-nitzschia* species [3][12], as well as on the balance between the mechanisms regulating DA uptake and elimination in bivalves. High inter-specific differences in DA accumulation capacity have been reported for various bivalve species [13].

The present studies examine the growth rate of toxic diatom *N. navis-varingica* in SWII medium [14] with added silicate content. The uptake rate of diatom cells in *A. granosa* tissue will be

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determined. *A. granosa* is a filter feeder that lives in the brackish water. It is chosen to examine the possibility of brackish water filter feeders to produce ASP toxin after consuming toxic diatom *N. navis-varingica.* The experiment was also designed to estimate the depuration rate of domoic acid in *A. granosa* by using HPLC-UV analysis.

MATERIALS AND METHOD

Mass Culture of *N. navis-varingica*

A clonal culture of *N. navis-varingica* (P22C7) was used in this study. The strain was isolated from Sungai Pendas (1° 22' 42.72" N; 103°38' 14.13E) and maintained in SWII medium. Filtered natural seawater with the salinity of 30 PSU was used as a medium base. The culture was kept in a light and temperature controlled incubator at 25°C under a 12:12h light: dark photoperiod. Mass culture of *N. navis-varingica* was cultivated in 5 L volume of SWII medium. The late exponential phase cultures were subsampled in 3 replicates for cell counting.

Determination of growth curve of *N. navis-varingica*

The subsamples for cell count were taken once every two days for 30 days. Cell count was carried out in a Sedgwick-Rafter chamber under a light compound microscope with a magnification of 10× (Olympus CX21, Philippines). Cell counts were performed in 3 replicates and recorded into a table then a graph was plotted by using the formula from [15] to determine the relative growth curve.

$$\frac{dN}{dt} = \mu N \quad \text{or} \quad \mu = \frac{1}{t} \ln \left(\frac{N}{N_0}\right) \quad (1)$$

$$N = N_0 2^{\mu t} \text{ or } \quad \mu = \frac{1}{t} \log_2 \left(\frac{N}{N_0}\right) \quad (2)$$

Preparation of bivalve

A. granosa (blood cockle) was used in the experiments. The bivalves were collected from Sungai Buloh, Jeram (3° 25' 23.97" N; 101°28' 81.14E) by using a metal rake. A total of 8 kg of *A. granosa* was collected. The bivalve and their sediments were brought back to the laboratory and their epibionts were cleaned off with a brush under running tap water to remove micro-organisms adhered to the shells. The shell weight of the bivalves was measured using a weighing balance. The salinity of seawater from their habitat was determined using a hand refractometer and filtered seawater was adjusted to the salinity by diluting with distilled water (dH_2O). Fifty individuals of *A. granosa* were placed evenly in 6 tanks inclusive of control tank. Each tank is filled with 4 litres of filtered seawater and equipped with the aeration system. The *A. granosa* were left to starve in the filtered seawater for 24 to 48 hours.

Determination of uptake rate

A. granosa will be left starving for 48 hours. *N. navis varingica* will be used to feed 5 batches of *A. granosa* while the control batch will be fed with non-toxic algae. A subsample of 3 replicates with 1.0 ml of seawater from the tank will be collected from cell count to determine the uptake rate. Cell count will be collected for every 3 hours in 24 hours. Cell count from zero hours will be counted as the initial data. A graph with the cell count data was then plotted.

Determination for depuration rate of ASP toxin by using HPLC-UV

DA content of culture

1 litre of late stationary phase clonal cultures will be harvested by manual filtration and resuspended in 1.0 ml Methanol/Water (1:1). The resuspension is vortexed for one minute followed by sonication for one minute. The sample is soaked for 30 min before repeating the previous homogenizing steps. The sample is vortexed for an additional one minute followed by incubation for 15 minutes at 60-70 °C. The sample is cooled down to room temperature and centrifuged at 10 minutes at 14,000 rpm. The supernatant is filtered through a 0.2 μm membrane filter and transferred to HPLC vial. The extracts were analyzed using SPDM10AVP HPLC-UV analysis (Shimadzu Corporation, Japan) according to the protocol for ASP toxin [16] in which an APEX ODS column (4.6 x 250mm, 5μm Cat#4M25310) (Argonaut Technologies Limited, UK) and the mobile phase of Acetonitrile/Water/Formic Acid (12.:87.8:0.2, v/v/v) were used. The commercial Canadian DA standard (DACS-1D, National Research Council Canada) with concentration 88 μg/mL was used as a calibration solution.

DA in shellfish meat

Three *A. granosa* was collected from each tank for consecutively four days. The shellfish meat was extracted and cut into smaller pieces. The sample was then resuspended in 1.0 ml Methanol/Water (1:1). The resuspension was sonicated by ultrasonic processor (CP 130PB-1, Cole-Partner Instruments, U.S.A) for one minute before being vortexed for one minute. The sample was then undergone sonication bath for 10 minutes. The samples centrifuged at 20 minutes at 4,500 rpm. The supernatant is then filtered through a 0.2 μm

membrane filter and transferred to HPLC vial. The extracts were analyzed using SPDM10AVP HPLC-UV analysis (Shimadzu Corporation, Japan) according to the protocol for ASP toxin [16] in which an APEX ODS column (4.6 x 250mm, 5µm Cat#4M25310) (Argonaut Technologies Limited, UK) and the mobile phase of Acetonitrile/Water/Formic Acid (12.:87.8:0.2, v/v/v) were used. The commercial Canadian DA standard (DACS-1D, National Research Council Canada) with concentration 88 µg/mL was used as a calibration solution.

RESULTS AND DISCUSSION

Growth Curve of *N. Navis-varingica*

When the toxic *N. navis-varingica* strain (P22C7) was transferred to the new media, the cells were in the resting phase where it adapts and restore energy for cell reproduction. The results have shown that the cell count increases drastically from Day 3 to Day 11 where the numbers multiplies to reach 3285 x 10^3 cells/ L^{-1} on day 11 (Figure 1). The highest average number of cells obtained was 9182 x 10^3 cells/ L^{-1} at day 23 (Figure 1). From day 3 to day 11, the cell density increases exponentially as they start to adapt into the new media and it provides ample of space for them to reproduce. The rate of growth during the slow phase was two cells every two days and reached 657 cells growth rate every two days during the exponential phase and 287 cells every two days in the stationary phase.

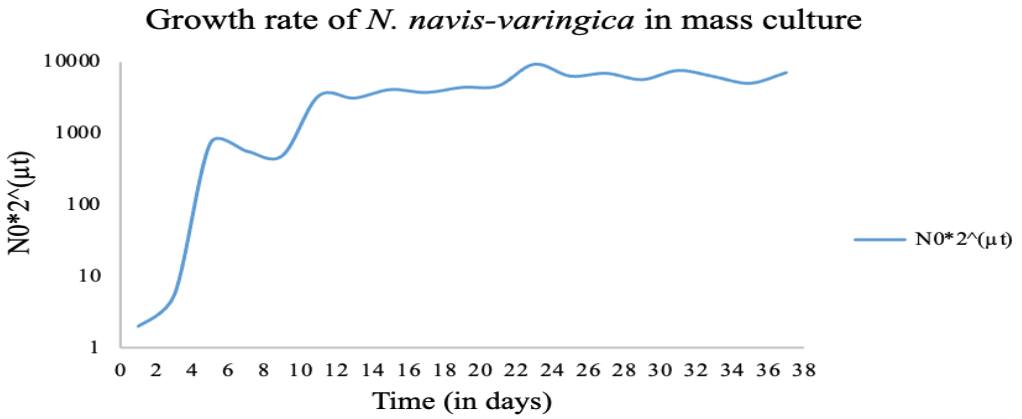


Figure 1: Growth rate of *Nitzchia navis-varingica* in SWII medium from day 1-37.

As comparison to [17], cell numbers did not increase between days 7 (the first sampling) and 16, but then a short period of exponential growth was observed between days 16 and 19, followed by the beginning of the stationary phase. In this experiment, the exponential growth happens from day 3 to day 13, followed by stationary phase starting from day 15 to day 37. Most DA is produced during the late-exponential to stationary phases [17]. In [18], [19] and [6]'s journal, *Nitzschia sp.* starts to grow exponentially between day 2 to day 10, followed by the beginning of stationary phase in Day 11 onwards whereby the cell density reaches 10^5 cell/ ml^{-1} and was maintained around this amount in in all three journals. In this experiment, the cell density fluctuates within 3080 to 9182 x 10^3 cells/ L^{-1} since day 13 to day 37 shows that it had reached stationary phase as the cell density no longer increases exponentially. Overall, as compared to the studies in [18], [19] and [6], the cell density is relatively lesser however the growth curve is stable in this experiment.

This experiment was conducted to determine the growth stages of *N. navis-varingica* for cell harvest during stationary phase. The accurate growth rate was not determined because the experiment was not conducted in a controlled environment. This finding is consistent with cells being arrested at the G1 or G0 phase, when they are still metabolically active, so that the DA can accumulate because cell division is stalled [3]. Microalgae require macro-, micronutrients and vitamins for growth. Macronutrients correspond to nitrogen (N) and phosphorus (P), while micronutrients correspond to trace metals (e.g. iron, manganese, cobalt, etc.). In addition, vitamins such as thiamine, biotin and cobalamin (vitamins B1, B7 and B12, respectively) are also needed since some microalgal species are not able to synthesize them [20]. One of the limiting nutrient is silicon. Diatoms require silicon (Si), which is involved in building the outer cell wall, or frustule [21]. Yet, silicate metabolism is tightly coupled with cell cycle [22] and appears linked to respiration [23]. Cells reach stationary phase at the lower temperatures later than at higher temperatures. The low-temperature cells had been

in stationary phase for less time than cells at higher temperatures, and therefore had less time to produce DA. Even though the concentrations and rates of DA production are low at low temperatures, the high cell yields attained could allow sufficient DA production to toxify molluscan shellfish [24][25].

Laboratory experiments have shown that depletion of silicate, phosphate and iron enhance the production of DA [4][19][26] whereas depletion of nitrogen in batch cultures results in a reduction in DA production [19]. The production of domoic acid stopped rapidly after the transition from the light to the dark period, and promptly resumed after the beginning of the light cycle [19].
Errors of the Sedgwick-Rafter counting chamber in the enumeration of *N. navis-varingica* cells could occur as the cells were overlapped in the chamber in 10x magnification under compound microscope (Olympus CX21, Philippines). The culture was place in the room condition near window to allow sufficient light for photosynthesis process, the culture was not placed in an incubator as there is not available in the laboratory hence it was not cultured under controlled environment with controlled (12:12) photoperiod.
This experiment can be improved by placing the culture in the incubator with controlled temperature and light intensity. An aerator pump can also be used to pump air bubbles into the culture to ensure enough air supply.

N. Navis-varingica Cells Uptake Of *A. Granosa*

Uptake rates in Tank A, Tank D and Tank E (Figure 2) decrease drastically to zero. Therefore, the graph cannot be plotted within the lines in the logarithmic graph. In Tank B, the rate of intake of *N. navis-varingica* diatom cells in the *A. granosa* tissue decreases drastically and fluctuates from ninth to 18th hours. The graph then goes down to zero. The rate of intake during the feeding experiment of Tank B is 12 cells per three hours in the first hour until the third hour and then one cell every 3 hours on the sixth hour until the 24th hour. Whereas in Tank C, the graph decreases drastically to zero in the first three hours and has a rate of up to ten cells every three hours in the first hour until the third hour.

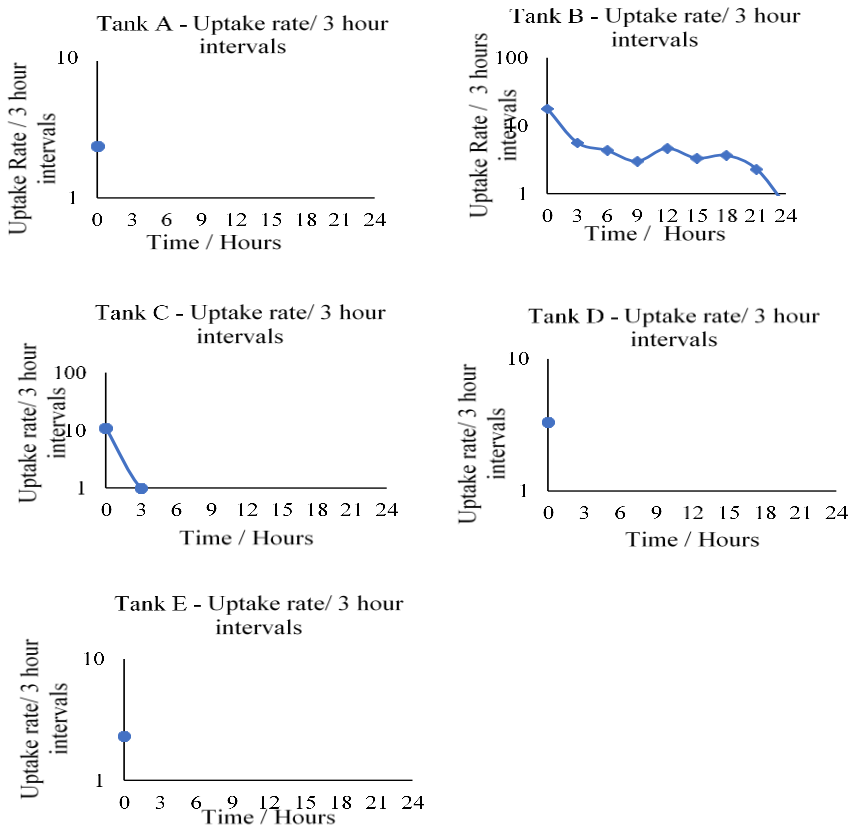


Figure 2: Indicates the rate of intake of diatom cells in *A. granosa* for three hours intervals in Tank A, B, C. D, and E. The blue graph line [- ● -] is a plot of the diatom cell uptake graph every three hours interval.

The five graphs above show variations uptake rate due to the possible reasons such as the stress level of *A. granosa* that affects the frequency of filter feeding in each tank. It is affected by several parameters such as food supply, temperature, population density, tidal change, pH and water depth [27]. Throughout the feeding process, the high mortality rate of *A. granosa* also might affect the uptake rate as averagely 5 to 9 cockles died in each tank during the feeding process. However, identification of the main causative factors is extremely difficult because these events involve a complicated interplay between the physiological condition of bivalves and environmental factors, so the cause of death can rarely be explained by a single factor [28]. The cockles size chosen for this experiment are medium-sized cockles and every cockle were confirmed alive before the feeding experiment started. The mass mortality of blood cockles is extremely likely upon exposure to a low-salinity environment of lower than 10 PSU for a week or more, which affects the filtering activity that cockles can exhibit at a salinity level of 16–19 PSU [29]. In this experiment, the seawater salinity was maintained between 20-25 PSU.

The effects of body size and exposure on DA accumulation by cockles may be significant as compared to oyster and mussels. [30] mentioned that weight-specific DA accumulation was significantly affected by body size in oysters but not in mussels. However, despite feeding on relatively high *P. multiseries* cell densities and toxicities, larger oysters never accumulated DA concentrations exceeding the regulation limit of 20 $\mu g/g^{-1}$ in whole tissues [29]. To support the statement above, [30] shows that all 3 bivalves (clams, mussels and oyster) have increasing order of actual feeding rate like: clam < oyster < mussel. Each type of bivalves has different shapes and sizes hence the feeding rate varies.

Another possible reason causing variations of uptake rate is due to low concentration of *N. navis varingica* culture is fed to the blood cockles. Only 100 ml of concentrated algae culture were fed to one tank of blood cockles that consists of 4 litres of seawater. The algae culture might be diluted or spread very far out causing *A. granosa* not able to filter feed efficiently. The relative feeding rates of all three bivalves were depressed at the lower food levels, and increased with higher food concentrations, until leveling off [30]. One of the ways to improve the accuracy and efficiency of this feeding experiment is to increase add a control tank of sediment and seawater to mimic the seawater condition without adding any cockles. This is to observe whether the algal cells were consumed by the cockles or the algae had died off before it was being consumed. [31] presented evidence that invertebrates exhibit a whole range of metabolic adaptations in response to a change in the quality and quantity of food available. These adaptations include depression of the metabolic maintenance requirement and an increase in the time food is retained in the gut, thereby allowing more effective assimilation.

A. granosa frequently lies in the mud such that the posterior end protrudes slightly above the surface. At other times, both inhalant and exhalant openings may be below the surrounding level of the mud surface in a small depression. In such a situation in the laboratory, during filtration surface mud often falls from the edges of the depression into the inhalant opening. Given this type of habitat, it seems that as long as the mud surface is easily resuspended then *A. granosa* is likely to be gaining its nutrition from a mixture of detritus (or microorganisms attached to detritus) and benthic microalgae [32]. However in this experiment, aerator pump had been installed in the tanks that ensure air bubbles were constantly pumped in and constant water circulation. These may cause *N. navis-varingica* unable to settle onto the sediment to be consumed by *A. granosa.* Hence, the uptake rate of *A. granosa* for *N. navis-varingica* is affected.

Determination of ASP Toxin Depuration Rate With LC-MS-MS

Multiple Reaction Monitoring (MRM**)** is the method used for LC-MS-MS that delivers a unique fragment ion that can be monitored and quantified during a very complicated matrix. This characteristic makes the MRM plot ideal for sensitive and specific quantitation. This experiment is accomplished by specifying the parent mass of the compound for MS/MS fragmentation and then specifically monitoring for a single fragment ion. The specific experiment in known as a "transition" and can be written (parent mass -> fragment mass), in this case for DA standard (DACS-1D) was (312.1 -> 266.1, 248.2, 161.1).

As compared to DA standard (DACS-1D) chromatogram (Figure 3 A), none of the retention peak from the sample chromatogram (Figure 3 C) belonged to domoic acid, as indicated by multiple chromatographic behaviours (different retention times). The LC-MS-MS chromatogram of cockles extract shows no detection of DA retention peak at 312.1 m/z. DA from the sample could not be detected by benchmarking DA standard (DACS-1D). Other than that, the low food concentration of algae culture being fed to the cockles may reduce the DA concentration and hence the toxicity decreases.

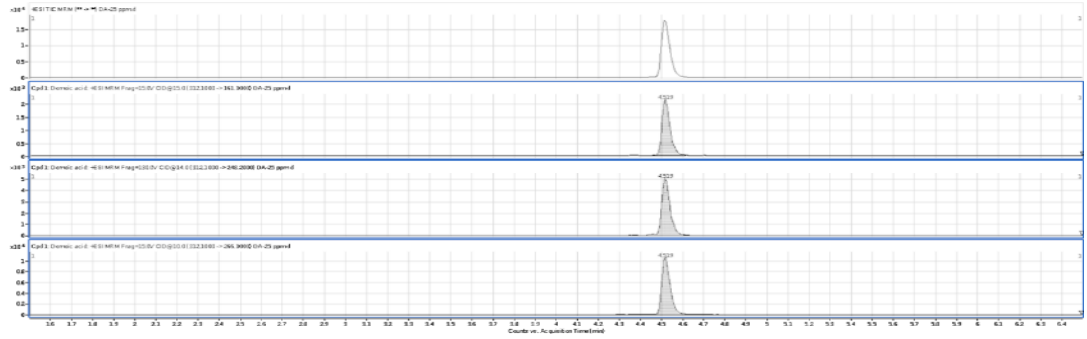
The mass culture of *N. navis-varingica* (P22C7) was obtained from the laboratory which was previously cultured from Sungai Pendas, Johor. In LC-MS analysis, the extracts showed a peak at the same retention time as DACS-1D standard [9]. The MS of the substance showed an ion peak at m/z 312 which is the $[M + H]^+$of DA (Figure 3.3 C and D). The cellular toxin content was calculated at 1.8 pg $cell^{-1}$. The LC-MS analyses verified that *N. navis-varingica* culture strains P22C7 produce domoic acid. [9]

There are also other unknown peaks in the chromatogram (Figure 3) which does not represent DA, it may be the protein derivatives or other unknown toxin that was already existed in the extract. According to [33], a quantitative Selective Reaction Monitoring (SRM) method was optimized for a range of toxins. Hence, this experiment can be improved by identifying the unknown peaks using MS/MS Library. A product ion spectral library can be constructed by acquiring QTRAP enhanced product ion (EPI) scans of $[M + H]^+$,$[M + NH_4]^+$, or $[M - H]^-$ ions of toxins at a range of collision energies (CEs) and collision energy spreads (CESs) [33]. Quantitative SRM method was not done in this experiment due to different method in sample extraction since the cockle sample was extracted for MRM method only and extra cockles was not collected for other methods.

ASP Jellet Test Kit was used in this experiment (Figure 4). It is a rapid test for ASP toxins that can be used with bivalves such as mussels, clams, oysters. This test involves converting the shellfish into a liquid form (known as the extract) which can then be applied to the test. The ASP test kit detects domoic acid well below the regulatory limit, at around 5 µg/g, but with dilution, can be used with a higher detection limit [34]. The sample was negative when two lines were shown which the second line intensity equals the control. First line represents the control, to make sure the test is working. If second line is absent, sample is strong positive [34].

During the sample collection process, the average mortality count of cockles sample were 3 to 4 per tank and this could affect the accuracy of extraction as the dead cockles may consume more toxic algae than the live ones. Reducing the amount of cockles in one tank may also be another solution to work on as 50 cockles in a 30cm x 40cm lab tray is too crowded and it caused stress to the cockles. The seawater used does not have continuous flow and aerator pumps were used for supplying air to the seawater, this might be one of the factor contributes to high mortality counts of cockles as clean seawater is essential to cockles aquaculture when in open seawater environment.

A.



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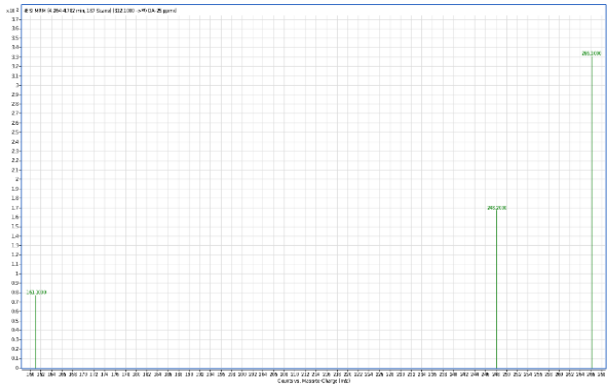

C

Figure 3: Results of liquid chromatography-mass spectrometry (LC-MS). Extracted ion chromatograms of MRMs from the sample against a domoic acid standard. A) Mass chromatogram of DACS-1D standard at a retention time of 4.51min; B) Mass spectra of DACS-1D standard derived from 4.3 (A) ; C) Mass chromatogram of sample extract from A. granosa that produce multiple peaks at various retention times.

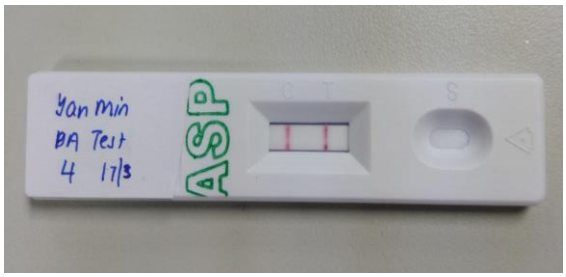

Figure 4: ASP Jellett Test Kit showed two obvious red lines which represents a negative results.

CONCLUSION

A study on the growth rate of *N. navis-varingica*, the intake of diatom cells by *Anadara granosa* and the rate of toxic depuration of domoic acid was carried out. The rate of growth has been determined using the relative growth count method for mass culture. The number of intake of diatom cells has been determined by the feeding experiment within 24 hours and the water sample will be taken into an inoculant containing 1ml of solid. The domoic acid toxicity depuration rate of *Anadara granosa* was determined using the Quadrupole Triple Mass Spectrometer (LC-MS / MS) method with Multiple Reaction Monitoring (MRM) and shellfish samples were taken after the 24-hour feeding experiment ended.

The results showed that the growth rate of *N. navis-varingica* mass cultures w high and had reached the exponential phase on the third day until the eleventh day. The growth rate of the mass culture has reached the stationary phase from day 13 until the 37th day. The rate of growth during the slow phase is two cells every two days and reaches the rate of growth of 657 cells every two days during the exponential phase and 287 cells every two days.

The findings on the study of diatomic cells were among the five aquaculture tanks, Tank B showed a clear diatomic intake graph in which the number of diatom cells in containers decreased from 18 diatom cells in inoculated one ml in the first hour to zero 24th hour. The rate of intake during the feeding experiment of Tank B was 12 cells every three hours during the first three hours interval and thereafter one cell every 3 hours at the sixth hour to 24th hour.

In terms of toxicity, the results of the study found that no domoic acid was detected in the sample of extracted shells since the first day of the depuration experiment. Jellett ASP test also shows two clear red lines that state a negative result means undetectable domoic acid. Both results can support that domoic acid cannot be detected in the extract of shellfish extract. Overall, the objectives of the first and second studies have been achieved. The third objective was not achieved because of the lack of provisions to carry out the LC-MS / MS Test of Selected Reaction Monitoring (SRM) and the way of preparation of different extract shellfish with Multi-

Reaction Monitoring (MRM). One of the factors is also due to uncertain food intake. The proposed future improvement to determine the mass growth rate of *N. navis-varingica* culture is to determine the growth of cell cells up to the phase of death to determine the expected future growth of *N. navis-varingica*. While for diatomic cell collection at *A. granosa* is to reduce the movement of water during the cultivation activity and to calculate diatom cells before feeding to *A. granosa*. To determine the level of toxic depuration of domoic acid, it is proposed to provide a sample of shellfish by Multi-Reaction Monitoring (MRM) and Selected Reaction Monitoring (SRM) or to use δ ^ 15N Isotope Analysis to determine the food source. In addition, the method of extraction of shellfish can also be converted into high heat extraction. This study will help the fishery industry in terms of food safety monitoring.

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