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RNA Isolation from Environmental Samples of a Harmful Algal Bloom for Metatranscriptome Next-Generation Sequencing

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ABSTRACT

During a harmful algal bloom (HAB), the seawater contains a high abundance of microorganisms and elemental ions. Such components can interfere with RNA isolation, leading to RNA degradation. The complex HAB seawater property makes isolating high-quality RNA for metatranscriptomic sequencing difficult, which is required for effective RNA sequencing and transcriptome profiling. This study used three isolation techniques to find the optimal strategy for isolating total RNA from bloom samples. One of the isolation techniques was the phenol-chloroform extraction method, which uses organic solvents to isolate RNA. The remaining two isolation techniques used the same commercial RNA extraction kit, *TransZol* Up Plus RNA kit (TransGen Biotech, China). One followed the extraction kit's protocol, while the other modified the protocol. Total RNA was extracted from three seawater samples of three occasions of HAB in Sepanggar Bay. The most effective approach used to extract high-quality RNA from the environmental samples of the HABs was the *TransZol* Up Plus RNA kit, with modified protocol. Results of the modified protocol generated a high-purity total RNA, ranging from 2.081 to 2.474 for both the absorbance ratios $A_{260/280}$ and $A_{260/230}$. The RNA integrity number value ranged from 6.2 to 7.6. All of the samples resulted in concentrations up to 91 ng/µl. We concluded that the modified protocol of *TransZol* Up Plus RNA kit yielded the highest quality total RNA for metatranscriptome next-generation sequencing (NGS). Apart from NGS, the high-quality RNA can also be used

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for various downstream applications, including real-time PCR, RNA cloning, and RNA microarray analysis.

Keywords: Harmful algal bloom, next-generation sequencing, RIN number, total RNA extraction

INTRODUCTION

Harmful algal bloom (HAB), also known as "red tide," is a common phenomenon in coastal waters worldwide. Under certain favourable environmental conditions, a unicellular microalga may proliferate and aggregate to form dense concentrations of cells or "blooms," resulting in a HAB outbreak. Such an outbreak severe affects the environment and human health and causes substantial economic losses to the aquaculture, fisheries, and tourism industries (Roberts et al., 2020). For example, since the first HAB report of the harmful alga, *Pyrodinium bahamense* var. *compressum*, in 1976, *Pyrodinium* blooms have occurred regularly for over four decades in the coastal waters of western Sabah, Malaysia (Roy, 1977; Anton et al., 2000; Law et al., 2020). Additionally, HABs caused by another harmful alga, *Margalefidinium* (formerly *Cochlodinium*) *polykrikoides*, have occurred every year since its discovery in 2005 (Anton et al., 2008). The former harmful alga produces saxitoxin, a neurotoxin that causes illnesses and fatalities in humans due to consumption of toxin-contaminated seafood (Usup et al., 2012). In contrast, the latter harmful alga causes water discolouration and mass fish mortality due to oxygen deficiency caused by the algae clogging the fish gills (Harun et al., 2015).

HABs in Sabah occur annually, usually from June to July (Northeast Monsoon) and December to January (Southwest Monsoon). The prevailing wind and surface current patterns may have contributed to the bloom's initiation (Usup et al., 1989). Another factor that contributed to the formation of HAB in Sabah was the accumulation of high nitrogen and phosphorus concentrations in the waters as a result of wastewater and pollutants being discharged from the mainland during heavy rainfall and surface runoff events. This factor helped promote the growth of the dormant harmful algae below the seabed (Anton et al., 2008). Compared to normal seawater, seawater during a HAB event usually comprises 90 % harmful algal species, produces a foul smell, and appears discoloured due to the pigmentation of the bloom-causing microalgae (Barsanti & Gualtieri, 2014).

Metatranscriptomic next-generation sequencing (NGS) is a relatively new field that attempts to investigate the functional properties of a complex environmental community (Jiang et al., 2011). The approach was designed originally to study bacteria and archaea, and its application to eukaryotic microorganisms has yet to be extensively documented (Lin et al., 2012). Furthermore, unlike metagenomics analysis, which provides information about the presence of microbes in an environmental community, the study of the metatranscriptome using NGS techniques provides information about gene activities, including gene diversity,

abundance, and differential gene expression between different conditions (Mutz et al., 2013). Therefore, isolation of total RNA from environmental samples with acceptable quality and intact RNA for effective RNA sequencing and transcriptome profiling is critical for acquiring good transcript data readings for metatranscriptomic analysis (Kukurba & Montgomery, 2015).

RNA quality is defined by the sample's RNA purity and integrity (Becker et al., 2010; Die & Roman, 2012). Total RNA is considered high-quality if it meets the NGS minimum criteria of (a) an absorbance ratio (A260:A280 and A260:230) of more than 1.8, (b) an RNA integrity number (RIN) score of greater than seven, and (c) a minimum concentration of 50 ng/µl (Die & Roman, 2012). The ratios of 260 nm to 280 nm and 260 nm to 230 nm absorbances are used to determine the purity of nucleic acids. For RNA, an A260:A280 ratio of approximately 2.0 is considered "pure". Protein, phenol, or other contaminants that absorb heavily at or near 280 nm may be present if the ratio is significantly lower. For the A260:A230 ratio, the value is often higher than the respective A260:A280 value, so the expected value is commonly in the 2.0 to 2.2. If the ratio is significantly lower than expected, it may indicate the presence of contaminants that absorb at 230 nm (Lucena-Agular et al., 2016).

In addition, the integrity of an RNA molecule is critical for investigations attempting to capture a snapshot of gene expression at the time of RNA extraction. The RNA integrity number (RIN) is the gold standard for determining the integrity of an RNA molecule. RIN is an algorithm for assigning integrity values to RNA measurements, traditionally evaluated using the 28S to 18S rRNA ratio (Schroeder et al., 2006). RNA integrity is measured using microcapillary electrophoresis that calculates the RIN value from 1 (fully degraded RNA) to 10 (intact RNA). The recommended RIN value for NGS is 7 to 8, depending on the type of samples (Thompson et al., 2007; Jeffries et al., 2014). The gradual degradation of ribosomal RNA is reflected by a continuous shift towards shorter fragment sizes (Schroeder et al., 2006). Furthermore, the concentration and amount of RNA depend on the type of RNA-seq library preparation method. Typically, the recommended RNA concentration is 10 to 100 ng/µl, while the total RNA amount is 5 ng to 10µg. It is important because using a more significant amount of starting total RNA can reduce the number of PCR amplification cycles during library preparation, which will result in more even distribution of mapped reads within target genes (Schmid et al., 2012; He & Jiao, 2014).

RNA extraction is frequently employed in molecular biology research to determine the metabolic activity of a sample. The input RNA's concentration and quality significantly influence gene expression consistency (Tavares et al., 2011). As a result, RNA purity and integrity have become the primary criterion for obtaining high-quality output data. It is critical to ensure that the extraction process is thoroughly managed because low-quality RNA can sabotage the results of NGS applications, such as RNA sequencing (Kukurba &

Montgomery, 2015). RNA extraction from a single species specimen is difficult enough, but environmental samples, such as harmful algal bloom (HAB) samples, are more challenging since they contain numerous species. The samples are also surrounded by various nutrients and ions, which can interfere with the RNA extraction process (Wang et al., 2012).

The RNA of HAB species is typically isolated by harvesting the monoclonal microalgal cultures that have reached their mid-exponential growth stage (Palani-Velu et al., 2013). However, RNA extracted from the environmental sample of HAB is unpredictable because HAB seawater contained many contaminants rather than a clean cell culture from *in-vitro* cell culture. Human activities, urbanisation, and industrialisation near the coastal area have resulted in the free flow of macronutrients, such as nitrogen and phosphorus, in abundance in a HAB environmental sample. Other factors like ions, sodium, debris, and suspended particulate matter will also significantly contribute to the instability of the RNA to be extracted from the HAB samples (Tan & Ransangan, 2016; Anderson et al., 2002). Due to some microalgae species in the HAB sample being made up of rigid cell walls, effective cell lysis for RNA isolation and retrieval of unbiased nucleic acid is substantially more challenging (Mäki et al., 2017).

Previous studies have reported using a variety of RNA extraction techniques to extract RNA from environmental samples of HAB for metatranscriptomic sequencing. For example, Cooper et al. (2014) used two different RNA extraction kits: the Ambion RNAqueous extraction kit (Life Technologies) to extract the total RNA of an environmental sample of *Prorocentrum minimum* bloom and the Nucleospin RNA Plant extraction kit (Machery-Nagel) to extraction the total RNA of clonal cultures of *P. minimum*. Meanwhile, total RNA from a *Levanderina fissa* bloom sample was extracted using the Ambion ToTALLY RNA kit (Life Technologies) after the sample was vortex with glass beads (Gong et al., 2017). In China, S. Xu et al. (2021) extracted the total environmental RNA of *Phaeocystis globosa* bloom by using the TRIzol Reagent Kit (Thermo Scientific) and then further concentrated the RNA sample with the RNeasy MinElute Kit (Qiagen). In addition, X. Xu et al. (2020) studied the microbial communities during HAB by extracting the environmental HAB sample using the RNeasy Isolation Kit (Qiagen).

This study compared three different RNA isolation techniques to extract the RNA from the environmental seawater samples during HAB: (a) phenol-chloroform method (Chomczynski & Sacchi, 2006), (b) *Transzol* Up Plus RNA kit (TransGen Biotech, China), and (c) *Transzol* Up Plus RNA kit (TransGen Biotech, China), with minor modification on the protocol. We aim to obtain high-quality RNA that meets the minimum NGS requirements, which include (a) RNA integrity number (RIN) value greater than 6.3, (b) minimum concentration of 50 ng/µl, (c) total RNA amount of greater than 1.3 µg, (d) absorbance ratio (A_{260}/A_{280}; A_{260}/A_{230}) of more than 1.8, and (e) two distinct intact 18S and 28S ribosomal RNA bands. Although there are other means for extracting RNA from

environmental HAB samples, this study was carried out to document other methods that may or may not be applicable. In addition, the study extends the knowledge on RNA extraction from the environmental sample of a HAB for metatranscriptomic NGS, as RNA extraction from HAB samples is not straightforward due to the presence of a large number of polysaccharides and polyphenols in microalgae.

MATERIALS AND METHODS

Sample Collection

Three HAB environmental seawater samples were used in this study. The HAB samples were collected around the Sepanggar Bay area, Kota Kinabalu, Sabah, Malaysia (Figure 1). Two litres of seawater samples from the HAB-affected area were collected directly from near-surface waters (approximately 0.2 m depth) using a water bucket and stored in a 10-litre water storage bottle. One bloom sample was obtained from each HAB event, as the bloom was confined to one area within the sampling site.

Table 1 details the bloom events that occurred throughout the study period. The first bloom event (BE1) occurred in November 2018, caused by the *Margalefidinium polykrikoides* species in UMS Jetty. The second bloom (BE2) was in July 2019, when the same causal species, *M. polykrikoides*, was identified in Sepanggar Bay (away from shore). The third bloom event (BE3) happened in October 2019, involving two HAB species, *M. polykrikoides* and *Pyrodinium bahamense* var. *compressum,* in Sepanggar Bay (near shore)

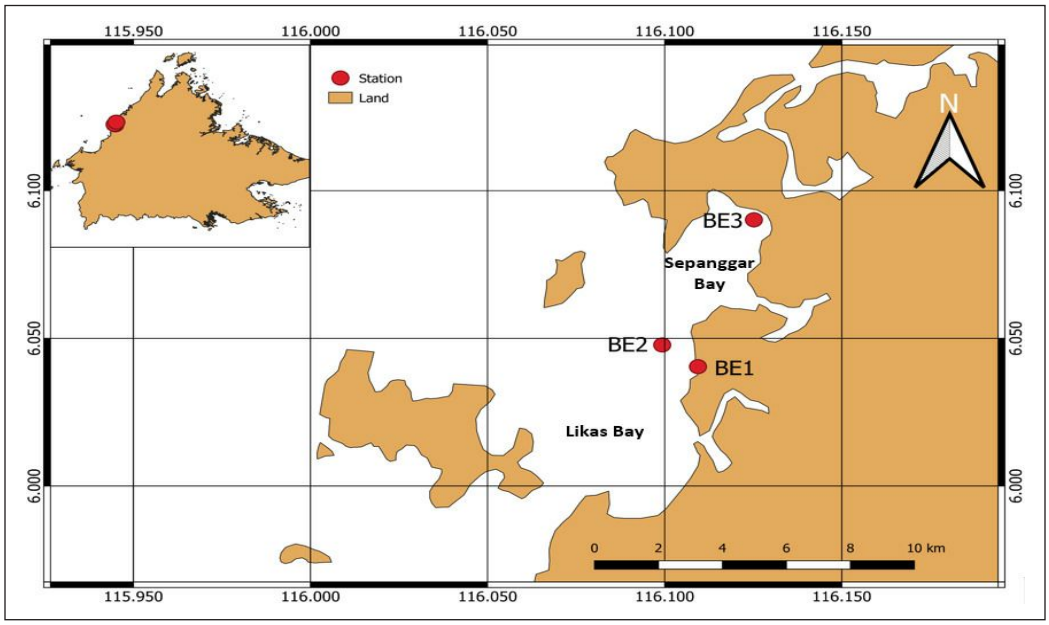

Figure 1. Map showing Sabah (inset) and Sepanggar Bay, Kota Kinabalu, Sabah. The three red points showed the location of the bloom events, BE1, BE2, and BE3, in the study area.

Table 1
Details of harmful algal bloom samples used in the study

Bloom Sample	Month, year	Cell Count (cells/l)	Species	Coordinates
BE1	November 2018	4.5×10^2	*Margalefidinium polykrikoides*	06°02'25.44" N 116°06'33.84" E
BE2	July 2019	1.2×10^2	*Margalefidinium polykrikoides*	06°02'51.9" N 116°05'57.6" E
BE3	October 2019	10.0×10^2	*Margalefidinium polykrikoides*	06°05'24.5" N 116°07'30.7" E
		4.2×10^2	*Pyrodinium bahamense* var. *compressum*	

Seawater Sample Preparation

The collected HAB seawater samples were promptly transported to the laboratory for filtration, which had to be completed within 24 hours of sampling. Cellulose nitrate membrane filter paper (0.45 µm) (Whatman, United Kingdom) was used to filter the two litres of water sample to retain eukaryotes. Each filter was individually wrapped in aluminium foil to minimize repeated thawing of the sample during RNA extraction and cross-contamination. The wrapped samples were placed in resealable bags and stored in a -80 ºC freezer (Thermo Fisher Scientific, USA). Right before RNA extraction, filters were briefly thawed on ice. Filtered samples of the same bloom event were placed in a 50 ml centrifuge tube and rinsed with sterile seawater. A total of five pieces of membrane filter paper were rinsed into the 50 ml centrifuge tube. The remaining sample on the cellulose nitrate membrane, which did not rinse off, was scrapped lightly to ensure all samples were ultimately rinsed into the falcon tube. The samples were then pelleted using a refrigerated centrifuge (Eppendorf, Germany) at 1,085 × g for 5 min at 4 ºC. After discarding the supernatant, the pelleted samples were ready for RNA extraction.

Total RNA Isolation Using Phenol-Chloroform Extraction Method (Chomczynski & Sacchi, 2006)

The complete procedure of the phenol-chloroform extraction method can be referred to in the original publication by Chomczynski and Sacchi (2006). After discarding the supernatant, the pelleted cells were lysed and homogenised with 1 ml of Solution D (Denaturing solution). Solution D contains 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5 % (w/v) *N*-laurosylsarcosine (sarkosyl) and 0.1 M 2-mercaptoethanol. The lysate was resuspended at least ten times with gentle pipetting, and the cell lysate was placed in a 4-ml polypropylene tube. In that order, 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml saturated phenol solution, and 0.2 ml chloroform/isoamyl alcohol (49:1) were added to the lysate. Before adding the chloroform/isoamyl alcohol, the tube was gently inverted

several times by hand for 10 s after adding each of the previously stated solutions. After thoroughly mixing, the tube was chilled on ice for 15 min before centrifuging at 10,000 × g for 20 min at 4 °C using a refrigerated centrifuge (Eppendorf, Germany).

Upon centrifugation, the solution in the tube was partitioned into three different phases: a lower cloudy white organic phase, pale brownish interphase and an upper colourless aqueous phase containing the RNA. The upper aqueous layer was transferred into a fresh 1.5 ml microcentrifuge tube before adding 1 ml of ice-cold isopropanol solution for RNA precipitation. The tube was incubated in a -20 °C freezer (Thermo Fisher Scientific, USA) for 1 h, followed by centrifugation at 10,000 × g for 20 min at 4 ˚C. The supernatant was discarded, and the RNA pellet was dissolved in 0.3 ml of solution D before being transferred to a new 1.5 ml microcentrifuge tube. A total of 0.3 ml of isopropanol solution was added into the tube, followed by incubation at -20 °C in a freezer (Thermo Fisher Scientific, USA) for 30 min. Next, the mixture was centrifuged at 10,000 × g for 10 min at 4 °C. After the supernatant was discarded, the RNA pellet was washed with 1 ml of 75 % ethanol solution. The RNA was gently mixed and incubated for 15 min at room temperature to completely dissolve any remaining residual salt. Next, the tube was centrifuged at 10,000 × g for 5 min at 4 °C, and the supernatant was discarded. The RNA pellet was air-dried at room temperature for 10 min before being dissolved in 150 µl of diethylpyrocarbonate (DEPC)-treated water. The sample was then incubated for 15 min at 60 °C before storing the RNA sample in a -80 °C freezer (Thermo Fischer Scientific, USA).

Total RNA Isolation Using *TransZol* Up Plus RNA Kit (TransGen Biotech, China)

The *TransZol* Up Plus RNA kit was used to isolate the total RNA content from the HAB environmental samples according to instructions provided by the manufacturer. The cell pellet was added with 1 ml of *TransZol* Up solution and gently pipetted until there were no visible residues in the lysate. After 5 minutes of incubation at room temperature, 0.2 ml of chloroform was added to the lysate according to the ratio (0.2 ml: 1 ml *TransZol* Up). The lysate was shaken vigorously for 30 s and incubated again for 3 min at room temperature before centrifuging at 10,000 × g for 15 min at 4 °C using a refrigerated centrifuge (Eppendorf, Germany). Three distinct phases of the lower pink organic phase, interphase, and a colourless upper aqueous phase containing the RNA were observed. The colourless, upper phase containing RNA was transferred into a fresh RNase-free tube, and an equal volume of 100% ethanol (25 µl) was added to the tube; the tube was inverted gently to ensure it mixed thoroughly.

All centrifugations in the following steps were carried out at 12,000 × g at room temperature using a refrigerated centrifuge (Eppendorf, Germany). Once mixed, the solution and precipitates were transferred into a spin column prior to centrifugation for 30 s, and the flow-through was discarded. A total of 500 µl of CB9 (Clean Buffer 9) was added into

the spin column and centrifuged for 30 s, and the flow-through was discarded. Next, the previous step was repeated, adding 500 µl of WB9 (Wash Buffer 9) into the spin column and centrifuging for 30 s. After that, the spin column was centrifuged for 2 min to remove any remaining ethanol. Once completed, the spin column was transferred to a clean 1.5 ml microcentrifuge tube. In the spin column, 50 µl of RNase-free water was added and incubated at room temperature for 1 min. Finally, the same tube was centrifuged for 1 min to elute the RNA and immediately stored in a -80 °C freezer (Thermo Fisher Scientific, USA) to avoid RNA degradation.

Total RNA Isolation Using a Modified Protocol of *TransZol* Up Plus RNA Kit (TransGen Biotech, China)

Minor modifications were made to the original protocol of the *TransZol* Up Plus RNA kit *TransZol* Up Plus RNA kit described by the manufacturer to improve RNA yield and quality. The detailed steps modified from the original protocol are listed in Table 2.

Table 2
Summary of modifications made to the original protocol of TransZol Up Plus RNA Kit

Components/Steps	Original Step	Modified Step
*Trans*Zol Up solution (Cell lysis)	1 ml *TransZol* Up solution was added and mixed by gentle pipetting until no residues were visible in the lysate.	If the sample still appeared saturated after adding the initial *TransZol* Up solution, additional 1 ml of *TransZol* Up solution was added to ensure complete lysis of the sample.
Clean Buffer 9 (CB9)	The washing step using CB9 was performed twice.	The washing step using CB9 was performed four times, where CB9 was re-added, and the sample was re- centrifuged four times.
Wash Buffer 9 (WB9)	The washing step using WB9 was performed twice.	The washing step using WB9 was performed four times, where WB9 was re-added, and the sample was re-centrifuged four times.
Washing step	CB9 and WB9 solutions were added directly to the bottom of the spin column.	CB9 and WB9 solutions were added entirely to the whole spin column.
RNA precipitation step	The RNA sample was precipitated for 30 minutes before the RNA elution step.	The RNA sample was precipitated for 2 hours before the RNA elution step.
RNA elution step	The RNA sample was directly eluted using RNAse-free water.	The spin column was air-dried for 10 minutes to remove the excess ethanol before the RNAse-free water was added for RNA elution.

RNA Quality Assessment

The total RNA's initial integrity and overall quality were observed with 1.5 % (w/v) agarose gel electrophoresis by inspection of the 28S and 18S rRNA bands. The extracted RNA sample was visually analysed following electrophoresis conducted at 75 V for 45 min, with

a 1 kb GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, USA). The concentration (absorbance at 260 nm) and purity (absorbance $A_{260/280}$ and $A_{260/230}$) of the RNA were measured with a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, USA). The RNA amount was measured using Qubit® 2.0 fluorometer (Life Technologies, USA). The RNA integrity number (RIN) was analysed with Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Table 3 summarises the required criteria for NGS RNA-seq for the eukaryotic sample.

Table 3
Criteria required for next-generation sequencing RNA-seq for microalgal sample: RNA concentration, RNA amount, RNA purity, RNA integrity number (RIN) and ribosomal RNA integrity on agarose gel

Sample Type	**RNA Concentration** (He & Jiao, 2014)	**RNA Amount** (He & Jiao, 2014)	**RNA Purity** (Die & Roman, 2012)	**RNA Integrity Number (RIN)** (Schroeder et al., 2006)	**Ribosomal RNA (rRNA)** (Die & Roman, 2012)
Total RNA (Microalgae)	≥ 50 ng/µl	≥ 1.3 µg	$A_{260/280}$ = 1.8- 2.2 $A_{260/230}$ ≥ 2.0	≥ 6.3 smooth base line	Two intact distinct rRNA bands

RESULTS AND DISCUSSION

The study area, Sepanggar Bay, Kota Kinabalu, is located on the south-eastern coast of the South China Sea and in the northwest part of Sabah, Malaysia (Figure 1). Sepanggar Bay receives freshwater inflows from the Inanam and Menggatal rivers and domestic sewage and factory wastes from an industrial area further inland (Anton et al., 2008). One of the eight international seaports in Sabah is located at Sepanggar Bay, where the port manages a few million tonnes of cargo annually (Goh & Lee, 2010). Besides that, resorts, coastal housing, and water villages can be found along the bay area. In addition, the bay supports fisheries' resources by providing farmed and fished seafood (Gallagher et al., 2016).

Three seawater samplings of bloom occurrences were conducted during the study period (October 2018 to November 2019), as shown in Table 1. *M. polykrikoides* was the primary cause of the first two HAB outbreaks, a dinoflagellate that clogs and damages the gills of wild and farmed fish, resulting in mass mortality due to lack of oxygen (Palani-Velu et al., 2013). Two harmful dinoflagellates, *M. polykrikoides* and *P. bahamense* var. *compressum,* were responsible for this study's most recent HAB event. *Pyrodinium bahamense* is a dinoflagellate that produces saxitoxin, a neurotoxin that causes paralytic shellfish poisoning in humans after consuming the toxin-contaminated shellfish (Jipanin et al., 2019). The HAB poses a severe threat to the Sabah's economy because of the state's dependency on marine and agricultural resources. Marine aquaculture accounts for more than 43% by volume and 73% by value of total aquaculture production in Sabah, equating to 11,846 metric tonnes valued at around USD$ 50 million (Liaw & Fung, 2000; Anton et al., 2008).

In Malaysia, *P. bahamense* var. *compressum* blooms were first documented in 1976 in Sabah, where the whole stretch of the west coast of Sabah, including Sepanggar Bay, was affected (Roy, 1977). Since then, *P. bahamense* bloom has been an annual occurrence and can be observed along the coastal waters of west Sabah, not exclusively at Sepanggar Bay (Usup et al., 1989; Usup & Azanza, 1998; Leaw et al., 2005; Chin et al., 2013; Anton et al., 2008; Law et al., 2020). Aside from *P. bahamense*, another two causative species were also identified, which were *M. polykrikoides* (Anton et al., 2008) and *Gymnodinium catenatum* (Mohammad-Noor et al., 2010). The *G. catenatum* and *M. polykrikoides* were first observed in Sepanggar Bay in 2003 and 2005, respectively, though no blooms of the former HAB species have been reported since then. Since the *M. polykrikoides* bloomed in 2005, the *P. bahamense* bloom has not occurred again until 2013 (Law et al., 2020). Blooms of *P. bahamense* and *M. polykrikoides* have frequently occurred solely or co-occurred since then. Understanding the time of occurrence and factors contributing to the HAB occurrence is still unknown.

The study initially performed the total RNA extraction using the phenol-chloroform method developed by Chomczynski and Sacchi (2006). Unfortunately, RNA extracted from HAB seawater samples using this method was inefficient, and the resultant RNA often appeared to be degraded or absent, as seen in Figure 2(a). The agarose gel photo in Figure 2(a) shows traces of degraded RNA in Lane 1 and no RNA in Lanes 2 and 3. Unsuccessful extraction of RNA by using this similar approach was also observed in *M. polykrikoides* monoalgal cultures, where RNA was frequently degraded or absent (Palani-Velu et al., 2013). RNA with low yield was obtained only a few times throughout the many trials performed by Palani-Velu et al. (2013), where barely visible large subunit (LSU) and small subunit (SSU) rRNA bands were observed on the agarose gel. The method failed maybe because the method was not suitable to eliminate the numerous impurities in the environmental samples, which were also accompanied by a high abundance of eukaryotic and prokaryotic organisms. Even though the phenol-chloroform method was unsuccessful for this study, this method is still widely used because of its high yield, purity, and speed, which makes it ideal for working with small numbers of samples (Shin, 2012).

After multiple failed attempts to extract total RNA using the phenol-chloroform extraction method (Chomczynski & Sacchi, 2006), a manufactured RNA extraction kit, *TransZol* Up Plus RNA Kit (TransGen Biotech, China), was used to extract total RNA from the HAB environmental samples. The *TransZol* Up Plus RNA kit is a ready-to-use reagent for isolating total RNA from cells and tissues. The silica-based spin column not only holds the RNA together but also prevents a significant volume of RNA from being spun out from the column, yielding a higher volume of RNA during the elution step (Karp et al., 1998). Attempts to extract RNA following the manufacturer's exact protocol have resulted in degraded and no visible RNA, as shown in Figure 2(b). However, in subsequent trials,

the RNA kit successfully produced two intact RNA bands with low yield, and some RNA degradation was observed at the bottom of the agarose gel [Figure 2(c)]. Figure 2(c) results showed that the RNA kit has the potential to extract RNA from the HAB environmental seawater samples; however, some modifications to the original protocol are required to improve the chances of isolating high-quality total RNA content.

The *TransZol* Up Plus RNA kit can be used on any organism, including humans, animals, plants, and bacteria. Therefore, it is not limited to a particular type of sample to be extracted. However, the main concern would be a mixture of organisms in one extraction process. Therefore, it is advisable to take several measures to change the protocols to suit the sample type better. HAB seawater environmental sample contains a combination of ions, planktons (phytoplankton, zooplankton), bacteria, and other contaminants or macronutrients. Therefore, HAB seawater samples' properties are vastly different compared to the cell-cultured samples, where the parameters of the cell cultures can be manipulated and controlled. Other factors such as excessive ions and pH might also become a restriction to yielding a good quality RNA (Tan & Ransangan, 2016; Anderson et al., 2002). As a result, modifications were focused on thoroughly removing all the impurities in the HAB seawater samples, ensuring a good quality RNA was obtained at the end of the extraction method. After several modifications to the *TransZol* Up Plus RNA kit (Table 2), total RNA was effectively recovered from the HAB environmental samples.

The successfully isolated RNA samples were of high quality and intact, as evidenced by visual observation of the ethidium bromide post-stained electrophoresed RNA samples (Figure 2d). Two distinct RNA bands were observed in Figure 2(d), representing the LSU rRNA and SSU rRNA, along with minor smearing at the lower molecular weight range. The LSU and SSU rRNAs were consistently observed in the RNA extracted from all HAB environmental seawater samples. The expected sizes of the rRNAs were 2,000 bp for SSU and 2,500 bp for LSU. However, based on the gel figure (Figure 2d), the sizes of LSU and SSU rRNAs observed were 750 bp and 500 bp, respectively. The size difference is because, in native agarose gels, the secondary structure of RNA affects its migration pattern, causing it to travel differently than its actual size. Native agarose gels separate RNA molecules not only by their size but also by the shape of their native structure (Wu & Tinoco, 1998; Buchmueller & Weeks, 2003). It was recommended to use a denaturing agarose gel system for better RNA quality assessment, as the denaturing gels run under the conditions that cause the natural structure of RNA to be disrupted, resulting in the formation of liner chains (Woodson & Koculi, 2009). In this study, the agarose gel electrophoresis was sufficient to judge the integrity and overall quality of the total RNA preparation by observing the SSU and LSU bands.

Due to the nature of the HAB seawater samples, the modifications made to the original protocol were to eliminate contaminants or impurities that would co-purified

together with the RNA. The modifications would therefore improve the possibilities of isolating high-quality RNA from the environmental seawater sample to be suitable for metatranscriptomic sequencing. Tan and Yiap (2009) documented that the target nucleic acid should be segregated from the contaminants, including protein, carbohydrate, lipids, or other nucleic acids. As a result, special attention was paid to the washing step of the protocol, in which RNA will bind to the silica. At the same time, impurities such as DNA, cellular proteins, ions, and metabolites are washed away by using the washing buffers, CB9 and WB9. The samples were washed twice with the washing buffers, yielding two RNA bands, but significant RNA degradation was observed on the agarose gel (Figure 2c). When the RNA quality was checked, the $A_{260/280}$ and $A_{260/230}$ ratios showed that all samples had a value less than 1.8. Following that, the number of washings was increased so that it was in stoichiometry with the volume of contaminants or impurities in the samples. The samples washed four times gave positive results (Figure 2d). The additional washings ensured that the RNA was cleaned thoroughly, and all the impurities were removed entirely through the flow-through of the spin column. In addition, when the washing solutions were added to the spin column, the solutions were added to the entire column rather than just the bottom of the spin column. This action was taken to ensure that all impurities on the interior wall of the column were also washed away, leaving no impurities in the column to be eluted out along with the RNA.

Other minor changes were also carried out to increase the extracted RNA's quality further. One of the significant changes was the extra addition of 1 ml *TransZol* Up solution when the sample lysate still appeared saturated due to incomplete lysis. The additional *TransZol* Up solution, a lysis solution, was necessary due to the high quantity of microorganisms in the HAB seawater samples. For example, sample BE3 (Table 1) contained a higher concentration of harmful algae. Also, it consisted of two types of microalgae, *M. polykrikoides* and *P. bahamense*, which required the extra addition of the lysis solution for the complete lysis of the sample. The second lysis solution would guarantee complete lysis of all the cells present in the samples. Complete cell lysis is essential for releasing RNA from the samples, where insufficient lysis will lead to low RNA yields (Islam et al., 2017).

Besides that, the incubation period during RNA precipitation by using absolute ethanol was also determined. The protocol of the RNA kit did not provide a specific time range for the RNA precipitation step. Usually, precipitation of RNA requires several hours to overnight, depending on the concentration (Green & Sambrook, 2020). Since the kit's protocol stated that the whole RNA extraction process could be completed within an hour, it was concluded that the RNA should only be precipitated for 10 to 30 minutes. The precipitation was initially carried out for 30 minutes; however, the RNA yield obtained was very low. Therefore, the precipitation incubation period was optimised, and the study

found that 2 hours of incubation time was sufficient to provide the needed RNA output. In the RNA precipitation process, the RNA needs to be recovered from the aqueous phase using alcohol; usually, isopropanol is preferred for RNA precipitation (Sambrook & Russell, 2006). Kaser et al. (2009) implied that there are different precipitation periods for different samples, and the optimal precipitation period for a given sample will succeed in producing the two rRNA bands. Complete precipitation will help retain and pool all the RNA molecules together, resulting in a much higher volume, which will reflect the quality of the samples (Johnson, 2012).

The last change on the kit's protocol was to air-dry the spin column before the elution step. This change ensured that any residual ethanol in the spin column would evaporate, preventing ethanol carryover during RNA elution. However, ethanol carryover could lead to the low performance of RNA in downstream applications. In addition, the presence of ethanol may interfere during library preparation, resulting in lower library concentrations than the acceptable threshold (Dehghani et al., 2019). Lastly, throughout the study, a precaution was taken to ensure that all reagents, such as chloroform and absolute ethanol (which were not included in the kit) and consumables, such as microcentrifuge tubes and micropipette tips used, were RNase-free.

After successfully obtaining two distinctive bands of 18S and 28S rRNA in the RNA extracted from all three bloom samples using the modified protocol of the *TransZol* Up Plus RNA kit, the quality of the RNA samples was evaluated. The quality of the extracted RNA was analysed using a UV-Vis spectrophotometer, showing a total RNA concentration of between 72 ng/µl to 91 ng/µl, with the RNA purity $A_{260/280}$ of 2.16 on average and $A_{260/230}$ of 2.08 to 2.47. The quality of the extracted RNA was further verified using an automated gel electrophoresis system, demonstrating a RIN ranging from 6.2 to 7.6 (Figure 3), with RNA amounts of 1.1 µg to 2.1 µg. The bioanalyzer also validated the RNA integrity by observing two peaks showing the 28S and 18S rRNA. Table 4 summarises the quality of the RNA extracted from the three HAB events using the modified protocol of the *TransZol* Up Plus RNA kit. The quality check of all three RNA samples fulfilled the requirements needed for metatranscriptomic next-generation sequencing stated in Table 3. According to Jiang et al. (2011), the RIN number should be the primary gold standard for ensuring the success of the sequencing.

Besides producing good-quality RNA, the *TransZol* Up Plus RNA kit was also preferred due to the shorter extraction time and the availability of a robust lysis solution (*TransZol* Up solution), which rapidly disrupts the cells without the help of a homogeniser. Therefore, thorough, rapid homogenisation of the sample and completing the RNA isolation process as quickly as possible to prevent further sample degradation is crucial in achieving successful RNA purification.

The modified protocol of the *TransZol* Up Plus RNA kit was optimised for extracting RNA from environmental samples of harmful algal bloom. Because the modifications to

the protocol were focused on eliminating contaminants and impurities from the environmental samples, the optimised RNA isolation technique may be useful for other environmental samples that are highly contaminated, such as soil samples and polluted seawater samples. Besides that, the optimised *TransZol* Up Plus RNA kit can also be applied to water-based environmental samples, like lake and river water samples. However, further optimisation may be needed to be carried out on the modified RNA isolation technique to suit the type of environmental samples.

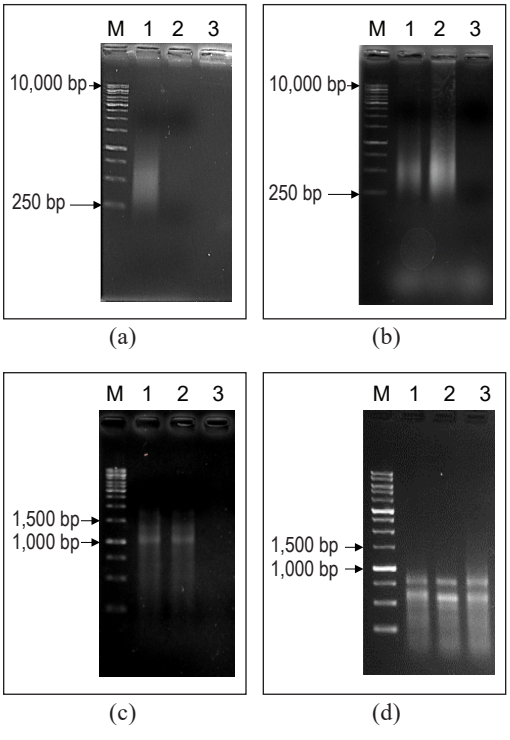

Figure 2. Electrophoresis of total RNA sample on 1.5 % agarose. (a) Phenol-chloroform method, (b) *TransZol* Up Plus RNA kit (Initial attempts), (c) *TransZol* Up RNA extraction kit (Subsequent attempts), (d) *TransZol* Up Plus RNA kit with modifications. Lane M: 1-kb DNA ladder, Lane 1-3: BE1, BE2, BE3.

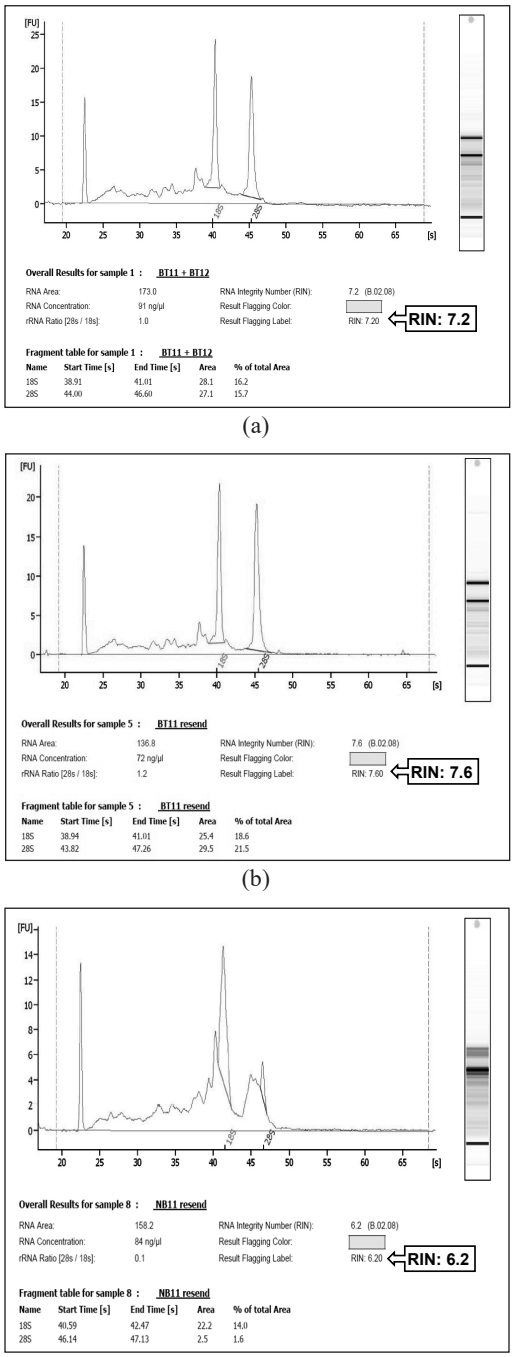

Figure 3. Profile image of intact 28S rRNA, 18S rRNA, and RIN of RNA sample extracted from three different bloom events, (a) BE1, (b) BE2, and (c) BE3, using the modified protocol of the *TransZol* Up Plus RNA kit.

Table 4
Quality assessment of RNA samples extracted using the modified protocol of the TransZol Up Plus RNA kit

HAB Environmental Sample	Nanodrop			Qubit		Bioanalyzer
	Conc. (µg/ul)	$A_{260/280}$	$A_{260/230}$	Volume (µl)	Total Amount (µg)	RIN
BE1	91	2.161	2.081	25	1.1	7.2
BE2	72	2.198	2.474	25	1.8	7.6
BE3	84	2.114	2.241	25	2.1	6.2

CONCLUSION

The first step in metatranscriptome sequencing is RNA isolation from an environmental sample. The focus of this study was to establish a suitable protocol for total RNA isolation from seawater environmental samples during a harmful algal bloom occurrence. The modified *TransZol* Up Plus RNA kit proved the most effective in providing reproducible, good-quality, and intact RNA that satisfied the standard criteria for RNA seq-NGS. The extracted RNA is of sufficient quality to produce a library, ensuring a successful RNA-seq experiment. In addition, the total RNA extracted in this study can also be used for other analytical analyses, such as gene expression profiling, cDNA synthesis, and differential expression study.

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