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Evaluation of RNA degradation in pure culture and field *Microcystis* samples preserved with various treatments

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ABSTRACT

RNA-based molecular technique (RT-qPCR) is a promising method for microcystin monitoring in lakes and reservoirs, but great lability of RNA in cyanobacterial samples limits its application. To date, no studies have investigated how to effectively preserve RNA in cyanobacterial samples. In this study, four different treatments (−80 °C freezer, −196 °C liquid nitrogen, 4 °C or 25 °C preservation after adding RNA protective fluid) were employed to preserve RNA in pure culture and field *Microcystis* samples, and RNA degradation in these treatments were systematically evaluated. Results showed liquid nitrogen was the most effective treatment to preserve RNA in pure culture and field *Microcystis* samples. RNA preservation using RNA protective fluid was temperature dependent. Low temperature (4 °C) could effectively slow down RNA degradation within a short time (1–7 d), since decay rate of *mcyH* mRNA (k = 0.00094 d^{-1}) was much lower at 4 °C than that at 25 °C (0.0549 d^{-1}) ($P < 0.05$). However, for field samples, RNA degradation was much faster than pure culture samples with the same treatment. Therefore, to better preserve RNA in field samples, a practical strategy for RNA preservation combining RNA protective fluid and liquid nitrogen, was proposed. Tests of field experiments showed it was more effective than individual treatment for RNA preservation in *Microcystis* samples during field sampling. Thus, this strategy could be employed to preserve RNA in cyanobacterial samples during field sampling, which will contribute to the application of RT-qPCR technique for microcystin monitoring in lakes and reservoirs.

1. Introduction

The frequent occurrence of cyanobacterial bloom in lakes and reservoirs has increasingly become a major environmental concern worldwide due to its great threat to water safety (Merel et al., 2013). Undesirable toxic metabolites microcystin (MCs) produced by a number of cyanobacteria (e.g., *Microcystis* spp., *Nostoc* spp., *Phormidium* spp., *Anabaena* spp., *Oscillatoria* spp. and *Planktothrix* spp.) (Chorus and Bartram, 1999; Jungblut and Neilan, 2006), are proved to be potent liver tumor promoter with > 90 variants, leading to a high risk of human health (Pearson et al., 2010; Zurawell et al., 2005; Niedermeyer et al., 2014). MCs biosynthesis is encoded by a *mcy* gene cluster, composed of 10 bidirectionally transcribed open reading frames arranged in two putative operons (*mcyA-C* and *mcyD-J*) (Tillett et al., 2000).

With the development of molecular biology technology, DNA-based molecular technique qPCR (quantitative polymerase chain reaction) (Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003; Fortin et al., 2010) have been applied to quantify microcystin biosynthesis genes (*mcy*) copies to predict potential toxin production in waters. Nonetheless, previous studies found nontoxic cyanobacterial mutants were produced when cells occurred with deletional or insertional mutagenesis of *mcy* genes, and these strains were incapable of expressing *mcy* genes (Kaebernick et al., 2001; Christiansen et al., 2008), suggesting the DNA-based technique may overestimate toxigenicity due to little insight of active microcystin biosynthesis gene transcription. Actually, MCs biosynthesis followed a series of steps starting with *mcy* transcription into mRNAs, translation of mRNAs into polyketide synthases,

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then nonribosomal peptide synthetases, and assembling into microcystin structure at last (Welker and Döhren, 2006). Therefore, RNA-based molecular technique (RT-qPCR, reverse transcription real-time quantitative polymerase chain reaction), which allows for measurement of *mcy* genes transcripts, is a promising method for microcystin monitoring.

However, the difficulties of RNA preservation in cyanobacterial samples limit its application. It may attribute to the greater lability of single-stranded RNA relative to double-stranded DNA. Majority of gene transcripts could merely remain for few minutes (Belasco and Brawerman, 1993). In *Escherichia coli*, exogenous and endogenous RNA enzymes (e.g., RNase P; RNase J; RNase E) could degrade RNA quickly (Luro et al., 2013; Hui et al., 2014), among which RNase E could cut RNA internally within single-stranded regions that are rich in AU sites (Mcdowall et al., 1994). Nonetheless, RNA degradation mediated by endonuclease and 5′-3′ exonuclease could be restrained with secondary structures (stem-loop, hairpin) involved in RNA molecules (Emory et al., 1992; Xu and Cohen, 1995). Besides, freezing and RNases inhibitors could prevent RNA degradation by inactivating RNases (Auer et al., 2014). It suggested RNA degradation is controlled via a complex mechanism. Hence, to slow down RNA degradation in cells, various treatments were employed to preserve biological samples.

Cryopreservation (−80 °C freezer and − 196 °C liquid nitrogen) has been widely utilized for long-term RNA preservation in cells and tissues samples in medical fields (Andreasson et al., 2013; Auer et al., 2014). Andreasson et al. (2013) demonstrated RNA integrity remained intact, although endocrine tissue samples were stored for 27 years at −80 °C. However, the −80 °C freezer is expensive up to $ 20,000, and requires tremendous amounts of energy. Liquid nitrogen stored in specially-made liquid nitrogen tanks is easily volatilizing and difficult to carry along for field sampling. Thus, cryopreservation of −80 °C and − 196 °C were not proper treatments for RNA preservation during field sampling.

Currently, a commercial easy-taking RNA protective fluid (e.g., TIANGEN RNAstore®; Invitrogen RNAlatter®) has been also used for short-term RNA preservation in samples. The solution is an aqueous tissue storage reagent that rapidly permeates most tissues to stabilize RNA in fresh specimens. It has been proven effective for RNA preservation in cells, bacteria, yeast and tissues (Grotzer et al., 2000; Rodrigo et al., 2002; Mutter et al., 2004; Dekairelle et al., 2007). Intact RNAs were obtained from bacterium *Escherichia coli,* which has been preserved at 4 °C after adding RNAstore® for 1 month, but it may not be effective in tissues that are poorly penetrated by the solution, such as waxy plant tissue and bone. Cyanobacteria is prokaryotic bacteria with particular cellular structure gelatinous sheath capsulated cells, consisting of pectic acid and mucopolysaccharideen (Jürgens and Weckesser, 1985). Additionally, cyanobacterial cells always form colonies with amorphous mucilage or sheaths loosely attaching to the cell surfaces in natural freshwaters (Reynolds, 2007; Ma et al., 2014). These structures may impede the permeation of RNA protective fluid. Therefore, whether RNA protective fluid is feasible to RNA preservation in pure culture and field cyanobacterial samples needs further investigated.

Until now, no studies have systematically evaluated these treatments (−80 °C freezer, −196 °C liquid nitrogen, 4 °C or 25 °C preservation after adding RNA protective fluid) to preserve RNA in cyanobacterial samples. In this study, RNA degradation in these treatments were assessed by RNA concentration, RNA quality, RT-qPCR of 16S rRNA and *mcyH* mRNA, respectively. The aim of this study was to find effective method to preserve RNA in cyanobacterial samples, especially during field sampling, which was strongly essential for the application of RT-qPCR techniques for microcystin monitoring in lakes and reservoirs.

2. Materials and methods

2.1. RNA preservation experiments for pure culture samples

The genus *Microcystis* is the most problematic, forming harmful cyanobacterial blooms worldwide. Thus, a pure culture of toxic strain *Microcystis aeruginosa* FACHB-915 was employed to conduct RNA preservation experiments. The strain was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. It was cultured in BG11 medium at 25 °C under constant light flux with a 12 h:12 h light–dark cycle. Prior to preservation experiments, *Microcystis* cells (cell density: 6.2 × 106 cells mL-1) were harvested at exponential growth phase by centrifugation at 6000 × *g* for 5 min, washed with 0.9% NaCl twice, and resuspend with phosphate buffer saline (PBS, 10 mM, pH 7.4).

Previous studies reported −80 °C freezer and − 196 °C liquid nitrogen were feasible to long-term RNA preservation in cells and tissues samples in medical fields (Andreasson et al., 2013; Auer et al., 2014). Thus, in this study, pure culture samples preserved in −80 °C freezer and liquid nitrogen, were set as controls. RNA extraction, determination of RNA concentration, RNA quality assessment, RT-qPCR of 16S rRNA and *mcyH* mRNA were also performed at 0, 3, 28 d. The details of methods were described below.

To explore whether RNA protective fluid was feasible to RNA preservation in pure culture *Microcystis* samples, samples were preserved at 4 °C and 25 °C after adding RNA protective fluid (samples: RNA protective fluid (*v*/v) = 1:5), respectively. RNA extraction, determination of RNA concentration, RNA quality assessment, RT-qPCR of 16S rRNA and *mcyH* mRNA were performed at 0, 1, 3, 7, 14 and 28 d. The details of methods were described below.

TIANGEN RNAstore® and Invitrogen RNAlatter® are most common commercial RNA protective fluid. According to the manufacturer, it eliminates the need to immediately process or freeze samples. The specimen could simply be submerged in solution and stored for analysis at a later date. RNAstore® ($ 75 per 100 mL) was cheaper than RNAlatter® ($ 235 per 100 mL). Thus, RNAstore®, which was purchased from TIANGEN Biotech, was utilized to preserve RNA in cyanobacterial samples in this study.

2.2. RNA preservation experiments for field samples

In natural freshwaters, *Microcystis* cells always form colonies with amorphous mucilage or sheaths loosely attaching to the cell surfaces (Ma et al., 2014). To investigate the effect of these colonies on the RNA degradation in *Microcystis* samples, colonial samples were collected in Maxi pond located in Shantou (China) (116.4° E 33.9° N), where a toxic *Microcystis* bloom occurred in 2018 (Fig. S2). These samples were stored in transparent bucket, and immediately transported to laboratory within 2 h. Then, colonial cells were centrifuged with 6000 × *g* for 2 min, and were preserved in these four treatments as described above in method section (RNA preservation experiments for pure culture samples). RT-qPCR of 16S rRNA and *mcyH* mRNA was employed to evaluate these four treatments for RNA preservation in field samples.

2.3. Field preservation experiments

The treatment of RNA protective fluid was more easily conducted for RNA preservation in *Microcystis* samples during field sampling than treatments of cryopreservation (−80 °C and − 196 °C). Thus, field preservation experiments for colonial *Microcystis* were conducted in Maxi pond, where a toxic *Microcystis* bloom also occurred in 2019. During field sampling, *Microcystis* samples were collected in situ by centrifugation at 3000 × *g* for 2 min using a portable M16 centrifuge (Cence, China), and preserved at low temperature (bubble chamber and ice bags) after adding RNA protective fluid. Then, after the preservation of 1, 2 and 3 days, samples were transferred to the preservation of liquid nitrogen for 28 days in laboratory, respectively. During treatment

process, copies of 16S rRNA and *mcyH* mRNA were estimated via RT-qPCR analysis to investigate RNA degradation after preservation of 1, 2, 3, 7, 14 and 28 days, respectively.

2.4. Analytical methods

2.4.1. RNA extraction from Microcystis samples

All experimental supplies were treated with DEPC RNAase-free water (Solarbio®). Cyanobacterial samples were pre-treated to disrupt cells by liquid nitrogen grinding. RNA was extracted using Spin Column Plant Total RNA Purification Kit (Sangon Biotech). Amending procedure was adding RNase inhibitor (ThermoFisher Scientific) to inhibit the activity of RNases, and DNAase I (ThermoFisher Scientific) to remove unwanted DNA from cell lysates.

2.4.2. RNA concentration determination and quality assessment

RNA concentration was determined by Nanodrop100 microspectrophotometer. RNA quality was assessed by RNA purity and integrity. RNA purity was evaluated by A_{260}/A_{280} and A_{260}/A_{230} ratio, and high purity RNA was 1.8–2.1 in A_{280}/A_{260} ratio and > 2.0 in A_{260}/A_{230} ratio, respectively (Sambrook et al., 1989; Manchester, 1995; Imbeaud et al., 2005). RNA integrity was assessed with 1% (*w/v*) agarose gel electrophoresis (Vendrely et al., 1968; Imbeaud et al., 2005), which was conducted by electrophoresis apparatus (JY600C, China).

2.4.3. RT-qPCR analysis

The primers of *mcyH* and 16S rDNA were designed by 'premier 5.0 software'. The optimum annealing temperature of primers of *mcyH* and 16S rDNA was 50 °C and 58 °C, respectively (Table S1). RNAs were transcribed to cDNAs using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) according to the manufacturer's protocol. The cDNAs were used as templates for RT-qPCR analysis. The qPCRs were performed in triplicates using a SYBR® Green I qPCR kit (Takara). Samples were run in a 96-well reaction plate on the ABI 7500 real-time PCR system (Applied Biosystems). The specific qPCR conditions were as follows: initial a hot start at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 35 s, annealing at 50 °C (*mcyH*) or 58 °C (16S rDNA) for 35 s; elongation at 72 °C for 45 s.

The target genes of 16S rDNA and *mcyH* were cloned into p7S6 cloning vector, which were used as plasmid standard substances. Eq. (1) is the calculation of gene copies (N). Standard curves for 16S rDNA and *mcyH* quantification were established using 10-fold serial dilutions of single copy plasmid and amplification efficiency of RT-qPCR was calculated using Eq. (2) (Fig. S1). Gene transcripts copies of 16S rDNA and *mcyH* in samples was calculated using the regression equation of plasmid standard curves (Fig. S1).

$$N = \frac{A \times C}{MW} \quad (1)$$

Where N = plasmid copies in copies mL^{-1}, A = constant value 6.02×10^{23} copies mol^{-1}, C = plasmid concentration in g mL^{-1}, MW = average molecule weight of plasmid standard substance.

$$E = 10^{-1/S} - 1 \quad (2)$$

Where E = qPCR amplification efficiency, S = slope of the regression equation of plasmid standard curve.

2.5. Statistical analysis

These experiments were conducted in triplicates, and error bars in the plots represent the standard deviation (SD) values. Results of RNA concentrations and mRNA copies over preservation time, were statistically analyzed using Student's *t*-test. Differences were considered significant at $P < .05$.

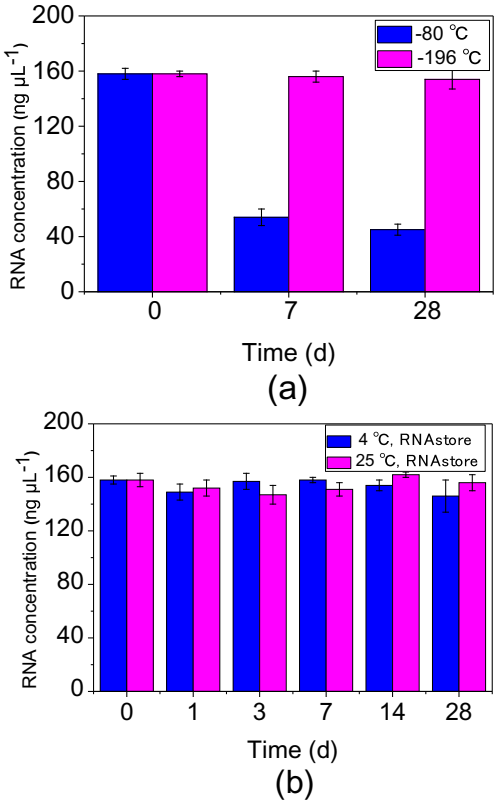

Fig. 1. Evolution of total RNA concentration in pure culture *Microcystis* samples with various treatments for 28 d. a: samples preservation of −196 °C liquid nitrogen and − 80 °C freezer, respectively. b: samples preservation at 4 °C or 25 °C after adding RNAstore®, respectively.

3. Results and discussion

3.1. Total RNA concentration

Total RNA was extracted from pure culture samples, and its concentration was determined. RNA concentration was 158 ng μL^{-1}at 0 d (Fig. 1). Samples were preserved in liquid nitrogen, RNA concentration remained about 155 ng μL^{-1} for 28 d ($P > .05$) (Fig. 1a). Nonetheless, RNA concentration declined from 158 ng μL^{-1}($t = 0$ d) to 48 ng μL^{-1} ($t = 28$ d) ($P < .05$) (Fig. 1a) when samples were preserved in −80 °C freezer. For RNAstore® treatments, samples were preserved at 4 °C and 25 °C, respectively, and RNA concentration was unaffected after 28 days of preservation ($P > .05$) (Fig. 1b). It suggested RNAstore® and liquid nitrogen were feasible for RNA preservation in pure culture samples.

3.2. RNA quality

RNA purity was assessed by A_{260}/A_{280} and A_{260}/A_{230} ratio. A_{260}/A_{280} and A_{260}/A_{230} ratio of total RNA extracted from pure culture samples in different treatments, was ranged from 1.8 to 2.1 and 1.9 to 2.1, respectively, as summarized in Fig. 2. It demonstrated RNA sample has high purity without DNA, proteins, organic solvent, carbon substance (e.g., carbohydrate, phenols) contamination and RNA extraction method was reliable.

RNA integrity was evaluated by agarose gels. Three bright bands (5S rRNA, 16S rRNA, 23S rRNA) on agarose gel were observed clearly at 0 d (Fig. 3a), indicating total RNA extracted from pure culture cyanobacterial cells was intact. A few dim bands were also observed, since total RNA have not been purified and residual little DNA existed in RNA samples (Fig. 3).

The majority of RNA extracted from samples preserved in −80 °C

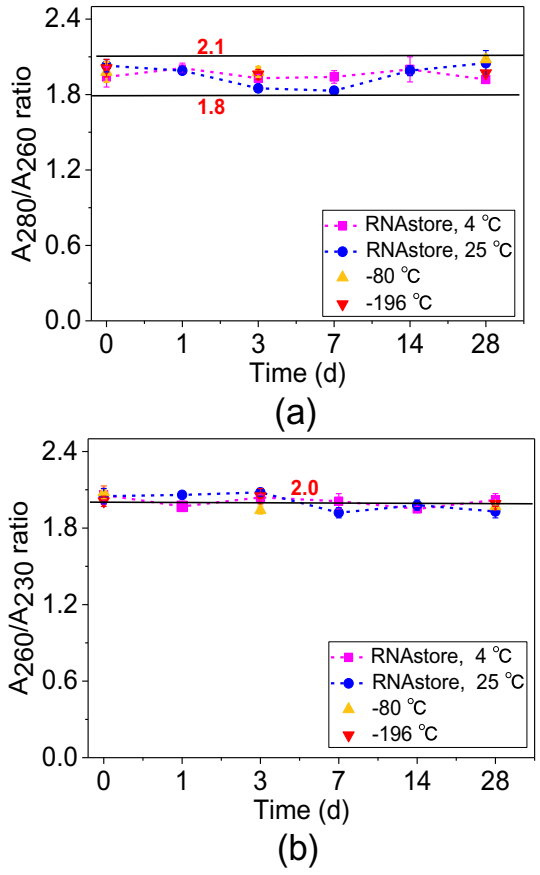

Fig. 2. RNA purity determination by A_{280}/A_{260} (a) and A_{260}/A_{230} (b) in pure culture *Microcystis* samples with various treatments (−80 °C freezer, −196 °C liquid nitrogen, 4 °C or 25 °C preservation after adding RNAstore®).

freezer was degraded rather rapidly, and no clear bands were observed at 3 d (Fig. 3b). When samples were preserved in liquid nitrogen, RNA was degraded partially, and 5S rRNA band remained bright and clear, but 16S rRNA, 23S rRNA bands become obscure at 28 d (Fig. 3c). The similar pattern was observed when samples were preserved at 4 °C or 25 °C after adding RNAstore® from 0 d to 7 d (Fig. 3d, e). However, all RNA bands became obscure, and other narrow bands dispersed on gels from 14 d to 28 d (Fig. 3d, e). It suggested all treatments were not feasible to RNA preservation in pure culture samples.

These results revealed by RNA concentration, RNA quality were paradoxical. Analysis of RNA concentrations showed RNAstore® and liquid nitrogen were feasible for RNA preservation, while assessments of RNA integrity reveled all treatments could not work well. It could be attributed to the difference of measurement principle of the two parameters.

Nucleotide consisted of phosphoric acid, ribose and base, is the basic unit comprising DNA or RNA. The benzene ring structure of base has maximum absorption peak 260 nm, and its absorbance (A260) is linear to DNA or RNA concentration (Manchester, 1996(Pearson et al., 2010)). The principle has been applied to determine DNA or RNA concentration by Nanodrop 100 microspectrophotometer. Here, RNA concentration declined with the treatment of −80 °C, implying the destruction of benzene ring structure. For other treatments, RNA concentration remained constant for 28 d, suggesting benzene ring structure in RNAs was intact. Nonetheless, RNA degradation was indeed observed by agarose gels in the four treatments. Literatures proposed RNA degradation go through two processes. Firstly, RNA is cut into fragments by RNase E, and into nucleotide by RNases (e.g., PNPase, Rhl B, RNase R, RNase II), then degrade completely (Carpousis, 2007). As evidenced in Fig. 3, dispersive bands occurred on gels in liquid nitrogen and RNAstore® preservation treatments, suggesting the RNAs were degraded into fragments with intact benzene ring structures rather than complete degradation. Therefore, RNA concentration parameter would overestimate the effectiveness of these treatments for RNA preservation in pure culture samples.

In comparison, RNA integrity evaluated by agarose gels is a strict index, which was supported by a hypothesis: mRNA is all degraded, once rRNA is degraded, since mRNA only share 3–5% and rRNA share 75–85% of total RNA in cells (Sambrook et al., 1989). Nonetheless, low proportion of mRNA do not signify high degradation potential. Furthermore, studies by Miller et al. (2004) and Imbeaud et al. (2005) revealed RNA integrity showed on gels, was proved to be a misleading indicator of the state of the mRNA for use in RT-qPCR. Thus, this parameter would exaggerate gene transcripts (mRNA) degradation, leading to underestimating the effectiveness of these treatments for RNA preservation in pure culture samples.

3.3. RT-qPCR analysis of 16S rRNA and mcyH mRNA copies in pure culture Microcystis samples with various treatments

Previous studies employed RT-qPCR to quantify target gene transcripts, and it was a direct and reliable parameter to evaluate the availability of RNA samples for downstream analysis (e.g., RT-qPCR and RNA sequencing) (Miller et al., 2004; Fleige et al., 2006; Carvalhais et al., 2013; Gallego-Romero et al., 2014; Seelenfreund et al., 2014; Campbell et al., 2016). 16S rDNA encoding 16S rRNA in genomes of

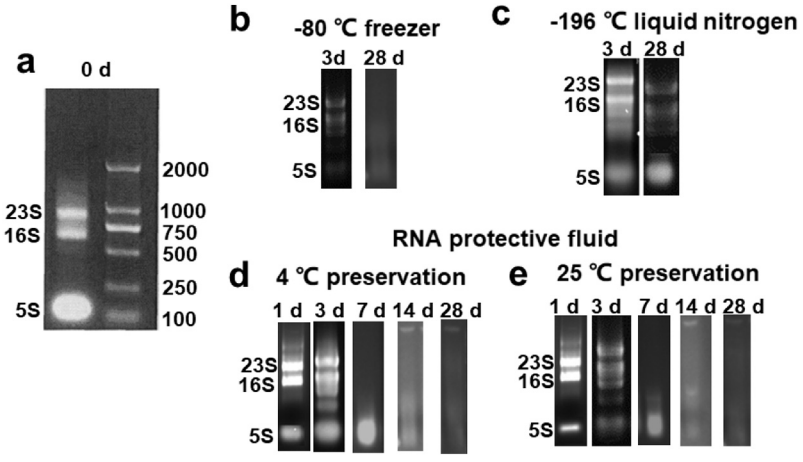

Fig. 3. RNA integrity presented on agarose gels during pure culture *Microcystis* samples preservation in different treatments (−80 °C freezer, −196 °C liquid nitrogen, 4 °C or 25 °C preservation after adding RNAstore®).

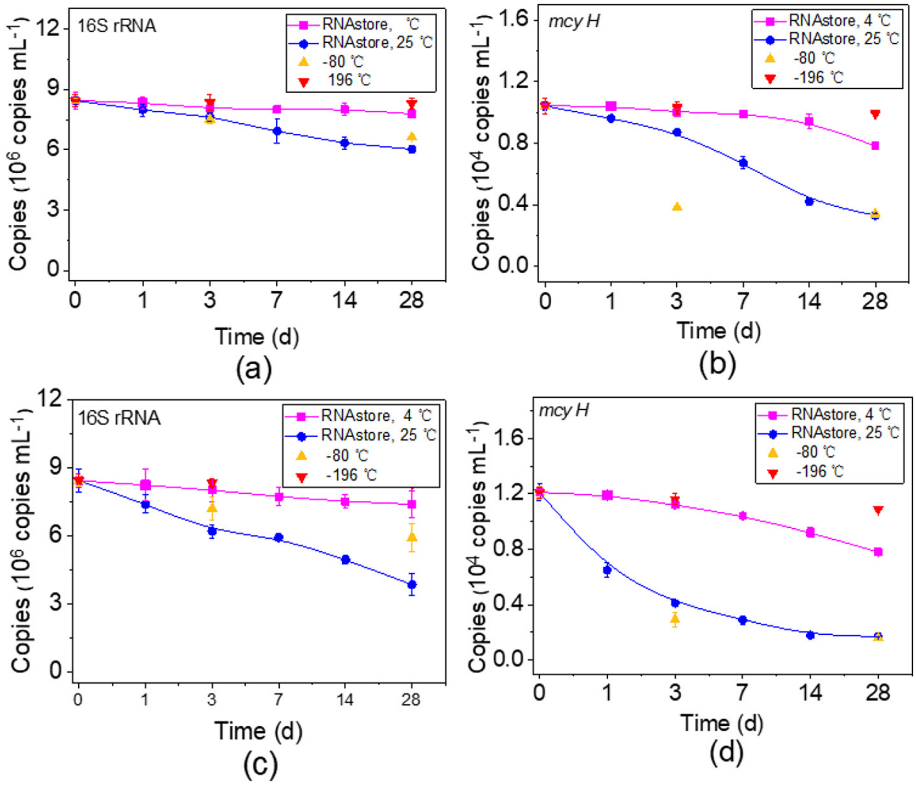

Fig. 4. Degradation process of 16S rRNA and *mcyH* mRNA copies in pure culture (a, b) and field *Microcystis* samples (c, d) with various treatments (−80 °C freezer, −196 °C liquid nitrogen, 4 °C or 25 °C preservation after adding RNAstore®). Error bars in the plots representing standard deviation of triplicates.

bacteria, is highly conservative and typed as house-keeping gene. Its gene expression level is less influenced by environmental factors. For *Microcystis*, a gene cluster (*mcyA-J*) encodes microcystin biosynthesis, and *mcyH* play a significant role in the thylakoid localization of microcystin (Tillett et al., 2000). Thus, RT-qPCR analysis of 16S rRNA and *mcyH* mRNA copies was employed to further assess RNA degradation in pure culture samples with various treatments.

The standard curves shown in Fig. S1, demonstrated a high correlation of CT value and gene copies of 16S rRNA (R^2 = 0.999) and *mcyH* (R^2 = 0.997). The qPCR amplification efficiency (E) of 16S rRNA and *mcyH* mRNA was > 90%, and the corresponding value was 93.4% and 90.5%, respectively (Fig. S1). It suggested that the RT-qPCR of 16S rRNA and *mcyH* mRNA was reliable.

The mRNA copies of 16S rRNA and *mcyH* was degraded to some extent in the four treatments, and correlated with preservation time (Fig. 4). For pure culture samples, a rapid degradation of *mcyH* mRNA (63.5% in 3 d), was observed in the preservation of −80 °C (Table S2), indicating this treatment was not feasible to RNA preservation in samples. In general, enzymatic reactions are considered to continue at −80 °C and cells do not remain viable when stored at −80 °C (Auer et al., 2014). However, the ultralow temperature may not completely inactive RNases in cells, leading to a great RNA degradation in *Microcystis* samples.

< 5% of 16S rRNA and *mcyH* mRNA in pure culture samples have been degraded after 28 d preservation in the treatment of liquid nitrogen (Fig. 4; Table S2), and this treatment has the lowest degradation percentage among the four treatments ($P < .05$) (Fig. 4). It suggested liquid nitrogen was the most effective approach for RNA preservation in pure culture *Microcystis* samples, due to RNases inactivation at this extremely low temperature (Hubel et al., 2014).

For RNAstore® treatments, the degradation process of 16S rRNA and *mcyH* mRNA was temperature dependent, among which the degradation percentage of 25 °C preservation was higher than that of 4 °C preservation after adding RNAstore® ($P < .05$) (Fig. 4). Only 5% of 16S rRNA and *mcyH* mRNA was degraded at 4 °C after 7 days of preservation (Table S2), suggesting low-temperature with RNAstore® could effectively slow down RNA degradation in pure culture samples within a short time (1–7 d). Other studies demonstrated 25 °C preservation with RNAstore® was effective for RNA preservation in cultured cells, bacteria, and yeast, and white blood cells within a week (Grotzer et al., 2000; Rodrigo et al., 2002; Mutter et al., 2004; Dekairelle et al., 2007), but a rapid RNA degradation was observed in pure culture *Microcystis* samples within 3 days (Fig. 4). It could attribute to the special sheath surrounding cells, which could impede the permeation of RNAstore®, and thus, residual RNases caused RNA degradation in *Microcystis* samples. In contrast, < 5% was degraded in 3 d during 4 °C preservation with RNAstore® (Table S2), due to the joint inhibition of RNase activity via low temperature and RNAstore®.

3.4. RT-qPCR analysis of 16S rRNA and mcyH mRNA copies in field Microcystis samples with various treatments

Similar degradation pattern of 16S rRNA and *mcyH* mRNA copies in field *Microcystis* samples was observed (Fig. 4) with various treatments. > 80% of *mcyH* mRNA was degraded at 7 d with the preservation of −80 °C (Table S2), suggesting this treatment was not feasible for RNA preservation in field samples. Although degradation percentage (9.9%) of *mcyH* mRNA in field samples with the treatment of −196 °C liquid nitrogen, was more than twice as high as that in pure culture samples (4.8%) at 28 d ($P < .01$) (Table S2), this treatment was the most effective method for RNA preservation in field samples. For RNAstore® treatments, RNA degradation in field samples was also temperature dependent, the same as the pure culture samples (Fig. 4). The 4 °C preservation after adding RNAstore® could effectively slow down the degradation of *mcyH* mRNA (Table S2), but the preservation time was shorter (1–3 d) than pure culture samples (1–7 d), since RNA degradation was much faster for field samples (Fig. 4).

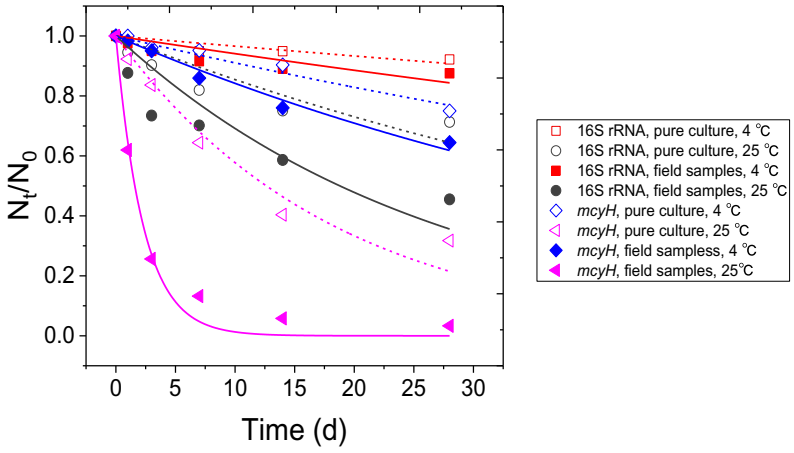

Fig. 5. Kinetics modeling of 16S rRNA and *mcyH* mRNA degradation in pure culture and field *Microcystis* samples preserved at 4 °C and 25 °C after adding RNAstore®, respectively.

3.5. A comparison of RNA degradation in pure culture and field samples with the RNAstore® treatments

To further compare RNA degradation in pure culture and field samples with the RNAstore® treatments,

a first-order model was developed to estimate decay rates of 16S rRNA and *mcyH* mRNA in pure culture and field samples during treatments (Fig. 5). Decay rates of 16S rRNA and *mcyH* mRNA was shown in the following equation (Eq. (3)):

$$N_t/N_0 = \exp(-kt) \quad (3)$$

Where N_t = 16S rRNA or *mcyH* mRNA copies after a given preservation time, N_0 = 16S rRNA or *mcyH* mRNA copies at 0 d, k = decay rate of 16S rRNA or *mcyH* mRNA, t = preservation time.

The models fitted well and R^2 were all > 0.9, except models of 16S rRNA (R^2 = 0.629–0.827) (Table 1). The fitted k was temperature dependent, and it was much lower at 4 °C (ranged from 0.0035 d^{-1} to 0.0172 d^{-1}) than that at 25 °C (ranged from 0.0157 d^{-1} to 0.4350 d^{-1}) (Table 1). Moreover, the fitted k of 16S rRNA (0.0035 d^{-1} at 4 °C, 0.0157 d^{-1} at 25 °C) and *mcyH* mRNA (0.0094 d^{-1} at 4 °C, 0.0549 d^{-1} at 25 °C) in field samples was all higher than that in pure culture samples ($P < .05$) (Table 1).

Microcystis species generally form colonial cells of varied sizes in natural freshwaters (Ma et al., 2014; Wang et al., 2015). The sizes of colonies become lager up to 500 μm during *Microcystis* blooming period (Wang et al., 2015). Unlike unicellular cells, colonies can be protected with amorphous mucilage or sheaths (up to 30 μm thick) (Reynolds, 2007; Pereira et al., 2009; He and Wert, 2016). The special surface structure of colonial cells prevented oxidant permeating when cells were treated with chlorine, leading to high capacity of standing up to oxidation pressure (He and Wert, 2016). In this study, these colonies could further prevent the permeation of RNA protective fluid, and RNases in cells could not be inactivated sufficiently, especially the inner cells in colonies. Consequently, a higher decay rate (k) of RNA was observed in field samples (Table 1).

Table 1
Decay rates of 16S rRNA and *mcyH* mRNA during RNA preservation in pure culture and field *Microcystis* samples, which were preserved at 4 °C or 25 °C after adding RNAstore®, respectively.

RNAstore®, preserved at 4 °C			RNAstore®, preserved at 25 °C		
Transcripts	k (d^{-1})	R^2	Transcripts	k (d^{-1})	R^2
Pure culture samples					
16S rRNA	0.0035	0.629	16S rRNA	0.0157	0.827
mcyH	0.0094	0.966	*mcyH*	0.0549	0.959
Field samples					
16S rRNA	0.0061	0.688	16S rRNA	0.0367	0.797
mcyH	0.0172	0.979	*mcyH*	0.4350	0.983

3.6. A practical strategy for RNA preservation during field sampling

The decay rates of RNAs were higher in field samples than that in pure culture samples, implying the greater difficulty in RNA preservation for field sampling. Besides, field samples were collected from lakes or reservoirs, and they were always far from laboratory and few days was required to be transported to laboratory. This process would lead to RNA degradation. However, intact RNAs was an important prerequisite for the application of RT-qPCR technique to monitor microcystin. Hence, during field sampling, effective methods for RNA preservation in cyanobacterial samples was essential.

Previous studies reported that cryopreservation (−80 °C freezer and − 196 °C liquid nitrogen) were effective to long-term RNA preservation in various samples. For example, RNA remained intact in endocrine tissue samples, which were stored for 27 years at −80 °C (Andreasson et al., 2013; Auer et al., 2014). However, for field *Microcystis* samples, > 80% of *mcyH* mRNA was degraded with the treatment of −80 °C after 3 days of preservation. Even with the most effective treatment of −196 °C, about 4.1% and 10% of *mcyH* mRNA were also degraded after 3 and 28 days of treatments, respectively. It suggested the treatment of −196 °C was ineffective to preserve RNA in field *Microcystis* samples for 28 d. Moreover, it is not convenient to take along for field sampling, since liquid nitrogen was easy to volatilize, and it must be stored in the specially-made liquid nitrogen tank. Thus, liquid nitrogen treatment was more applicable for samples in lab than for field sampling.

In comparison, RNAstore® is more convenient for field sampling than liquid nitrogen treatments. The 4 °C preservation after adding RNAstore® could effectively slow down RNA degradation in field samples, but was time-limited (< 3 d) (Fig. 4). Thus, to better preserve RNA in field *Microcystis* samples, a practical strategy combining RNA protective fluid and liquid nitrogen was proposed. During field sampling, samples could be preserved in situ at low temperature (bubble chamber and ice bags) after adding RNAstore® to slow down RNA degradation in field samples, and then these samples should be transported to laboratory for fast RT-qPCR analysis, or transferred to the preservation of −196 °C liquid nitrogen as soon as possible to prolong preservation time (Fig. 6).

To test the effectiveness of the strategy for RNA preservation during filed sampling, field preservation experiments were conducted. Field samples were preserved in situ at low temperature after adding

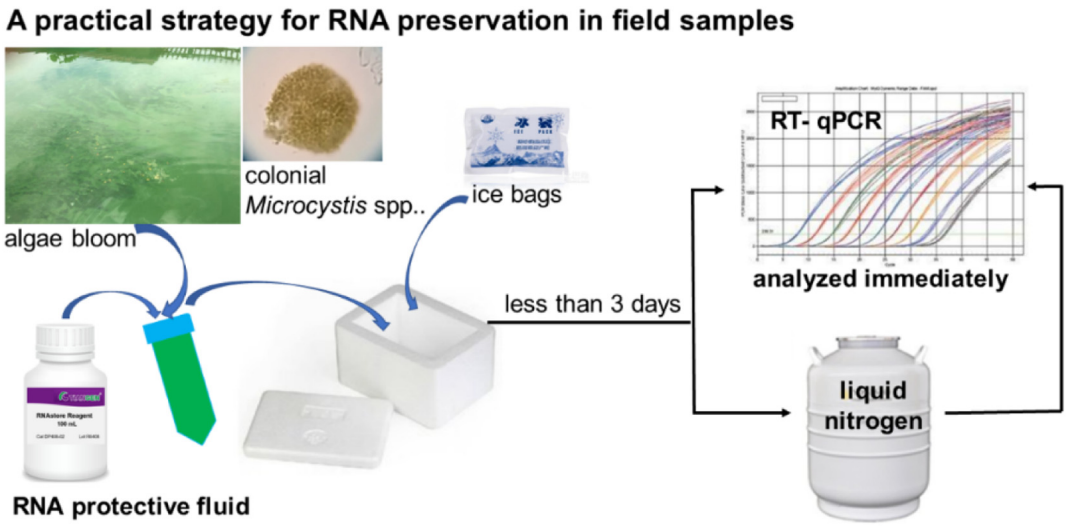

Fig. 6. A practical strategy for RNA preservation combining RNA protective fluid and liquid nitrogen for field *Microcystis* sampling.

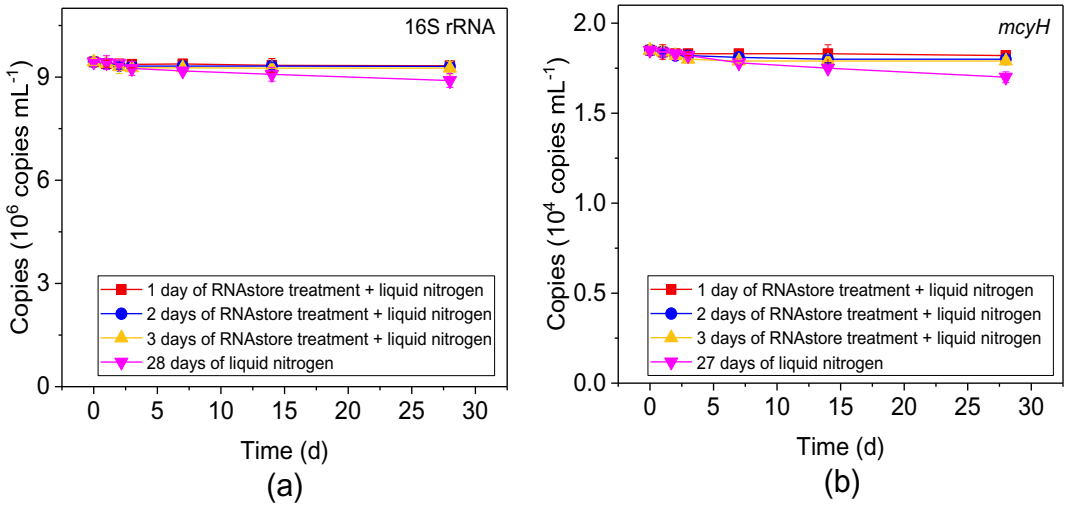

Fig. 7. Degradation process of 16S rRNA (a) and *mcyH* mRNA (b) in field *Microcystis* samples, which were preserved in situ with the treatment of combining RNAstore® and − 196 °C liquid nitrogen, and individual treatment of liquid nitrogen for 28 d as controls.

RNAstore®. After 1–3 days of this treatment, these samples were transferred to the preservation of liquid nitrogen for 28 days, results showed there was no significant difference of the degradation percentage of 16S rRNA and *mcyH* mRNA copies in these treatments ($P > .05$) (Fig. 7). In comparison with the most effective treatment of −196 °C liquid nitrogen, the degradation percentage of 16S rRNA and *mcyH* mRNA copies with this strategy was much lower ($P < .05$) (Fig. 7). It may attribute to the joint inhibition of RNases by the treatments of RNAstore® and liquid nitrogen. Despite colonial *Microcystis* cells impede the permeation of RNAstore®, subsequent treatment of liquid nitrogen could further inactivate residual RNases in colonial cells. Thus, this strategy combining RNAstore® and liquid nitrogen for RNA preservation was more effective than individual treatment for *Microcystis* samples, and it could be employed to preserve RNA in cyanobacterial samples during field sampling.

4. Conclusions

This study revealed liquid nitrogen was the most effective method to preserve RNA in *Microcystis* samples, but it was more applicable in laboratory than for field sampling. Low temperature (4 °C) preservation with RNA protective fluid could effectively slow down RNA degradation within a short time (1–7 d). However, RNA degradation was much faster in field samples than in pure culture *Microcystis* samples. Therefore, to better preserve RNA in field samples, a practical strategy combining RNA protective fluid and liquid nitrogen for RNA preservation was proposed and further demonstrated it was more effective than individual treatment for *Microcystis* samples during field sampling. Thus, it could be employed to preserve RNA in cyanobacterial samples during field sampling, which will contribute to the application of RT-qPCR technique for microcystin monitoring in lakes and reservoirs.

Declaration of Competing interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105684.

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