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Determination of glycolic acid in natural seawater by liquid chromatography coupled with triple quadrupole mass spectrometry

Haizheng Hong, Weiying Li, Xiaolin Li, Bangqin Huang, Dalin Shi*
State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, Fujian, People's Republic of China

Abstract

Glycolic acid, which is produced during photorespiration, is one of the major components of dissolved organic matter released by phytoplankton, comprising up to 40% of their daily primary productivity. However, the sensitivity of existing methods does not allow for accurate measurement of glycolic acid levels in oligotrophic seawater. In this study, we present a straightforward, robust, and sensitive liquid chromatography-mass spectrometry (LC-MS) method to quantify glycolic acid in natural seawater following liquid-liquid extraction, requiring only a small sample size (25 mL seawater). The method has a highly sensitive detection limit of 9 nM, at least fivefold lower than existing methods. This approach was successfully applied to the analysis of seawater samples collected from both eutrophic and oligotrophic marine environments. The results showed that glycolic acid concentrations in natural seawater are positively correlated with chlorophyll *a* concentrations, indicating that phytoplankton contribute significantly to glycolic acid production in marine environments. As glycolic acid is an important carbon and energy source for marine bacteria, the method developed within this study allows further investigation into the rates of its release and uptake by phytoplankton and heterotrophic bacteria, respectively, as well as the role it plays in phytoplankton–bacteria interactions.

Photorespiration, which is important and prevalent in marine phytoplankton (Beardall 1989), occurs when O_2 outcompetes CO_2 for binding with RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), a Calvin Cycle enzyme. The fixation of O_2 rather than CO_2 by RubisCO results in the formation of 2-P-glycolate, which is then further metabolized to form various small organic compounds, such as glycolic acid, glycine, and serine (Beardall 1989; Fig. 1). Increases in levels of photorespiration, due to factors such as high irradiance, high temperature, or high O_2 concentrations, can stimulate the release of fixed carbon, of which glycolic acid is one of the major components (Al-Hasan and Fogg 1987; Parker et al. 2004).

Previous culture experiments have demonstrated that glycolic acid can constitute a substantial proportion of algal excreted dissolved organic matter (DOM) in the media of a variety of marine species including *Chrysophyceae*, *Bacillariophyceae*, *Chlorophyceae*, *Cyanophyceae*, etc., with high values observed in *Chaetoceros pelagicus*, *Skeletonema costatum*, and *Chlorococcum* sp. (Hellebust 1965). Field measurements have also showed that glycolic acid is a significant component (10–50%) of phytoplankton excreted DOM in marine environments (Wright and Shah 1975; Edenborn and Litchfield 1987). Excreted glycolic acid and other phytoplankton extracellular products have been estimated to account for 5–40% of the total fixed carbon, with levels reported to be in the same order of magnitude as net primary production in oligotrophic tropical oceans (Fogg 1983; Edenborn and Litchfield 1987; Leboulanger et al. 1997).

Excreted glycolic acid is taken up and utilized rapidly as an energy source by marine heterotrophic microorganisms, thereby playing a significant role in phytoplankton–bacteria interactions, which are important for the cycling of marine organic matters (Fogg 1983; Leboulanger et al. 1997; Lau and Armbrust 2006). Due to the essential role of glycolic acid in marine ecosystems, it is critical to investigate the dynamics of glycolic acid in the seawater environment. Leboulanger et al. (1997) observed that the concentration of glycolic acid in seawater increased during day time due to phytoplankton photorespiration and decreased rapidly to very low concentrations at night time due to fast uptake by bacteria. The quantification of glycolic acid in seawater during a diel cycle could thus be used to estimate its release and uptake rates by phytoplankton and heterotrophic bacteria, respectively (Leboulanger et al. 1997). Other studies, however, have reported that in situ glycolic acid concentrations often remain at very low and constant levels (Billen et al. 1980; Lau et al. 2007). Assuming steady-state conditions, the release rate of glycolic

*Correspondence: dshi@xmu.edu.cn

acid by phytoplankton is estimated to be equal to the rate of uptake, which is measured using the ^{14}C-technique combined with glycolic acid concentration measurement (Billen et al. 1980; Fogg 1983; Edenborn and Litchfield 1987). In any case, the development of a sensitive and reliable method to accurately quantify glycolic acid in seawater is an essential step in understanding its dynamics in marine environments.

Several chromatographic methods have been previously developed for the determination of glycolic acid in natural seawater or culture media. For example, Leboulanger et al. (1994) analyzed seawater samples using ion exchange HPLC, detecting glycolic acid by ultra-violet (UV) absorption at 210 nm, with a detection limit of glycolic acid in seawater of 53 nM, when 250 mL of sample was used for extraction (Table 1). The UV absorption spectroscopy method utilized, however, contains non-specific interference from many other organic compounds with absorption at similar wavelengths. Therefore, glycolic acid was not fully separated at the baseline from interfering compounds in the ion exchange chromatogram, with the interference becoming more significant when measuring analytes at trace levels in open ocean water samples, thereby compromising the sensitivity and accuracy of the method (Leboulanger et al. 1994). Subsequently, Leboulanger et al. (1998) developed a gas chromatography-mass spectrometry (GC-MS) method for the analysis of glycolic acid in marine samples, requiring post extraction derivatization and generating a moisture- and heat-sensitive product, with a detection limit of 105 nM when 250 mL of sample was processed. Another method by Albert and Martens (1997) derivatized low molecular weight organic acids using 2-nitrophenylhydrozide reagent followed with analysis by HPLC-absorbance, achieving a sensitivity in the submicromolar range. The method was then further modified by the introduction of a concentrating column within the injection loop for the determination of glycolic acid in algal culture media (Parker et al. 2004). Rigobello-Masini et al. (2012) reported improved separation of glycolic acid from co-occurring organic acids, using hydrophilic interaction liquid chromatography (HILIC) and UV detection at 220 nm, resulting in detection limit of 39 μM (after taking the extraction process and injection volume into consideration). More recently a reverse phase liquid chromatography-mass spectrometry (LC-MS) method has been developed for quantification of the concentration of glycolic acid in algal culture media, in which a pentafluorophenylpropyl (PFPP) column was utilized for separation (Shi et al. 2015). Literature has shown that that PFPP columns can often perform better than HILIC columns in achieving complete separation and reliable quantification of organic acids with similar physicochemical properties, such as glycolate/glyoxylate and malate/fumarate (Bajad et al. 2006; Yoshida et al. 2007; Yang

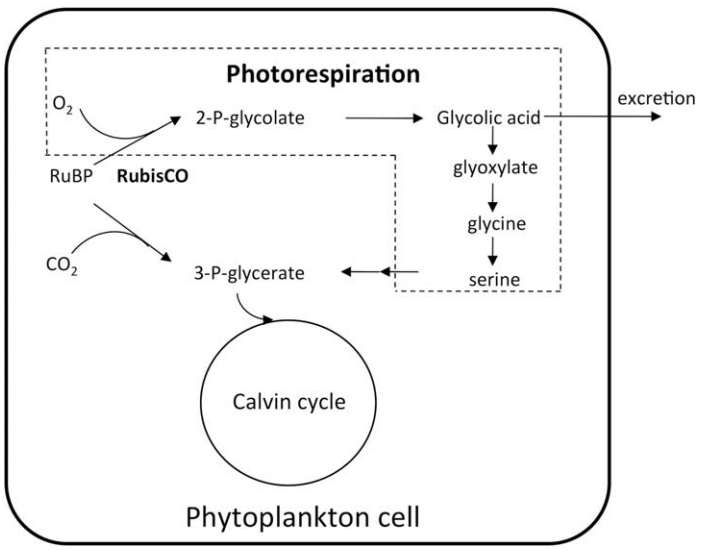

Fig. 1. Scheme indicating photorespiration processes and the Calvin cycle in phytoplankton cells, where glycolic acid is generated and excreted to the surrounding seawater environment. Adapted from Leboulanger et al. (1994).

Table 1. Summary of the sample processing conditions, sample size, analytical instrument method, and sensitivity of various methods for analyzing glycolic acid in marine water.

Sample processing	Sample size (sample type)	Instrument method	Detection limit	References
Alumina adsorption	1 L (seawater)	Colorimetrical method	–	Shah and Wright (1974)
Ethyl acetate extraction	250 mL (seawater)	Ion exchange HPLC-UV	53 nM	Leboulanger et al. (1994)
Ethyl acetate extraction and derivatization	250 mL (seawater) 100 mL (culture medium)	GC-MS/FID	105 nM	Leboulanger et al. (1998)
Derivatization	10–30 mL (culture medium)	RP HPLC-UV/VIS with a concentrating column in the injector	–	Parker et al. (2004) Parker and Armbrust (2005)
Ethyl acetate extraction	25 mL (culture medium)	HILIC HPLC-UV	39 μM	Rigobello-Masini et al. (2012)
Ethyl acetate extraction	30 mL (culture medium)	LC-MS	–	Shi et al. (2015)
Ethyl acetate extraction	25 mL (seawater)	LC-MS	9 nM	This study

Table 2. Concentrations of glycolic acid in natural marine water.

Sampling sites		Glycolic acid concentration (nM)	References
Gulf of Maine		n.d.*–1000	Shah and Wright (1974)
Liverpool Bay		190–810 (surface)	Al-Hasan and Fogg (1987)
Western Mediterranean Sea		260–1100 (vertical profile)	Leboulanger et al. (1994)
Eastern tropical Atlantic Ocean	Eutrophic	$< 53^{\dagger}$ −740 (vertical profile)	Leboulanger et al. (1997)
	Mesotrophic	$< 53^{\dagger}$ −1000 (vertical profile)	
	Oligotrophic	$< 53^{\dagger}$ −220 (vertical profile)	
Xiamen Bay (coastal)		$335 \pm 17.9^{\ddagger}$ (surface)	This study
Taiwan strait		$49 \pm 0.8^{\ddagger}$ (surface)	This study
South China Sea		22–210 (surface and vertical profile)	This study

*Not detected. The method detection limit was not reported.
†The method detection limit was 53 nM (Leboulanger et al. 1994), below which quantification was not reliable.
‡Data show mean values ± 1 SD ($n = 3$).

et al. 2010). However, the study by Shi et al. (2015) did not provide sensitivity data or full validation of the LC-MS method, as it was only applied to the quantification of glycolic acid in algal culture media which is often at levels of at least several hundred nM.

The concentrations of glycolic acid in natural seawater vary considerably. In eutrophic coastal waters, concentrations vary from below the detection limits (depending on the method used) to up to 1100 nM, with values often observed in the range of 200–800 nM (Table 2). Therefore, the LC-UV and GC-MS methods with detection limits of 53 nM and 105 nM, respectively, can practically be applied to quantify glycolic acid in most of eutrophic seawater samples, although some samples are still below detection limits. In contrast, very few studies have measured and reported glycolic acid concentrations in oligotrophic oceans, where it constitutes a much higher fraction of phytoplankton excreted DOM than in eutrophic seawater (40% vs. 5%, Fogg 1983). Leboulanger et al. (1997) measured glycolic acid concentrations in the oligotrophic eastern tropical Atlantic Ocean using the LC-UV method, with many reported values being below the detection limit (< 53 nM), suggesting that the existing methods do not effectively or accurately measure low glycolic acid concentrations in oligotrophic seawater.

Direct analysis of organic acids by LC-MS provides a fast and sensitive approach that requires no derivatization process and does not require large sample sizes. Therefore the LC-MS method reported by Shi et al. (2015) can potentially be developed into a sensitive and straightforward method for the accurate quantification of glycolic acid in natural seawater, especially from oligotrophic regions. In the present study, we modified and validated the LC-MS method and show that it is a fast, sensitive and reliable method for the analysis of glycolic acids in natural seawater, which is essential for better understanding of phytoplankton–bacteria interactions and the cycling of organic matters in marine environments.

Materials and procedures

Chemicals and reagents

The glycolic acid standard was obtained from Ehrenstorfer Quality (Augsburg, Germany); hydrochloric acid (HCl, analytical grade) and ethyl acetate (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany); and acetonitrile was obtained from TediaCo. (Fairfield, Connecticut, U.S.A.). $^{13}C_2$-glycolic acid (99% ^{13}C-labeled) and all other reagents were purchased from Sigma-Aldrich Chemical (St. Louis, Missouri, U.S.A.).

Sampling and pretreatment

The natural seawater samples were collected using Niskin bottles coupled with CTD rosettes, from the South China Sea in July 2014 (stations C8, E3, H6, and J3) and June 2015 (stations A6 and X43) (Fig. 2). Niskin bottles were pre-washed using 0.5% Micro, 0.3 M HCl and Milli-Q water and then thoroughly rinsed with in situ seawater prior to sampling. Surface water was collected at stations C8, A6, J3, and X43, while seawater samples for vertical profile analysis were collected at stations E3, H6, and J3 from the depths of 5 m, 25 m, 50 m, 75 m, 100 m, and 125 m. For all samples, 25 mL of seawater was filtered through 0.22-μm polycarbonate membranes (Millipore), acidified immediately using concentrated HCl to a final concentration of 0.1 M and stored in precombusted (400°C, 4 h) brown glass vials at −20°C for less than 1 month prior to sample extraction.

Sample extraction

The pretreated samples were further acidified using concentrated HCl, forming a final concentration of 0.4 M. Meanwhile, ^{13}C-glycolic acid was added as the internal standard (IS), forming a final concentration of 130 nM.

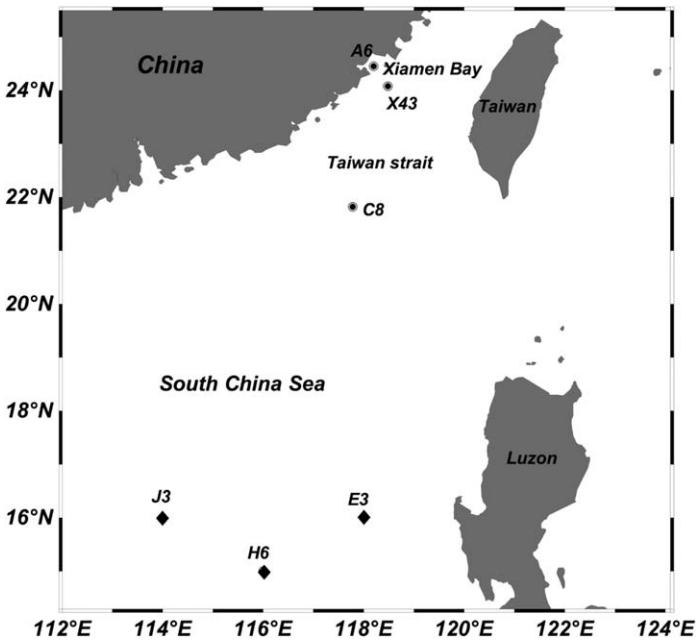

Fig. 2. Sampling stations at Xiamen Bay (A6), Taiwan Strait (X43), and the South China Sea (C8, J3, E3, and H6) in July 2014 and June 2015. Surface water was collected at stations A6, X43, and C8 (solid circles), and marine water samples for vertical profiles were collected at stations J3, E3, and H6 (diamonds) from the depths of 5 m, 25 m, 50 m, 75 m, 100 m, and 125 m.

Extraction was performed by adding 10 mL of ethyl acetate to 25 mL of samples in a pre-combusted (400°C, 4 h) 65-mL borosilicate glass bottle. Following vigorous shaking and phase separation, the organic fraction was transferred to a pre-combusted 30-mL glass centrifugation tube, with repeated extraction of the aqueous fraction performed using another 10 mL of ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness under a constant N_2 stream at room temperature for about 45 min, with residues re-dissolved in 250 μL of 0.1% formic acid aqueous solution.

LC-MS/MS

A 2.1 × 150 mm Supelco Discovery® HS F5-3 column (particle size 3 μm, Sigma-Aldrich Chemical) was used, with a flow rate of 250 μL min^{-1} achieved using an Agilent 1290 UPLC binary pump (Agilent Technologies, Palo Alto, California, U.S.A.) and an injection volume of 20 μL. A gradient elution mobile phase was utilized, containing mobile phase A (0.01% formic acid, pH 5.6) and mobile phase B (100% acetonitrile), with a linear gradient program implemented as follows: hold isocratic elution at 0% B (0–6 min); linear gradient elution from 0% to 70% B (6–7 min); hold isocratic elution at 70% B (7–9 min); re-equilibrate at 0% B (9–14 min). The effluent from the LC column was delivered to an Agilent 6490 triple-quadrupole mass spectrometer, equipped with an electrospray ionization source operating in negative-ion mode, except for the LC eluent of the first 1.4 min, which was diverted to waste to avoid contamination of the mass spectrometer with unretained salts. The mass spectrometry parameters were optimized for maximum glycolic acid sensitivity, with conditions set to: gas temperature 160°C; sheath gas temperature 360°C; sheath gas flow 11 L min^{-1}; nebulizer 30 psi; capillary 3 kV; nozzle voltage 1.5 kV; collision energy 12 V, and cell accelerator voltage 3V. The selected multiple reaction monitoring (MRM) transitions for the quantification of unlabeled and labeled glycolic acid were m/z 75→45 and 77→48, respectively. The MRM transition event of m/z 75→47 was also monitored simultaneously to verify the glycolic acid peak.

Calibration and analytical variation of the method

Standard solutions for injection were prepared at 0.026 μM, 0.066 μM, 0.26 μM, 0.66 μM, 1.3 μM, and 6.6 μM, with ^{13}C glycolic acid added as the IS forming a final concentration of 1.3 μM. Calibration curves were plotted as the ratios of analyte peak area to the IS peak area, vs. analyte concentration. The linearity of the mass spectrometer response was confirmed when the coefficient of determination (R^2) of the calibration curve was > 0.99. Limits of detection (LOD) and limits of quantification (LOQ) were defined as the amount of analyte required to give a signal-to noise (S/N) ratio of 3 and 10, respectively.

Accuracy and precision of the LC-MS analysis method were determined by analyzing the standard samples at 0.26 μM and 0.66 μM. Intra-day and inter-day precision was confirmed with repeated analysis of four replicate samples, with precision expressed as the relative standard deviation (RSD%).

For the determination of the overall method recovery rate, concentrations of glycolic acid in open ocean marine water collected from the surface of station J3 with a salinity of 34‰, coastal marine water collected from the surface of Xiamen Bay (station A6) with a salinity of 30‰, and river freshwater collected from the Jiulong River at Xiamen estuary with a salinity of 0‰ were determined as outlined above after being spiked with the IS forming a final concentration of 130 nM. Triplicate samples were included in the extraction and analysis. Another three aliquots of each of the three types of seawater were spiked with the IS added at a final concentration of 130 nM along with glycolic acid standard at final concentrations of 33 nM, 132 nM, and 263 nM (Table 3), respectively, according to the predicted glycolic acid concentrations. After quantification of the total concentration in the spiked samples, the method recovery rates were calculated using the following the equation:

$$\text{Method recovery rate} = (C_{\text{total}} - C_{\text{actual}})/C_{\text{spike}} \times 100\%$$

where C_{total} is the measured total concentration of glycolic acid in the spiked samples, C_{actual} is the mean of the three measured actual concentrations in the non-spiked samples ($n = 3$), and C_{spike} is the nominal spiked concentration.

The method detection limit (MDL) is defined as the minimum concentration of a substance which can be measured

Table 3. The recovery rate of the LC-MS method determined using three different sample matrix with varying salinity. Data show mean values ± 1 SD ($n = 3$).

	Salinity (‰)	Actual glycolic acid conc. without spiking (nM)	Nominal glycolic acid spiked conc. (nM)	Recovery rate (%)	RSD (%)
Oligotrophic surface seawater (South China Sea, Station J3)	34	21.6 ± 4.7	33	103.2 ± 2.7	2.6
Coastal surface seawater (Xiamen Bay, Station A6)	30	335 ± 17.9	132	104.4 ± 8.6	8.3
River surface water (Jiulong River, Xiamen estuary)	0	71.4 ± 2.7	263	106.2 ± 4.7	4.4

and reported with 99% confidence that the analyte measured concentration is greater than zero (40CFR Appendix B to Part 136, revision 1.11, United States Environmental Protection Agency, USEPA). MDLs are statistically calculated concentrations used to qualitatively identify the lowest reliable detection levels for a target analyte in a specific laboratory, and they are widely used by a variety of agencies and programs, e.g., USEPA and United States Geological Survey (USGS). Briefly, the MDL of the current method was determined by analyzing seven replicate samples of deep seawater collected from the West Pacific Ocean at the depth of 2000 m following the sampling procedure described above, which were spiked with glycolic acid at the concentration of 33 nM, as per standard methods, and calculated based on the following equation (USEPA):

$$\mathrm{MDL} = s \times t_{(n-1,1-\alpha)}$$

where n is the number of replicates; s is the standard deviation of the measured concentrations of n spike determinations; α is level of significance (0.01); and t is Student's t value at n-1 degrees of freedom and 1-α (99%) confidence level. The background concentration of glycolic acid in the deep seawater was also measured prior to sample spiking.

Stability

The stability of glycolic acid in the acidified seawater stored at −20°C was evaluated, with triplicate samples processed and analyzed by LC-MS at 1 d, 3 d, 7 d, and 30 d. The stability of glycolic acid in extracts stored at −20°C was also evaluated for up to 15 d.

Measurement of chlorophyll *a* (Chl *a*) concentration

For analysis of Chl *a* concentration, 100–500 mL of seawater was filtered onto 25 mm GF/F filters (Whatman®) under a low vacuum (< 150 mm Hg). Filters were then extracted in 90% acetone overnight at −20°C in the dark, with fluorescence measured using the non-acidification method (Welschmeyer 1994) and a Turner Designs Fluorometer (San Jose, California, U.S.A.).

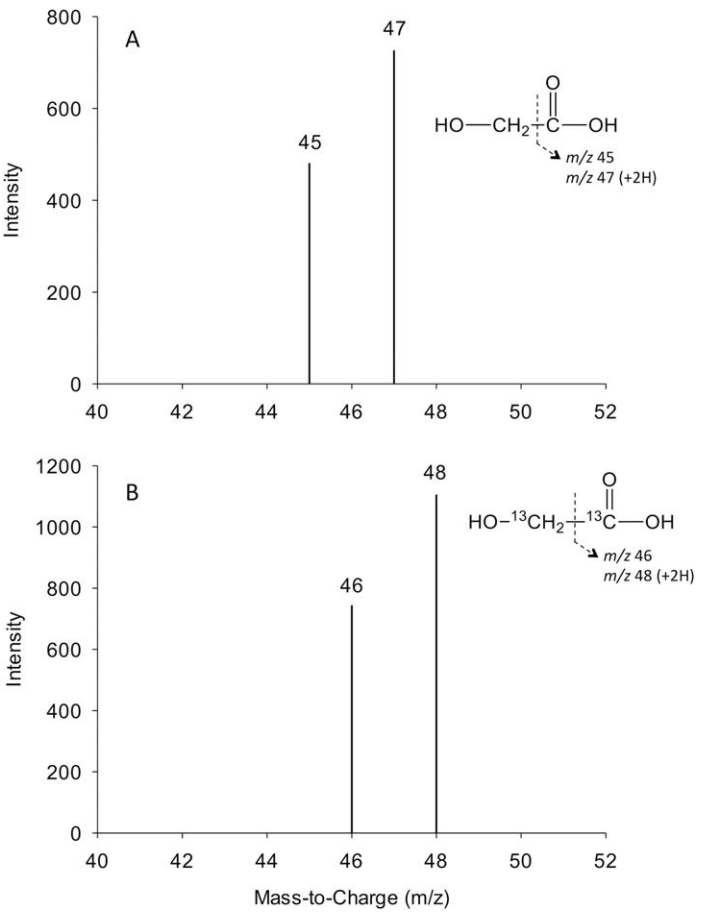

Fig. 3. The product ion spectra of the deprotonated molecular ions of (**A**) glycolic acid (*m/z* 75) and (**B**) $^{13}C_2$-glycolic acid (*m/z* 77). Inserts show the cleavage reactions for the formation of the major fragment ions.

Assessment

Optimization of LC-MS condition

Glycolic acid had a deprotonated molecular ion $[M\text{-}H]^-$ at *m/z* 75 and two product ions at *m/z* 45 and 47, resulting from the cleavage of the C—C bond (Fig. 3A). Following the same fragmentation mechanism, the $[M\text{-}H]^-$ of $^{13}C_2$-labeled

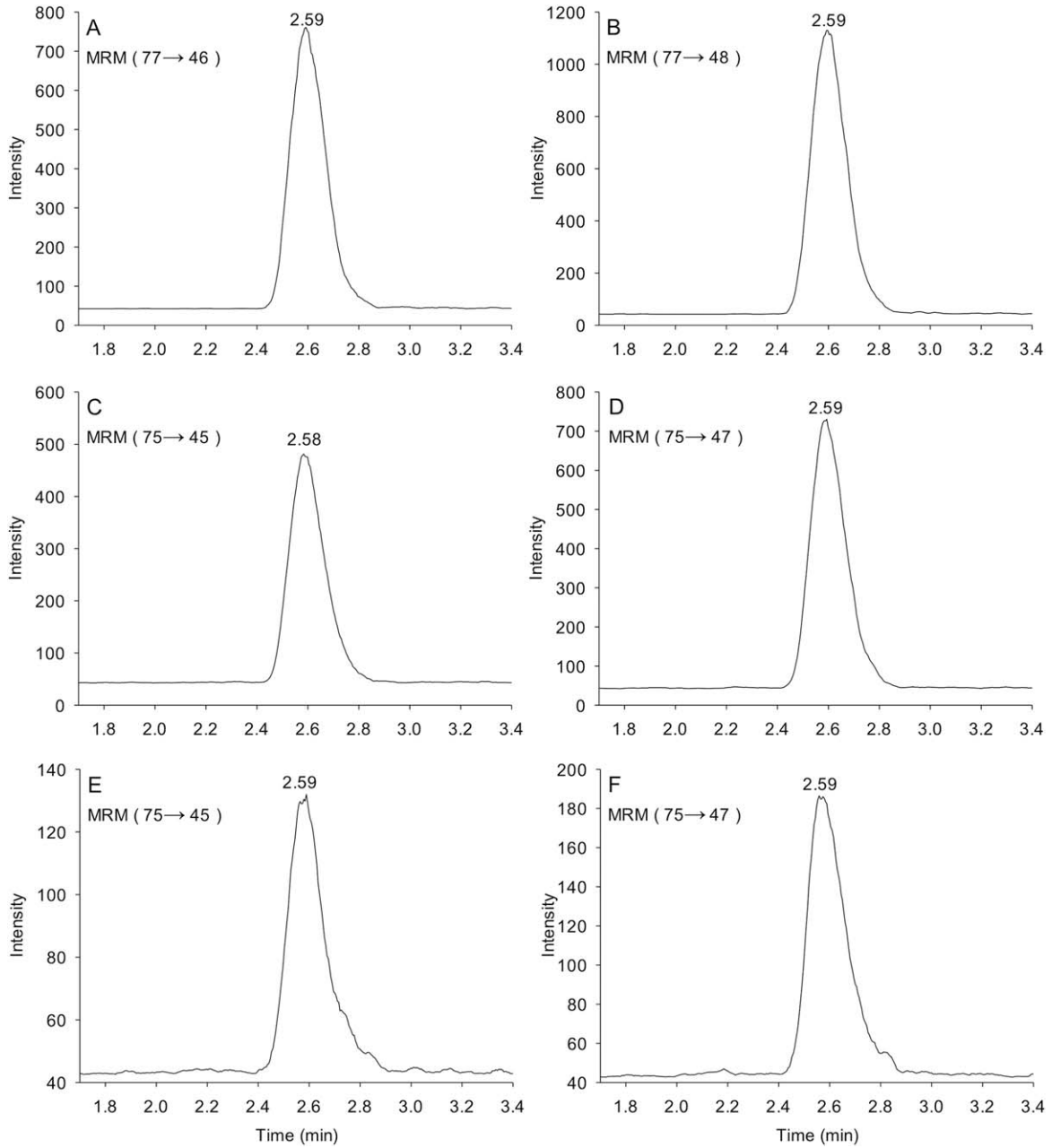

Fig. 4. Extracted ion chromatograms of the MRM events 77→46 and 77→48 for $^{13}C_2$-glycolic acid (**A**, **B**), 75→45 and 75→47 for glycolic acid in the standard (**C**, **D**) and 75→45 and 75→47 for glycolic acid in marine water sample extracts (**E**, **F**).

glycolic acid (*m*/*z* 77) generated two product ions, *m*/*z* 46 and 48 (Fig. 3B).

Extracted ion chromatograms (EICs) of the glycolic acid standard (Fig. 4C,D) and the marine water sample extract (Fig. 4E,F) using MRM events 75→45 and 75→47 showed only one chromatogram peak at a retention time of 2.59 min. The EICs of the ^{13}C-labeled IS (77→46 and 77→48, Fig. 4A,B) showed identical retention times to the analyte. In addition, retention times of the analyte and IS were consistent with multiple injections. The peak ratios of 75→47/75→45 for the glycolic acid standard and 77→48/77→46 for the IS were 1.54 ± 0.03 and 1.56 ± 0.03, respectively, confirming an equal MS response to both analyte and the IS. The peak ratio between the two product ions can be used to ensure the identity of glycolic acid in samples. Although the absolute ion intensity and peak area of 75→47/77→48 was higher than 75→45/77→46, the S/N of 75→45 was slightly better than 75→47 in seawater samples with low glycolic acid concentrations and the S/N for 77→48 and 77→46 were consistently similar (data not shown). Hence, for

quantification purpose, the MRM transitions of m/z 75→45 and 77→48 were selected for the analyte and the IS, respectively.

Based on current reported literature and HPLC methods commonly used for hydrophilic compound separation, it was established that reverse phase gradient elution, using an aqueous formic acid/acetonitrile mobile phase, achieved good separation and reliable quantification of organic acids on PFPP columns, in particular for the separation of glycolic acid and glyoxylic acid, which very often co-elute due to chemical structural similarities (Yoshida et al. 2007; Yang et al. 2010). Use of this method allowed glyoxylic acid elution 0.35 min prior to glycolic acid (data not shown) in our LC-MS system, avoiding ion suppression effects that may occur if the glyoxylic acid concentration is much higher than glycolic acid.

Calibration, sensitivity, recovery, and stability

Linear calibration was achieved over a range of 0.026–6.6 μM in the injection solution, with a coefficient of determination (R^2) of 0.998 ± 0.001. Injection of the lowest standard concentration (0.026 μM) generated a chromatographic peak at S/N of 6, establishing an LOD and LOQ of the injection solution to be 0.013 μM and 0.043 μM, respectively, with an LOD and LOQ on column of 0.26 pmol and 0.86 pmol, respectively, given an injection volume of 20 μL. The accuracy and precision of LC-MS analysis were determined using the standard solution at the glycolic acid concentrations of 0.26 μM and 0.66 μM, showing accuracy to be between 78.1% and 101.4%. The intra-day and inter-day RSD were 0.74–1.09% and 4.45–6.53%, respectively, both below the 15%, suggesting acceptable range for repeatability (intra-day precision) and reproducibility (inter-day precision).

Preliminary experiments showed that in the extraction process, increasing the volume of ethyl acetate from 5 mL (per Shi et al. 2015) to 10 mL (used in the present study) increased extraction efficiency by about 20% without significantly extending sample processing time. In addition, for accurate quantification, the introduction of an isotope labeled IS to the samples prior to extraction allows concentration corrections for the variations arising from the ion matrix effect and for losses due to sample preparations, and provides a method for reliable quantification by mass spectrometry (Siekman 1979; Yang et al. 2010). Therefore ^{13}C-glycolic acid was added to the seawater samples prior to extraction as the IS, making a final concentration of 130 nM, which was half the concentration used in Shi et al. (2015).

After optimizing the extraction and LC-MS analysis conditions, the overall method recovery rate was evaluated in three types of sample matrix, i.e., the open ocean marine water, coastal marine water, and the river freshwater (Table 3). All samples were spiked with the glycolic acid standard at the similar levels as their actual glycolic acid concentrations (Table 3) and with the IS at a final concentration of 130 nM. The actual concentrations without spiking were determined to be 21.6 ± 4.7, 335 ± 17.9, and 71.4 ± 2.7 for surface water samples collected from J3, A6 and the Jiulong River, respectively (Table 3). Following extraction and analysis, the resulting recovery rates were between 103% and 106% (Table 3), demonstrating method recovery rates to be consistently high despite variations in salinity or the environmental matrix being analyzed.

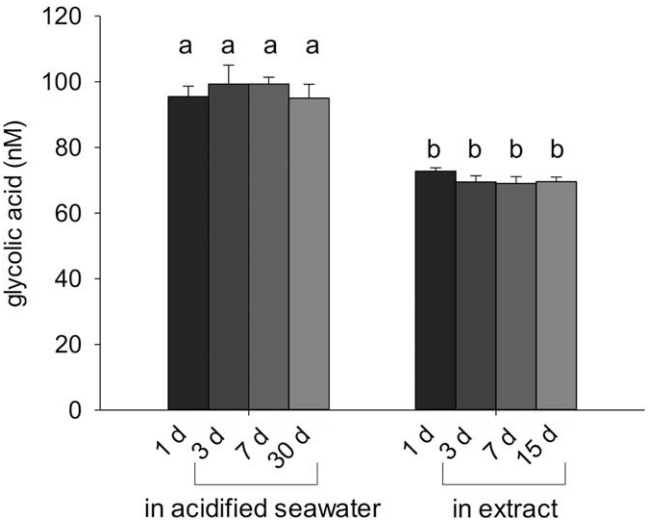

Fig. 5. Stability of glycolic acid in acidified marine water and in sample extract stored at −20°C. Data show mean values + 1 SD ($n = 3$). No significant difference in glycolic acid concentration was found among different sampling times ($p > 0.05$, one-way ANOVA). AQ

The MDL of the current method was established as 9 nM, as determined by multiple analyses of the spiked sample at 33 nM. The spike level was higher than the calculated MDL and lower than 5 folds of the MDL, suggesting that the selected spike level was appropriate (40CFR Appendix B to Part 136, revision 1.11, USEPA). In addition, due to the high sensitivity of the mass spectrometry instrument with a sub-pmol LOD on column, detection of native glycolic acid in the deep seawater samples used in this study occurred occasionally, although the measured background concentration in the deep seawater samples were consistently extremely low and were never higher than the calculated MDL, and thus it can be used as a qualified blank for the determination of MDL (USEPA).

The calculated MDL of the current method shows significant improvement to previously reported LC-UV and GC-MS methods for analyzing glycolic acid in natural marine water, which had detection limits of 53 nM and 105 nM, respectively (Leboulanger et al. 1994, 1998). It is of note that in addition to significantly higher sensitivity, the LC-MS method developed in the present study only requires 25 mL of sample, much smaller than the 250 mL minimum sample

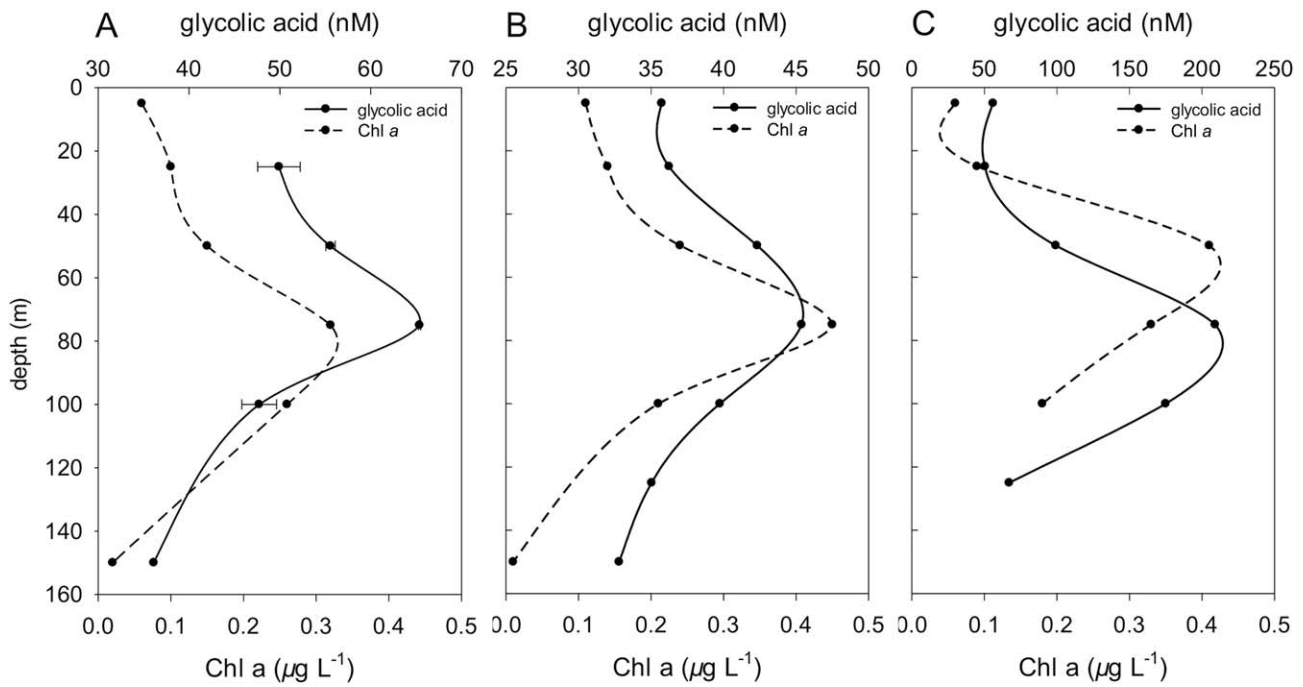

Fig. 6. Vertical profiles of the concentrations of glycolic acid (solid line) and Chl *a* (dash line) at stations J3 (**A**), H6 (**B**), and E3 (**C**). Triplicate samples were extracted and analyzed for the concentration of glycolic acid at station J3 and only one sample at each depth was available for analysis at stations H6 and E3.

volume required in previously described methods (Leboulanger et al. 1994, 1998) (Table 2).

As shown in Fig. 5, glycolic acid was stable in acidified marine water samples stored at −20°C for at least 1 month ($p > 0.05$), and glycolic acid in the extract showed stability at −20°C for at least 15 d ($p > 0.05$).

In situ results

The concentrations of glycolic acids in the marine surface water samples collected from Xiamen Bay (A6), Taiwan Strait (X43), and the South China Sea (C8) were 335 ± 17.9 nM, 49 ± 0.8 nM, and 33 ± 2.5 nM, respectively (Fig. 2), with all measurements based on extraction and analysis of three replicate samples. The coastal marine water from Xiamen Bay is eutrophic, with Chl *a* concentrations of 4.0 ± 0.6 μg L^{-1}, much higher than those detected at the mesotrophic station X43 and the oligotrophic station C8, which were 0.55 ± 0.26 μg L^{-1} and 0.48 ± 0.33 μg L^{-1}, respectively. Consistently, we observed much higher glycolic acid concentrations in Xiamen Bay than in either the Taiwan Straits or the South China Sea.

Vertical profiles were established for glycolic acid concentrations at stations J3, E3, and H6 in the South China Sea (Fig. 2). Triplicate analyses were performed to establish glycolic acid concentrations at station J3, while single analysis was performed for stations H6 and E3 due to limited sample availability. The maximal zone for glycolic acid concentrations was at the depth of 75 m, which was also the maximal zone in the vertical profiles of Chl *a* concentrations of the stations J3 and H6, or close to the Chl *a* maximum for station E3, suggesting that marine phytoplankton play a key role in the active excretion of glycolic acid (Fig. 6).

The concentrations of glycolic acid in marine water samples collected at various depths in the present study were within a similar range as those measured in other locations worldwide, such as Liverpool Bay, the Mediterranean Sea, and the eastern tropical Atlantic Ocean, by colorimetric methods, LC-UV and GC-MS, respectively (Table 2) (Al-Hasan and Fogg 1987; Leboulanger et al. 1997, 1998). It is of note that these reported studies utilized sample volumes of 250–1000 mL, whereas the smaller sample size (i.e., 25 mL) implemented in the present study makes it more feasible to collect sufficient sample volume for repeated analytical measurements, as well as making the extraction procedure less labor intensive.

Discussion

The LC-MS approach for analysis of glycolic acid in natural seawater presented here provides a significant increase in sensitivity, of at least fivefold, as compared to previously reported LC-UV and GC-MS methods, and greatly reduces the required sample volume to 25 mL (Table 1). The LC-MS method is simple and direct, without requiring additional sample derivatization steps that may introduce interference or variation, while the use of a stable-isotope labeled internal standard assures accuracy and robustness.

Glycolic acid consists of a substantial portion (10–50%) of the phytoplankton excreted materials in the marine environment, serving as an important nutrient and energy source for marine heterotrophic microorganisms (Fogg 1983; Leboulanger et al. 1997). The glycolic acid released during photorespiration could account for $\sim$ 5% of the total fixed carbon in eutrophic waters and $\sim$ 40% of that in oligotrophic seawaters (Fogg 1983). However, inaccuracies in analysis due to the high detection limits of the previously established analytical methods, such as LC-UV and GC-MS (53 nM and 105 nM, respectively), have hindered the accurate measurement of glycolic acid concentrations in the vast regions of the oligotrophic oceans (e.g., as low as 20–30 nM in the oligotrophic South China Sea as shown in this study, Tables 2, 3). The sensitivity of the LC-MS method in the present study allows accurate and simple quantification of glycolic acid concentrations in the oligotrophic marine water.

Better understanding of phytoplankton–bacteria interactions, which play an essential role in the cycling of marine organic matters, involves the identification of bacterial responses to specific organic substances produced by specific phytoplankton processes (Lau and Armbrust 2006). Glycolic acid is considered as an important energy source for marine bacteria in the uptake and assimilation of other compounds (Fogg 1983). Given its abundance in the marine environment, the metabolism of glycolic acid may provide an energetic advantage to bacterial species that can utilize it as a substantial source of energy, and therefore the ability to utilize glycolic acid may considerably influence bacteria community structures (Wright and Shah 1975; Lau and Armbrust 2006). Previous studies have shown that the examination of the dynamics of glycolic acid in seawaters based on accurate quantification coupled with the ^{14}C-technique will help understand the bacteria–phytoplankton interactions (Billen et al. 1980; Edenborn and Litchfield 1987). However, it should be noted that a steady-state condition is often assumed in assessing the release and uptake rates of glycolic acid (Billen et al. 1980), without taking into account the complexity of the marine environment where other processes such as advection are occurring simultaneously and might affect its pool size. Therefore, aside from accurate quantification and rate measurement, the laboratory mechanistic investigation of glycolic acid production, excretion and utilization by marine phytoplankton and bacteria, in concert with the determination of phytoplankton and bacteria community structures and the examination of the related genes (e.g., glycolate oxidase) in the field, will help to provide a holistic view of the dynamics of glycolic acid in the oceans and hence enhance our understanding of the bacteria–phytoplankton interactions.

It is known that different species of marine phytoplankton have a varying capacity for concentrating CO_2 intracellularly (Badger et al. 1998), which may result in variations in RubisCO carboxylase/oxygenase activity, consequently affecting photorespiration rates and thus overall levels of production and excretion of glycolic acid. For example, the diatom *Phaeodactylum tricornutum* is reported to contain and excrete more glycolic acid per cell unit than the chlorophyte *Tetraselmis gracilis* (Rigobello-Masini et al. 2012), which is likely attributable to differences in RubisCO carboxylase/oxygenase activity and photorespiration rates. However, to the best of our knowledge, variations in rates of release of glycolic acid among phytoplankton species have not yet been systematically examined. To tackle this, the LC-MS quantification method, together with radioisotope-labeling technique, provides an approach to measure glycolic acid released by representative phytoplankton species in laboratory axenic monocultures. In addition, the relationship between glycolic acid excretion and photorespiration could be examined species-specifically, if the measurement of release rate is combined with assessment of the activities or gene expression levels of photorespiration enzymes (Parker et al. 2004; Parker and Armbrust 2005; Shi et al. 2015).

In the present study, we observed a similar Chl *a* vertical profile at stations J3, E3, and H6, all within the range of 0.1–0.4 μg L^{-1} at a depth of 5–125 m (Fig. 6). Conversely, the concentrations of glycolic acid at stations J3 and H6 (35–65 nM) were considerably lower than those at station E3 (50–210 nM) (Fig. 6), which may be because that the phytoplankton community composition at E3 varied from those at J3 and H6, displaying higher rates of glycolic acid excretion, and/or that the rate of glycolic acid utilization by various heterotrophic bacteria communities at different stations may vary considerably. In addition, varying levels of environmental stress (e.g., light intensity) may also exist at different sampling stations.

Marine phytoplankton are expected to encounter elevated CO_2 concentrations, as more anthropogenic CO_2 dissolves into the ocean, increased temperatures, as the greenhouse effect intensifies, and enhanced irradiance, as warming augments stratification of surface waters (Behrenfeld et al. 2006; Doney 2006). All these factors are known to affect photorespiration and hence the release of glycolic acid (Parker et al. 2004; Shi et al. 2015). Therefore, the bacteria community structures and the phytoplankton–bacteria interactions will also be affected, and variations may be expected in the cycling of marine organic matter, which may have far reaching and high impact effects on marine ecosystems. Accurate measurement of low concentration glycolic acid provides the basis to quantitatively evaluate glycolic acid release and uptake rate by phytoplankton and bacteria, respectively, which is essential for the better understanding of phytoplankton–bacteria interactions in the future oceans.

Comments and recommendations

The reported method was developed to measure low concentrations of glycolic acid, one of the important components of phytoplankton released DOM, in oligotrophic

seawater. The whole method, including extraction and analysis, is sensitive, robust, and easy to conduct. The LC method employed here also has a good separation capacity for other organic acids, such as lactic acid, acetic acid, and formic acid (Albert et al. 1995; Yoshida et al. 2007), other notable DOC components in seawater. The addition of compound specific MRM events for these organic acids into the ESI-triple quadrupole mass spectrometry system method could enable the quantification of multiple organic acids in the same samples, given that these organic acids have similar physical and chemical properties and can be co-extracted by liquid-liquid extraction methods. The LC-ESI can also be interfaced with a high-resolution mass spectrometer, such as an Fourier Transform Ion Cyclotron Resonance (FT-ICR) or Orbitrap mass spectrometer, which can readily distinguish organic acids from background matrices and may further improve analytical sensitivity.

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Conflict of Interest

None declared.

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