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Measuring Oceanic Respiration with a Unique High-efficiency, Low-energy Electron Ionization Approach

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Introduction

We present a novel low energy EI approach to better understand the diel cycle of glycolate, a metabolite which fuels the oceanic microbial loop. Glycolate is a waste product of photorespiration and is subsequently consumed as both a carbon and energy source for heterotrophic microbes.[1] Existing protocols for measuring oceanic concentrations of glycolate have been plagued by low recoveries, high analytical variance, as well as chromatographic interferences.[2] We propose that applying a novel high efficiency EI source will allow high analytical sensitivity measurement of microbial metabolites at environmentally relevant concentrations while concurrently reducing chemical interferences. Moreover, using a standardized metabolic workflow allows for greater coverage of known biological pathways and gives deeper insight into the complexity of marine microbial community metabolism.

Glycolate is found in seawater in the 1 nM to 100 nM range (10^{-9} mol/L).[3] While not inherently difficult to detect at these levels, the seawater matrix has proven to be a difficult analytic for small, polar metabolites such as glycolate.[4]

Experimental

Preparation of standards and matrix blanks

Standards and vehicle blanks were prepared in synthetic seawater (Ricca Chemical Company) containing only the salts and trace minerals typically found in seawater. The glycolate stock solution was prepared in ethyl acetate at 1 mM. All the reagents were obtained from Sigma Aldrich.

Sampling and preparation of natural samples

Natural seawater samples were collected from the Gulf of Mexico (Perdido Key, and Tampa Bay). The littoral zone samples were stored at 4 °C in Polyethylene terephthalate bottles. Ideally the samples would have been shipped frozen over dry ice and kept frozen until analysis to reduce enzymatic processes. Subsequent samples will be handled in this manner.

Experimental

Extraction and concentration of glycolate

Aliquot 10 mL of each ocean sample into a 20 mL headspace vial. Spike every sample with internal standard. headspace vial. Spike every sample with internal standard. Acidify with 2 ml of 1 N HCl to reduce the pH well below the pKa of glycolic acid. Extract twice with ethyl acetate and combine the organic fractions. Then add base to increase the pH and form ammonium glycolate. Converting from the free acids to the salt form reduces potential for evaporative losses. Take to dryness with a nitrogen evaporator at room temperature. Transfer to a 2mL vial with 3 x 500 µL ethyl acetate and again bring to dryness.

While not necessary for the analysis of glycolate, we wanted to make the method as universal as possible by matching the Fiehn derivatization protocol.[5] This allows us to apply a global profiling approach and identify other small polar metabolites such as pyruvate. Therefore we used methoxyamine hydrochloride to oximate ketones. Then derivatize with MSTFA (1% TMCS) to form the silyl derivatives. The final volume is 100 µL which means that there is a hundred fold concentration factor.

Instrumental Analysis

The study was performed using an Intuvo GC with guard chip and planar column technology. The guard chip is effectively a 0.7 m x 0.53 mm inner diameter deactivated channel that captures contaminants that would otherwise reach the analytical column. The guard chip can be heated independently from the inlet and can be used to capture late eluting compounds or operate as an extension of the inlet. In this case we used it as an extension of the inlet because further optimization wasn't necessary.

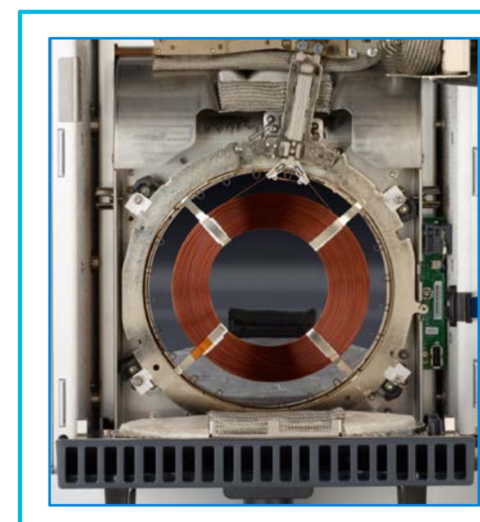

Figure 1. Intuvo GC with planar column and guard chip technologies.

Experimental

The study also took advantage of the latest generation of high-efficiency electron ionization (EI) source technology available with the 5977B GC/MSD. This source generates an ion flux typically 30 fold higher than traditional design under standard source conditions. Due to the novel way it generates and propagates electrons, it can be run at lower emission voltages and electron energies than traditional sources. This mode of operation maintains a relatively high ion flux while reducing fragmentation and enhancing the generation of the higher molecular weight fragments.

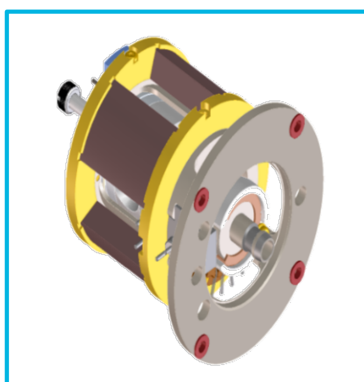

Figure 2. High Efficiency EI source technology.

The instrument performed well enough that only minimal optimization was required to achieve the goal of high sensitivity of glycolate in scan mode.

GC and MS Conditions:	
Column	DB-5msUI-INT 30 m x 0.25 mm x 0.25 µm with integrated Guard Chip (0.7 m x 0.53 mm)
Injection volume	1 µL
Split ratio	5:1 Pulsed split, 25 psi for 0.5 min
Split/ Splitless inlet temp	250 °C
Column flow	1.2 mL/min
Oven temperature program	60 °C for 1 min 10 °C/min to 325 °C, 3.5 min hold
Transfer line temperature	325 °C
Ionization mode	HES EI at 70 eV
Source temperature	275 °C
Quadrupole temperature	150 °C
Mass range	50 to 600 Da, Threshold 150
Electron multiplier gain setting (0.1 to 20)	1

Table 1. GCMS Parameters.

The LLOQ will be 1 nmol/L the effective concentration will be 100 nmol/L. When we convert this to analyte on column it is around 1 pg. So the real challenge is not detecting glycolate but reducing matrix interferences. Two simple approaches are to further reducing the aliquot size and/or reducing the fragmentation through source optimization. For example, we should be able to reduce the fragmentation by reducing the source temperature to 200 °C, drop the electron voltage to 30 eV, and lower the emission current to 20 mA.

Results and Discussion

Source optimization

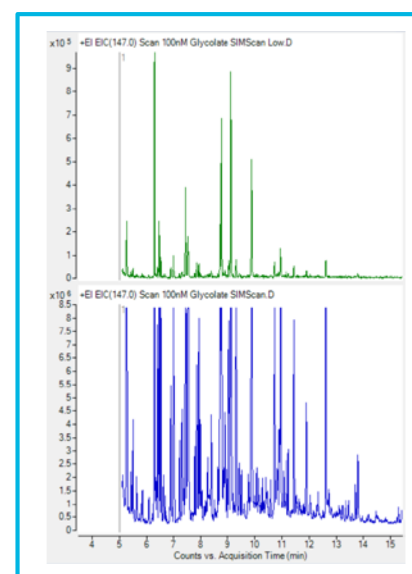

Figure 3. The loss of two TMS groups is reduced significantly at lower ion energies (green). Further optimization would be needed to needed to detect the pseudomolecular ion peak at 220 Da.

A quick adjustment of the source parameters as described at the end of the experimental section reduced fragmentation. As we see in figure 3, the low mass fragmentation is reduced. However, additional optimization that includes the gain settings would be needed to detect the pseudomolecular ion at 220 Da or even significantly improve the response for the M-15 (loss of methyl) fragment ion that we use for quantification.

A simpler approach would be to use the high ion flux to further reduce the initial aliquot size or increase the split ratio.

Quantification

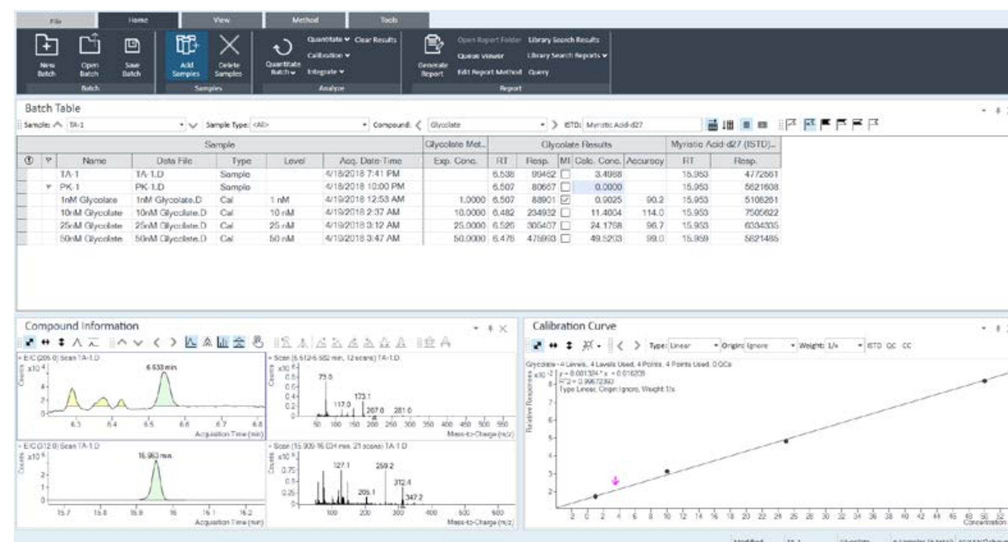

Figure 4. The target ion is reasonably free of interferences. There are several ways to increase the selectivity of the approach: further reduce the aliquot size, use single ion monitoring (SIM), transfer the method to a Tandem Quad, or switch the silylating agent from MSTFA to MTBSTFA.

Results and Discussion

The trophic role of glycolate in marine metabolism.

During oxygenic photosynthesis, the oxidation rather than carboxylation of ribulose bisphosphate, is an inevitable consequence. While most photosynthetic organisms have one or more pathways to recover some of this loss, the most abundant primary producer in the oligotrophic subtropical gyres, *Prochlorococcus*, has lost this pathway through genome streamlining, or perhaps delegated the salvage to its heterotrophic counterparts. Instead, photorespired glycolate is excreted and subsequently consumed by heterotrophicmicrobes. The kinetics of uniformly labeled ^{14}C-glycolate assimilation and respiration in natural seawater samples, measured over several diel cycles, indicated rapid consumption by heterotrophic microbes.[6] The dynamics of glycolate uptake and subsequent assimilation and respiration were light dependent and time-of-day dependent, though physiological changes, also operating on the diel cycle, could not be distinguished from ambient concentrations.

Past approaches relied on large aliquot volumes being concentrated by a factor of a thousand or more. This lead to a complicated sample preparation protocol that aimed at removing interferences while hopefully obtaining reasonable recovery of the target analytes. Recent development in instrumentation have provided us with two new techniques that allowed us to reduce matrix interferences as well as increase the instrument's analytical sensitivity. Our goal is to establish bounds on the magnitude of this exchange of carbon and to improve our understanding of the coupling of photosynthesis with respiration in the marine environment.

Applying the approach to metabolic flux

The partitioning of metabolic tasks, as in the case of photorespiration, seems widespread in marine microbial communities. However, much of our suspicions of division of labor is cursory, deriving from molecular evidence of complementary pathways rather than direct observation. This knowledge gap is largely due to difficulty in the quantitation of extracellular 'intermediates' in these pathways.

Detailed metabolic flux models of the most abundant representatives of the oligotrophic marine microbial community, currently being developed, resolves these complementary pathways. This new analytical approach should allow us to quantify the dynamics of extracellular metabolites, especially low molecular weight organic acids, nucleotides, and sugars which are thought to mediate microbial community interactions. Time-resolved quantitation of these exchanged metabolites will provide flux balance constraints to be implemented in *in silico* microbial community simulations.

Conclusions

Our long-term goal is to develop a multi-Omic approach that includes measurements from cultivated and natural microbial communities to develop a metabolic flux model for oceanic respiration. Quantitative analysis of glycolate is a key step in developing accurate models. This current work demonstrates several steps:

- We were able to quantitate glycolate using the classical Fiehn metabolomic approach.
- We successfully applied the Fiehn approach with a novel gas chromatography instrument that combines the speed of direct column heating with an integrated guard chip for capturing interfering matrix.
- We also employed a novel high-efficiency EI source to boost the ion flux 20 to 30 fold. This gave us a strong signal response for glycolate, an assay that has been traditionally very difficult at the low nM concentration range.

We plan on applying the insight from this study to other small organic acids that have been classically hard to analyze but are metabolically important.

References

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