Environmental Metabolomics: Analytical Strategies

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ABSTRACT

Microbial metabolism plays a primary role in shaping the marine carbon cycle through processes of carbon fixation and remineralization. Many metabolic intermediates pass through the reservoir of marine dissolved organic matter (DOM), as compounds move among microbes as part of complex ecological networks of interactions. Environmental metabolomics can be used to identify and quantify these compounds, and thus will provide insight into the chemical underpinnings of microbial networks at the foundation of global biogeochemical cycles. Here we present methods for metabolite profiling (untargeted metabolomics) and for relative quantification (targeted metabolomics) of intracellular and extracellular metabolites from marine microbes. We describe our approach to method development with regard to metabolite extraction and instrumental analysis, culminating in the methods currently in use in our laboratory.

1. INTRODUCTION

Marine microbes play a key role in global biogeochemical cycles through their metabolic interactions with dissolved organic matter (DOM) in the ocean. The suite of compounds produced by a microbe reflects its metabolic state, which is a function of its genetic potential and its environment. Interactions with neighboring organisms, the availability of nutrients, and environmental conditions all exert an influence on the expressed metabolic profile and hence on microbial contributions to the DOM pool (Carlson, 2002; Nagata, 2000). Heterotrophic and many autotrophic microorganisms also rely on the low molecular weight (<600 Da) fraction of DOM for their growth and metabolism (reviewed in Kujawinski, 2011). Identifying metabolites that pass through the pool of DOM and that interconnect microbial species is an important step in understanding the impact of microbes on the global carbon cycle. New insights into microbe-DOM interactions within the marine ecosystem can be gained through the use of tools from the field of metabolomics.

Metabolomics is the study of the low-molecular weight metabolites within a biological system (e.g. cells, tissues, or biofluid) (Viant, 2007). The purpose of environmental metabolomics is the characterization of an organism's metabolic response to natural or anthropogenic stressors in its environment (Viant, 2007). Metabolomics experiments generally fall into two categories, untargeted and targeted.

Untargeted metabolomics seeks to detect and describe as much of the metabolome as possible without bias (Patti et al., 2012). By contrast, targeted metabolomics studies are typically focused on quantifying a specified set of compounds through the use of authentic standards (Lu et al., 2008). The suite of target compounds is often derived from one or more metabolic pathways of the biological system of interest (Patti et al., 2012).

Metabolomics methods can be applied to the study of microbial communities in their environment, including in marine systems. Here, untargeted metabolomics enables the discovery of new or unexpected metabolites, thereby expanding the metabolic pathways that can be queried in microbial consortia. For example, analysis of metabolites exuded by microorganisms into the surrounding dissolved phase (i.e. extracellular metabolites) can be used to elucidate cooperative relationships among microbial consortia members. Targeted metabolomics provides an important quantitative complement to untargeted methods; and in environmental applications, many of these targets are not currently studied in standard organic geochemistry investigations. For example, quantitative profiles of intracellular metabolites from targeted metabolomics can provide a snapshot of how microorganisms respond to environmental stress, such as nutrient limitation.

The application of metabolomics methods to the marine environment is, however, still in its infancy. In contrast to the large number of metabolomics studies in plants (Quanbeck et al., 2012; Tolstikov and Fiehn, 2002), human systems (Dunn et al., 2008; Kind et al., 2007; Want et al., 2010; Wikoff et al., 2009), and model microorganisms (e.g. yeast or *Escherichia coli*, Bajad et al., 2006; Lu et al., 2010; Rabinowitz and Kimball, 2007; Yanes et al., 2011), fewer metabolomics profiles have been collected for marine microbes. Studies to date include measurements of specific metabolite classes in seawater, such as B vitamins (Heal et al., 2014; Sañudo-Wilhelmy et al., 2012), as well as broader assessments of primary and secondary metabolites within marine microbial cultures (Baran et al., 2010; Bennette et al., 2011; Fiore et al., in press; Longnecker et al., 2015; Paul et al., 2009; Romano et al., 2014).

Marine environmental metabolomics follows in the footsteps of seminal organic geochemistry studies that explored the sources and sinks of biochemicals in the oceans such as amino acids and carbohydrates (Benner, 2002; Lee et al., 2000). These compounds are common to most microbes and participate in numerous metabolic reactions. For example, amino sugars are components of cell walls, peptides and iron-binding ligands (Kaiser and Benner, 2008) and are catabolized by a variety of bacteria in the ocean. The ratios of individual molecules and their stereoisomers track the rate of organic matter degradation during transport to the deep ocean (Kaiser and Benner, 2009). Compound-class centric studies, such as these, have provided fundamental

insights to specific research questions within biogeochemistry. With the advent of new analytical approaches, environmental metabolomics can significantly broaden the range of metabolites under study by probing many compound classes simultaneously. Importantly, these complex datasets complement other systems biology tools such as meta-genomics, meta-transcriptomics and meta-proteomics, and together these tools enable systems-wide understanding of microbial metabolism and organic carbon cycling in the environment.

A variety of analytical platforms have been used for metabolomics experiments, including liquid (LC) or gas (GC) chromatography coupled with mass spectrometry (MS) (Fiehn, 2008; Lu et al., 2008; Want et al., 2007; Zelená et al., 2009), NMR spectroscopy (Barton et al., 2008), direct infusion MS (Southam et al., 2007), and capillary electrophoresis-MS (Monton and Soga, 2007). Of these, chromatography coupled with MS has emerged as a preferred approach for global metabolic profiling and metabolite quantification (Bennette et al., 2011; Dunn et al., 2011; Patti et al., 2012; Want et al., 2010).

Creating a mass-spectrometry based metabolomics method presents several challenges. Metabolites vary widely in their chemical properties and dynamic range, making the selection of a comprehensive extraction protocol and chromatographic separation technique a considerable task. The choice of ionization method can be an

additional source of bias. Ionization techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption/ionization (MALDI) select for certain analyte physical and chemical properties (Nordström et al., 2008). ESI is particularly well suited for polar metabolites that would normally carry a charge in solution; many metabolites of interest, in fact, have ionizable basic or acidic groups. Many neutral compounds, however, will not ionize efficiently (or at all) with ESI. For example, myo-inositol, a small carbohydrate, does not ionize by ESI. Marine microbial samples pose additional analytical challenges due to the high levels of salt in growth media and in the ocean itself. Salt in the sample matrix can lead to ion suppression of mass spectrometry signal during electrospray ionization, and the build-up of salt over time in the mass spectrometer and ESI source can decrease instrument performance and data quality. Metabolomics methods must also be able to make reproducible measurements, and include strategies to monitor that reproducibility. To this end, the particular strengths of the mass analyzer must be well matched to the experimental goals (i.e. quantitative analysis or global profiling).

The purpose of this manuscript is to review our approach over the past five years to the development of methods for marine environmental metabolomics. We present both untargeted and targeted metabolomics methods, in which we extract intracellular and extracellular metabolites from marine microbial samples and measure their composition and relative abundance with ESI coupled to liquid chromatography – mass

spectrometry (LC-ESI-MS). Here, we detail our decisions in developing our analytical strategies with regard to experimental design, metabolite extraction, and LC-MS analysis, culminating with the methods currently in use in our laboratory. We address data analysis methods in a companion paper in this issue (Longnecker et al., in press).

2. EXPERIMENTAL SECTION

2.1 Materials and Chemicals

Metabolite standards at the highest grade available were obtained through Sigma Aldrich (MO, USA), with the exception of: dimethylsulfonylpropionate (DMSP), purchased from Research Plus Inc. (NJ, USA); 2,3-dihydroxypropane-1-sulfonate (DHPS) and n-acetyltaurine, which were generously donated by Dr. Mary Ann Moran at the University of Georgia; and S-(1,2-dicarboxyethyl)glutathione, purchased from Bachem (CA, USA). Isotopically labeled standards were purchased from Cambridge Isotopes (d3-glutamic acid), from CDN Isotopes (d4-4-hydroxybenzoic acid), and from Toronto Research Chemicals (d5-sodium taurocholate) through Fisher Scientific. Solvents and additives used for metabolite extraction and LC-MS analysis were acetonitrile (Optima LC/MS, Fisher Chemical), methanol (Optima, Fisher Chemical), formic acid (eluent additive for LC/MS, Fluka), hydrochloric acid (Optima, Fisher Chemical), and ammonium hydroxide (ACS Plus, Fisher Chemical). Water purified by a Milli-Q system (Millipore) was used throughout (resistivity 18.2 MΩ•cm @ 25 °C, TOC < 1 μM).

2.2 Samples Used for Method Testing

We used a series of laboratory and field samples for testing the metabolomics methods. Marine microorganisms *Ruegeria pomeroyi* (heterotrophic α -proteobacterium), *Micromonas pusilla* (autotrophic pico-eukaryote), and *Thalassiosira pseudonana* (autotrophic diatom) were grown in laboratory cultures. Surface seawater samples were collected from the University of Rhode Island's Narragansett Bay Plankton Time Series site. Net trap samples were collected in the Atlantic Ocean during a cruise on the R/V *Knorr*. Details pertaining to these samples can be found in Table S1 of the Supplemental Information.

2.3 Metabolite Extractions

All glassware was acid-washed and combusted at 460°C for at least 5 hours. Water samples obtained from laboratory cultures were filtered through 0.2 μ m PTFE filters (Omnipore, Milipore). Field samples were filtered in series onto 1.6 μ m glass fiber filters (pre-combusted size GF/A, Fisher Scientific) and 0.2 μ m PTFE filters. The volume of water filtered typically ranged from 50-4000 mL, depending on anticipated cell abundance (see section 3.2). Filter blanks were used as negative controls by filtering culture media without cells. Filters were stored in cryovials (Nalgene Cryoware™) at -80°C until extraction. The filtrate was acidified to pH 2-3 with 12 M HCl and stored at 4°C until extraction.

Extracellular metabolites were extracted from the acidified filtrate using solid phase extraction (SPE) with 1 g/ 6 cc PPL cartridges (Bond Elut PPL, Agilent), as previously described (Dittmar et al., 2008). We kept flow rates to less than 40 mL min⁻¹, following the original protocol. We have not tested the effect of flow rate on our extraction efficiencies. The cartridges were rinsed with at least two cartridge volumes of 0.01 M HCl (aqueous solution) and eluted with 8 mL of methanol. The eluent was reduced to near dryness with vacuum centrifugation. The samples were re-dissolved in 495 µL of 95:5 water:acetonitrile with 5 µL of 5 µg/mL of biotin-(*ring*-6,6-d₂) and divided into two aliquots to be analyzed with the targeted and untargeted metabolomics methods. The deuterated biotin served as an autosampler injection standard.

Intracellular metabolites were extracted from the filters using a method adapted from Rabinowitz and Kimball (2007). Working quickly throughout to minimize degradation, immediately prior to extraction the filters were removed from the freezer, weighed and cut. A combusted aluminum foil surface was used for all weighing and cutting, and scissors and tweezers were rinsed with methanol before use and between samples. Either a portion or the full filter was cut into small pieces and placed at the bottom of an 8 mL amber glass vial. We determined the amount of filter to extract based on the number of cells filtered (see section 3.2). Cold (-20°C) extraction solvent was added to each amber vial to adequately cover the filter pieces (500 µL to 1 mL of 40:40:20 acetonitrile: methanol: 0.1M formic acid). In addition, 25 µL of a 1 µg/mL

deuterated internal standard mix (d3-glutamic acid, d4-4-hydroxybenzoic acid, and d5-sodium taurocholate) was added to monitor the intracellular extraction recovery. The vials were gently vortexed to ensure solvent coverage of all filter surfaces and then sonicated for 10 minutes to lyse the cells. Extracts were transferred by pipette to centrifuge tubes (Fisherbrand™ Premium Microcentrifuge tubes), and the remaining filter pieces were rinsed with 200 µL of extraction solvent; the rinse was added to the tubes. The extracts were spun at 20,000xg for 5 minutes at 4°C and the supernatant transferred to new 8 mL amber glass vials without disrupting any particulates at the bottom of the tube (filter or cellular materials). The extracts were neutralized with 6M ammonium hydroxide and reduced to near dryness using vacuum centrifugation. The intracellular extracts were re-dissolved as described above for the extracellular samples and divided into two aliquots for targeted and untargeted analysis.

Additional samples for quality control (QC) were analyzed in all targeted and untargeted experiments. These QC samples included a mix of the authentic metabolite standards described above and a pooled sample consisting of aliquots of each sample. Samples were analyzed in randomized order.

2.4 Untargeted Metabolomics Instrumentation

Intracellular and extracellular metabolite samples for untargeted analyses were run within 24-48 hours of extraction using a liquid chromatograph (Thermo Micro AS autosampler and Surveyor MS Pump Plus) coupled via ESI to a hybrid linear ion trap-

204 Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (7T LTQ FT
205 Ultra, Thermo Scientific). Metabolite separation was accomplished on a Synergi Fusion
206 RP column (4 μm , 2.0 $\times$ 150 mm, Phenomenex), equipped with a guard column and pre-
207 column filter and maintained at 35°C with a Hot Pocket column heater (Thermo
208 Scientific). The column was eluted with (A) 0.1% formic acid in water and (B) 0.1%
209 formic acid in acetonitrile at a flow rate of 0.25 mL min⁻¹. The column was held at 5% B
210 for 2 min, ramped to 65% B over 18 min, quickly ramped to 100% B in 5 min, held at
211 100% B for 7 min and then equilibrated at 5% B for 8 min prior to the next injection
212 (total run time = 40 min). Separate autosampler injections of 20 μL each were made for
213 positive and negative ion modes.

214 The mass spectrometer was externally calibrated weekly in positive and negative
215 modes using solutions provided by the manufacturer. The capillary temperature and
216 ESI voltage were optimized at 330°C and 4.2 kV in positive mode and at 365°C and 3.8
217 kV in negative mode. Sheath gas, auxiliary gas, and sweep gas flow rates were
218 maintained at 35, 5, and 2, respectively (arbitrary units) for both polarities. Both high
219 resolution MS and data dependent MS/MS scans were collected. The MS scan was
220 performed in the FT-ICR analyzer from 100-1000 m/z , with mass resolving power set to
221 100,000 (defined at m/z 400). In parallel to the FT acquisition, the four most abundant
222 ions in each cycle were fragmented via collision-induced dissociation in the linear ion
223 trap. MS/MS spectra were collected under dynamic exclusion with an exclusion time of

20 seconds. Dynamic exclusion allows for greater depth of MS/MS coverage because the instrument will not repeatedly trigger an MS/MS scan of the same ion. Data were collected in profile mode as XCalibur RAW files (XCalibur 2.0.7) and then converted to open source formats using ProteoWizard (Chambers et al., 2012). Each data set may contain thousands of features, where a feature is defined as a unique combination of a mass-to-charge (m/z) ratio and a retention time. For more details on data processing see Longnecker et al. (in press) in this issue.

2.5 Targeted Metabolomics Instrumentation

Concentrations of intracellular and extracellular metabolites were measured using an LC system (Thermo Accela Open Autosampler and Accela 1250 Pump) coupled via a heated ESI (H-ESI) source to a triple quadrupole mass spectrometer (Thermo TSQ Vantage). The instrument was calibrated in positive and negative ion modes using a polytyrosine-1,3,6 solution infused directly into the mass spectrometer. Selected reaction monitoring (SRM) conditions for each compound (s-lens, collision energy, parent and fragment ions) were optimized by infusion of authentic metabolite standards. For each metabolite parent mass, quantification and confirmation SRM transitions (a parent and product ion pair) were selected primarily to maximize signal-to-noise. Polarity switching in the H-ESI probe was employed, thereby combining positive and negative SRM transitions in a single run. The SRM transitions were scheduled such that each compound was monitored over a 2-4 min window around its

244 retention time. This ensured adequate sampling across chromatographic peaks (at least
245 10 data points over a 10-24 second baseline peak width). H-ESI parameters were:
246 capillary temperature 275°C, vaporizer temp 375°C, sheath gas pressure 40 units, and
247 auxiliary gas pressure 25 units. Spray voltage was 4 kV and 3.2 kV for positive and
248 negative ion modes, respectively. Data was collected in centroid mode as XCalibur
249 RAW files.

250 The LC column, mobile phase and gradient conditions were the same as those used
251 in the untargeted metabolomics analysis. Stock solutions of each metabolite standard
252 were prepared in either Milli-Q water or a 1:1 mix of methanol and water at 1000
253 µg/mL, unless constrained by solubility. Certain standards required the addition of
254 either ammonium hydroxide or formic acid for dissolution in the stock solution. Those
255 that required formic acid include: glutamic acid, guanine, cysteine, riboflavin,
256 guanosine, and aspartic acid. Standards that required ammonium hydroxide include:
257 orotic acid, biotin, xanthosine, folic acid, xanthine, and tyrosine. Stock solutions were
258 stored in the dark at -20°C. Prior to metabolomics analysis, individual stock solutions
259 were combined and diluted in Milli-Q water to create 6 metabolite standard mixes each
260 at 10 µg/ml; metabolites were grouped together in these mixes based on chemical
261 compatibility. For the targeted method, these 6 mixes were then combined to generate a
262 full mix of all metabolite standards ("standard mix") and diluted in Milli-Q water to
263 concentrations between 0.5 - 1000 ng/mL (0.5, 1, 5, 10, 25, 50, 100, 250, 500, and 1000

ng/mL). The current method does not use isotopically-labeled internal standards for each target analyte to account for matrix effects, but rather uses external calibration for relative quantification. External calibration curves for each compound were generated by plotting peak area against concentration for each metabolite standard, and then used to determine relative metabolite concentrations in the intracellular and extracellular extracts.

3. RESULTS AND DISCUSSION

In this section we discuss key issues involved in several aspects of our marine metabolomics methods: experimental design, sample collection, metabolite extraction, and LC-MS analysis.

3.1 Experimental design:

In designing a metabolomics experiment it is essential to incorporate strategies for assessing data quality and measuring analytical system variability. For this purpose, we include in every study (targeted or untargeted) several types of quality control (QC) samples: pooled samples, standard test mixes, cell-free controls (i.e. growth media without cells processed identically to culture samples), and biological replicates. We also include internal standards for monitoring metabolite extraction and autosampler injection.

The pooled QC sample is an important component of our metabolomics methods and serves two main purposes. First, it is used to condition the analytical system, in particular the LC column; after several injections of the pooled sample (~5), chromatography conditions stabilize, improving reproducibility of the ensuing measurements (Dunn et al., 2011; Want et al., 2010). Second, the pooled sample is used to assess variability within a data set, and provides a means to correct the data for drifts in retention time or signal (Dunn et al., 2011). (For more details, see Longnecker et al. (in press) in this issue.) Ideally, the pooled sample is representative of all samples in the study that will be subject to statistical analyses. We create the pooled sample by mixing aliquots of each extract (~45 μ L) and inject it after every 6 sample injections.

In our culture studies, samples are generally taken at several time points across the growth cycle of a microorganism. The duration of the experiment is determined by microbial growth rate, often ranging from days to weeks. On this time scale, the need for a pooled sample that encompasses the entire sample set may conflict with the desire to filter, extract, and analyze samples rapidly after they are generated to minimize metabolite degradation. Given the value of a fully representative pooled sample for evaluating data quality, we choose to store the filters (and filtrates) generated at each sampling time point and later extract and analyze them together as a group after the final time point. This approach affords two advantages: (1) a pooled QC sample is generated from aliquots of each metabolite extract and therefore reflects their

composition; and (2) samples are analyzed in close succession, thereby reducing the effects of instrument variability over time.

In addition to the pooled sample, we analyze a mix of metabolite standards at the beginning and end of each sample set. In the untargeted method this mix is limited to a set of 22 standards that preferentially ionize in either positive or negative ion modes and span the LC gradient. In the targeted method, the full suite of standards is used (Table 1). By monitoring for shifts in retention time, peak shape, and signal intensity, the standard mix serves as a rapid performance check of the analytical system. Because the metabolite standard mix is in highly aqueous solution (> 95% Milli-Q), it is not injected during the analysis of a set of samples to avoid disrupting the matrix-equilibrated column.

For studies involving microorganisms cultured in the laboratory, we also include cell-free control samples (media blanks) treated identically to cultures with cells. In a time course experiment, for example, we generally have a cell-free control at each time point in addition to biological replicates. These control samples allow us to subtract features from the data set corresponding to the growth media components and any contaminants from the processing method. In marine field studies, however, there is no clear way to define a cell-free control, and so such blanks are not used. Rather, we include procedural blanks by processing and analyzing aliquots of the Milli-Q water

used in the field. At a minimum, the procedural blanks allow us to detect contaminants that are by-products of the filtration and/or extraction methods.

Replicate measurements are an important means of assessing variability. We use the repeated injections of the pooled sample and metabolite standard mix to define instrument variability and thus do not typically include technical replicates in the analytical sequence. In the laboratory, we assess biological variability through the use of replicate growth flasks containing both media and cells. At least three biological replicates are preferable in order to perform statistical analyses. Figure 1 compares technical and biological variability in the untargeted method as measured by the percent of peaks shared across three replicate samples for: the metabolite standard mix in pure solvent ("Standard mix"), "Field samples A", and "Lab experiment B" (see Table S1 for sample details). The proportion (%) of peaks shared was defined as the total number of peaks shared between the three replicates divided by the average number of peaks found in the three replicates. For the "Standard mix", three repeat injections of the metabolite standard mix were made. For "Field samples A", replicates were obtained by processing three water samples collected per day on 5 different days. For "Lab experiment B", points represent samples from three replicate growth flasks at multiple times along the growth curve of *M. pusilla*. From these measures of experimental variability, we find that technical variability is generally lower than biological variability. However, note that #2 in "Field samples A" was as variable as the

342 repeat injections of the standard mix. These results emphasize the importance of
343 biological replication in metabolomics experimental design, in order to appropriately
344 resolve treatment effects.

345 The accumulation of sample components in the ionization source (e.g. salt) can
346 reduce instrument sensitivity over time. To limit this potential source of instrument
347 variability we perform preventative maintenance in the ion source between sample sets.
348 This includes flushing the ESI probe with Milli-Q water and organic solvent and
349 changing the ion capillary tube. In addition to preventative cleaning, we randomize the
350 order in which samples are run relative to the order in which they were processed, to
351 avoid introducing systemic bias into the data (Dunn et al., 2011; Want et al., 2010).

352 We use a small set of isotopically labeled metabolite standards as a qualitative check
353 of recovery through the multi-step intracellular extraction protocol. Three standards,
354 d3-glutamic acid, d4-4-hydroxybenzoic acid, and d5-sodium taurocholate, are added to
355 the extraction solvent at the beginning of the filter extraction process. Consistency in the
356 measured response levels (peak areas) of these internal standards is then used as a
357 criterion for removing any samples from the set prior to data analysis. Table 2 shows
358 the percentage of the three deuterated standards recovered during filter extraction of
359 intracellular metabolites (Lab experiment E, Table S1). Another standard, deuterated
360 biotin, is added at the same level into each sample prior to LC-MS analysis as an

autosampler injection standard, and can quickly alert the analyst to injection issues or other instrumental problems.

3.2 Sample Collection and Filtration

We have developed procedures for the collection and filtration of marine samples from both the field environment and laboratory cultures. Our sample volumes have typically ranged from 50 to 4000 mL, with laboratory cultures at the lower end and samples from the open ocean at the higher end. Ultimately, the choice of sample volume depends on the carbon concentrations required for acceptable metabolite signal levels on our analytical platforms. From our culture experiments, we have found that a carbon concentration of 0.3 mg C on filter is an ideal target, leading to high quality signal from both high and low abundance metabolites. Filtering less carbon can still produce acceptable metabolite signals, but detection of the lowest abundance metabolites suffers. We use this value, 0.3 mg C, to calculate a target cell number based on the type of organism and its size. As an example, for coastal bacteria, 0.3 mg C requires filtration of approximately 10^{10} cells, assuming 30 fg C per cell (Fukuda et al., 1998). In our culture studies, we count cells using microscopy or flow cytometry and adjust the volume filtered to reach $\sim 10^{10}$ cells. To collect this number of cells in the surface ocean where microbial concentrations are $\sim 10^6$ cells/mL, the ideal sample volume would be 10 L. In the field, however, there is often a trade-off between sample volume and the

381 number of samples that can be collected and processed. During a recent field campaign,
382 for example, we limited our sample size to 4 L of seawater, less than the ideal estimated
383 volume, in order to obtain a sample set with comprehensive spatial and depth coverage.
384 Collecting more water, and therefore more cells, is always better for the detection of low
385 abundance metabolites, but this is not always practical given the significant time and
386 human resources required to filter and process each sample at sea.

387 Our filters of choice are either glass fiber or PTFE. We have tested various other
388 materials and have found that glass fiber and PTFE filters produce the least
389 downstream contamination for LC-MS analysis. The ideal filter pore size depends upon
390 the scientific question at hand. We have chosen different filtration methods for use in
391 the laboratory and the field, primarily for logistical reasons. In the laboratory, we use
392 gentle vacuum filtration with an all-glass apparatus that can be acid-cleaned and
393 combusted. Water is pulled through a PTFE filter (typically 0.2 μm) at vacuum pump
394 pressures less than 10 mmHg. We find that this filtration speed balances the need to
395 work quickly to limit metabolite degradation and the desire to avoid cell lysis.
396 Transporting an all-glass vacuum apparatus into the field, however, is neither
397 convenient nor practical. Furthermore, while it is possible to perform vacuum filtration
398 with a plastic apparatus, this would lead to unacceptable levels of plastic contamination
399 in the samples for mass spectrometry analysis. For these reasons, while at sea we have
400 chosen to use a peristaltic pump filtering apparatus equipped with Masterflex PharMed

BPT peristaltic tubing, a PTFE filter holder, and PTFE tubing. Water is pumped through both glass fiber and PTFE filters in series. Figure S1 illustrates the potential impact of plastics on mass spectrometry data. The direct infusion mass spectrum of DOM extracted (by the SPE method) from a seawater sample is shown in Figure S1A. The prominent series of peaks spaced 44 mass units apart are characteristic of polymeric interference from polyethylene glycol (PEG)(Keller et al., 2008). Such contamination is absent from the spectrum shown in Figure S1B of a seawater sample collected and extracted in the field using Teflon-based materials and Pharmed BPT peristaltic pump tubing. To avoid contamination of the mass spectrometry data, we prefer to be as conservative as possible in our use of plastics when collecting and processing water samples, and use glass or PTFE materials whenever possible. We have, however, found that collecting water in polycarbonate bottles can be acceptable.

3.3 Metabolite Extraction Methods

Metabolite extraction protocols for cells or tissues all have steps to quench metabolism, release metabolites from cells, and remove remaining cellular material. However, the choice of extraction method can have a significant impact on the number of features observed in a metabolomics experiment as well as on their concentration, largely due to the broad chemical and physical diversity of the metabolome. Given this complexity and the heterogeneous nature of organic matter, no single extraction

protocol will fully capture all metabolites. Our initial intracellular extraction tests were performed with *E. coli* using protocols optimized for organisms grown in low saline environments (Bolten et al., 2007; Winder et al., 2008). However, upon applying these methods to marine microorganisms such as *Ruegeria pomeroyi*, few features were distinguishable from the cell-free media controls. We found that the method developed by Rabinowitz and Kimball (2007) using an ice cold (-20°C) solution of acidic acetonitrile/methanol/water to quench marine microbial cells resulted in the most features distinct from the media controls (Figure 2).

In our initial experiments using this extraction protocol, we harvested cells of *T. pseudonana* by centrifugation of 1.5 mL of culture for 30 minutes. Intracellular metabolites were then extracted from the resulting cell pellet and analyzed by our untargeted metabolomics method (Longnecker et al., 2015). To increase the biomass in each extraction, we now use filtration to collect cells and, as expected, this has increased the number of detected intracellular features. We have used this approach for cultures of marine microbes as well as for field samples taken from the marine environment. We can typically extract ~18 filters per day; vacuum centrifugation to reduce sample volume at the end of the extraction protocol is generally the limiting step. We conspicuously avoid bringing the samples to complete dryness during vacuum centrifugation to limit metabolite degradation and evaporation.

We investigated two approaches for preparing the intracellular filter extracts for LC-MS analysis: 1) desalting the intracellular extract using solid phase extraction to enable a head-to-head comparison with the extracellular extract, or 2) injecting the intracellular fraction “as is” onto the targeted and untargeted LC-MS platforms. Neither option is without concern. In the first case, the bias of solid phase extraction methods results in selective metabolite loss. In the second, build-up of salt from the intracellular extracts requires more frequent cleaning of the mass spectrometry system and replacement of consumable parts. Whenever possible, we forgo additional SPE processing of the intracellular extract. However, we have found that very large sample volumes, as in the case of field samples, may require desalting of the intracellular extract.

We are also interested in the metabolites exuded by marine microorganisms into their environment. Extraction of some kind is necessary to isolate these dilute compounds from the highly saline matrix of seawater or culture media. Unlike urine, seawater is not rich enough in metabolites to simply dilute prior to analysis (Want et al., 2010). Furthermore, freeze-drying samples generates a salt-rich sample and compounds cannot be detected due to lower abundance and excessive ionization suppression in the LC-ESI MS analysis. Thus, methods to extract the extracellular metabolites must remove salts while retaining organic matter, as well as concentrate metabolites, which otherwise would be too dilute in the collected sample volume for detection by our methods. Several approaches meet these criteria, including SPE with PPL, XAD and other resins,

460 and reverse-osmosis/electrodialysis (RO/ED) (Aiken et al., 1992; Dittmar et al., 2008;
461 Kim et al., 2003; Vetter et al., 2007). An intercomparison study of three DOM extraction
462 methods, RO/ED, SPE with PPL, and SPE with XAD-8/4, showed that while RO/ED
463 captures more of the organic material than extraction by PPL (75-82% vs 61%) it has
464 considerably lower sample throughput (Green et al., 2014). As such, the authors
465 recommend extraction by PPL when large numbers of samples are to be processed.
466 Solid phase extraction with PPL is in fact, currently the most popular choice for high-
467 throughput isolation of DOM (Arnold et al., 2014; Flerus et al., 2012; Lawson et al., 2014;
468 Roth et al., 2013). We have chosen PPL for extracellular metabolite extraction so that the
469 LC-MS metabolomics data sets from our field and laboratory samples are comparable,
470 and so that they can dovetail with other DOM measurements. We estimate the PPL
471 extraction efficiencies for organic carbon as described in Bhatia et al. (2010). Briefly, we
472 measure the DOC concentrations in bulk seawater prior to extraction and then measure
473 the DOC concentration in the resulting extract. From this, we can estimate the percent
474 of organic carbon we have recovered. Our metabolomics studies involving the marine
475 microbes *T. pseudonana* and *Synechococcus elongatus* in culture observed lower than
476 expected PPL extraction efficiencies of organic carbon (between 3 and 41%, with
477 increased extraction efficiencies at later growth stages) (Fiore et al., in press; Longnecker
478 et al., 2015), compared to those measured by Dittmar et al. (2008) for marine and

estuarine water samples. Our values are, however, comparable to dissolved organic carbon recoveries reported from phytoplankton cultures (Becker et al., 2014).

Solid phase extraction isolates DOM molecules based on their chemical composition and is known to be susceptible to extraction bias. The literature gives some insight into compounds types that are not likely to be isolated well by PPL extraction. Hertkorn et al. (2013) found that samples from the South Atlantic Ocean isolated with PPL showed lower extraction efficiencies for dissolved organic nitrogen than for dissolved organic carbon, with retention values ranging from 10.2% to 28.3%, depending on depth. Other reports found that highly polar compounds such as urea and amino acids were not retained by PPL (Bronk, 2002). Similarly, DMSP, a polar sulfur-containing compound, was not efficiently recovered by PPL extraction from the dissolved phase of cultures of *T. pseudonana* (Longnecker et al., 2015). Another class of compounds, sugar alcohols (polyols), showed low PPL extraction efficiencies from Atlantic and Pacific surface DOM samples (Gonsior et al., 2014). In light of the extraction biases inherent in SPE methods, we have conducted a thorough investigation of the PPL extraction recoveries from a variety of chemical matrices of each metabolite in our targeted method. Full description of these data is beyond the scope of this paper but will be presented in a forthcoming publication (Johnson, Kido Soule & Kujawinski, unpublished results).

3.4 Chromatographic Method

Selection of a chromatographic method for metabolomics is challenging due to the well-recognized chemical diversity of the metabolome. Unlike the proteome, metabolites can range widely in size, polarity, and charge. In fact, there is currently no single chromatographic approach that is ideal for all compound classes (Lu et al., 2008). Sample matrix also influences the choice of LC method; not all approaches are ideal for marine organisms due to the presence of abundant salts. Other considerations include sample throughput, processing time and instrument availability. Our approach for both targeted and untargeted metabolomics platforms aims to balance these issues.

Most analytes of interest in a metabolomics study are hydrophilic in nature. We therefore chose existing LC approaches from the metabolomics literature shown to have enhanced retention for polar compounds and optimized separation parameters for each using a metabolite standard mix. These methods included reversed phase chromatography with ion-pairing (RIP), hydrophilic interaction chromatography (HILIC), and reversed phase (RP) chromatography with a polar embedded C18 phase.

An ion-pairing chromatography method for the separation of metabolites was initially applied to cellular extracts of *E. coli* (Lu et al., 2010; Xu et al., 2015). A slightly modified RIP method has also been used in a metabolomics study of the marine cyanobacterium *Synechococcus* sp. PCC 7002 (Bennette et al., 2011). Our tests of the ion-pairing method, run in negative ion mode with tributylamine (TBA) as the ion-pairing

agent on a Synergi Hydro RP column (Phenomenex, 150 x 2 mm, 4 μ m), showed excellent retention and separation of polar compounds with good peak quality. However, TBA contaminates surfaces in the HPLC and MS systems and is difficult to remove. In positive ion mode, the intense signal from TBA resulted in significant ion suppression and was particularly problematic in the case of full scan MS operation of the FT-ICR MS. Thus, the RIP chromatography method is an ideal choice, but only feasible when dedicated instrumentation is available to operate solely in negative ion mode.

We also tested an aminopropyl HILIC column (Phenomenex Luna NH₂, 150 x 2mm, 3 μ m) in positive ion mode with an ammonium acetate mobile phase at high pH (pH 9.4), as reported by Bajad et al. (2006). These conditions resulted in good retention of polar compounds, particularly amino acids, but lower peak quality in comparison to the RIP method. As shown in Figure S2, we encountered unacceptably high chromatographic background levels and difficulties with source cleanliness. This particular HILIC method has been used to analyze metabolites from a variety of non-marine sources, including *E. coli*, mammalian cells, bodily fluids, and tissues (Bajad et al., 2006; Bennett et al., 2008; Onorato et al., 2008; Yuan et al., 2012). Thus, the high salt content of our metabolite extracts may have contributed to challenges with this method. Bajad et al. (2006), for example, observed ion suppression in their aminopropyl HILIC method when sodium chloride was spiked at ~ 100 mM into a standards mix.

Our current LC method uses a polar embedded RP C18 column with polar end-capping (Synergi Fusion, Phenomenex). Such stationary phase modifications offer improvements for polar retention over a traditional RP C18 column (Lu et al., 2006). We optimized LC conditions for this column based on peak quality and signal-to-noise levels. The resulting chromatography method produces overall very good peak shape for most of the 92 compounds, with relatively narrow peak widths ranging from ~10 to 24s at baseline (Figure S3). As expected with a RP method, non-retained inorganic salts elute as a peak at or near the column dead volume, around 1.2 minutes. We use the same LC conditions for our targeted and untargeted metabolomics methods, which enables ready comparison between platforms. The chromatography is imperfect, however, notably in the retention of very polar compounds; 45 out of the 92 metabolite standards tested elute within the first 2 minutes (see Table 1). Salt from the sample elutes from the column in this window as well, along with metabolites with the earliest retention times (e.g. putrescine, spermidine, ornithine, arginine, s-adenosyl-L-methionine, and thiamine). The potential consequences of co-elution are so-called matrix effects, or the suppression (or enhancement) of the instrumental response due to interference from matrix constituents (Niessen et al., 2006). Co-elution is a particular issue for ESI due to its competitive nature; more abundant and/or strongly ionizing compounds may suppress the formation of species with less abundance or lower ionization efficiency.

One way to improve chromatographic resolution, and thereby potentially reduce matrix effects, is to reduce peak width with sub-2 μ m particles in the stationary phase and ultrahigh performance liquid chromatography (UPLC). The UPLC approach has gained popularity in metabolomics studies as it can improve metabolite separation as well as lead to more sensitive and faster analyses (Buescher et al., 2010; Dunn et al., 2008; Evans et al., 2009; Want et al., 2010; Zelena et al., 2009). Narrow peak widths, however, require mass spectrometers capable of collecting a sufficient number of data points across a chromatographic peak. The triple quadrupole MS used for targeted experiments has the requisite acquisition speed for UPLC measurements, but the FT-ICR MS does not. Other MS platforms with fast enough acquisition speed for coupling with UPLC include Orbitrap or time-of-flight (TOF) mass analyzers.

3.5 Untargeted LC -MS Metabolomics Method

Mass spectrometers with high resolution and accurate mass are desirable for use in untargeted metabolic profiling studies. These attributes facilitate both detection of co-eluting species in a complex matrix (possibly having the same nominal mass) and identification of unknown compounds. The most popular mass analyzers for this task include TOF and Q-TOFs, Orbitraps, and FT-ICR. We use a hybrid instrument (LTQ-FT), which combines a low resolution linear ion trap on the front end with a FT-ICR MS on the back end. This design affords data-dependent MS/MS acquisition in the ion trap

of the most abundant ions present in the high resolution full scan. The combination of accurate parent mass and nominal mass fragmentation data is advantageous for downstream data analysis and metabolite annotation using informatics tools.

For our untargeted metabolomics experiments we typically collect data in both positive and negative ionization modes. This requires separate sample injections and produces two data files per sample. Each data set may contain thousands of features. For example, in an untargeted analysis of intracellular and extracellular metabolites retained or exuded by *T. pseudonana*, we detected > 4,000 features in negative ion mode and > 9,000 features in positive mode after subtraction of the controls (Longnecker et al., 2015). Nonetheless, we find analyses in both ion modes valuable for profiling as much of the metabolome as possible, given the ionization preference of most compounds for one polarity or the other.

Figure 3 shows an example of LC/FT-MS results from *T. pseudonana* intracellular metabolite extracts analyzed by our untargeted metabolomics method (Longnecker and Kujawinski, unpublished). With our method, we are able to collect high-resolution and high mass accuracy MS data for parent ions in parallel to MS/MS fragmentation spectra at unit mass resolution. Both types of data are used in the downstream data analysis for metabolite putative identification. We show an example of the MS/MS spectrum (Figure 3C) corresponding to the feature indicated with the orange arrow in Figure 3A with m/z

555.2846 and a retention time of 31.74 minutes. In this case, we used MetFrag (Wolf et al., 2010) to compare the measured MS/MS fragments with fragments calculated *in silico* for compounds with mass matches to the parent ion found in public databases (e.g. KEGG, PubChem, ChemSpider). The structures of potential fragment matches are shown in Figure 3D along with the feature putatively identified as a sulfoquinovosyl monoacylglycerol with a C16:0 fatty acid tail (Figure 3E).

Other data processing steps include peak detection and peak alignment, the latter of which is necessary to account for any retention time drift over the course of an experiment. (For data analysis details see Longnecker et al. (in press) in this issue.) Once processed, the LC/FT-MS dataset is a list of features. Each experimental data set may contain thousands of features, though some do not correspond to metabolites of interest but instead represent matrix or other background components and multiple ion adducts. Here, experimental control samples such as method blanks are useful for narrowing the list of features. More features are often observed in positive than negative ion mode, likely due to greater variety of adducts generally formed by electrospray ionization in positive mode (Huang et al., 1999). We examined the positive-ion mode adducts formed in our untargeted method for 11 metabolite standards, shown in Figure S4. All of the standards form the $[M+H]^+$ adduct, and some metabolites are also measured as other adducts, notably sodium.

Given the marine origin of our samples, it is reasonable to expect significant sodium adduct formation in positive ion mode. In Figure 4, we compare the abundance of non-deuterated biotin (extracted ion chromatogram peak area) detected as $[M+H]^+$ and $[M+Na]^+$ in three sample sets: two laboratory experiments (Lab experiment C and D, see Table S1), and one set of water samples from the Atlantic Ocean (Field Samples B, see Table S1). Each point within the figure is data from a single sample, and multiple samples are shown from each sample set. With the exception of samples where biotin levels are low, the peak areas for biotin observed as $[M+H]^+$ are greater than the corresponding measurement of biotin as $[M+Na]^+$, that is, the points fall above the one-to-one line. Therefore, despite the salt in our samples, sodium adduct formation does not overwhelm adducts formed with hydrogen, likely due to our LC conditions which include the addition of formic acid to the mobile phase. The presence of both sodium and hydrogen adducts can help in the feature annotation process, and we use freely available informatics software to help define features with multiple adducts (e.g. CAMERA, Kuhl et al., 2012).

3.6 Targeted LC-MS Metabolomics Method

In contrast to global profiling with untargeted metabolomics, the goal of targeted metabolomics experiments is to quantify a well-defined set of known compounds. The most widely used instrument for quantification in mass spectrometry is the triple

634 quadrupole MS, which affords sensitive and specific measurements when operated in
635 selected reaction monitoring (SRM) mode. In SRM mode, fragment ion(s) from a
636 particular parent ion is identified, and this parent-product pair of m/z values (called an
637 SRM transition) is monitored throughout the LC run. The mass spectrometer scans
638 through a list of SRM transitions and detects the product ion, generating an ion-specific
639 chromatogram for each compound. Peak areas in these chromatograms are related to
640 metabolite abundance and used for quantitative analysis across a sample set.

641 LC-MS can be used for either relative or absolute metabolite quantification. Absolute
642 quantification requires the use of internal standards in order to account for variations of
643 instrument response (from matrix effects, drifts over time) and for analyte losses during
644 extraction and sample processing (Boyd et al., 2008). The ideal approach uses an
645 isotopically labeled internal standard corresponding to each target metabolite.
646 However, the commercial availability and cost of such labeled standards is prohibitive
647 for a method such as ours that seeks to quantify a large number of metabolites. We
648 therefore use relative quantification in our targeted method, in which relative ion
649 intensities are used to assess changes in metabolite concentrations across samples
650 within an experiment (Crutchfield et al., 2010). A limited set of internal standards are
651 used to monitor performance of the analytical method only; isotopically-labeled
652 metabolites are added during the filter extraction of intracellular material and into each
653 sample prior to LC analysis (injection standard).

One consideration in implementing an SRM mass spectrometry method is the need to obtain purified standards for each metabolite of interest. These standards are used in order to determine the fragments produced by a given compound (as this is not typically known a priori) and to optimize collision energies and other instrument parameters for producing a selected product ion. Many desired standards are not commercially available and/or are costly, particularly in environmental metabolomics where non-human metabolic pathways are of interest. Table 1 shows the list of 92 compounds monitored in our targeted method, compiled largely based on their importance to central carbon and nitrogen pathways of marine microbes. This list includes a variety of metabolite classes: nitrogenous bases, nucleosides/nucleotides, phosphorylated sugars and amines, vitamins and derivatives, carbohydrate derivatives, carboxylic acids, osmolytes and derivatives, amino acids, peptides and amino acid derivatives, and intermediates in 3-C metabolism and lipid synthesis.

Structural isomers abound in nature and exist for some of the metabolites in Table 1. Such isomers are often indistinguishable by MS alone due to their same exact mass and similar fragmentation patterns (Xu et al., 2015). Adding an orthogonal step, such as chromatographic separation, may discriminate between isomers provided that they can be resolved. For example, two isomers of adenosine monophosphate, 5'-AMP and 3'-AMP, analyzed by our targeted method produce the same fragment ions but elute at different retention times. By contrast, we cannot separate isomeric pairs such as

674 leucine/isoleucine and threonine/homoserine by our current methods, and therefore
675 these isomers cannot be quantified individually. Table S2 lists isomers that may be
676 detected by our targeted method. We have not yet confirmed chromatographic
677 retention times for all compounds in this list with authentic standards to enable specific
678 quantification.

679 Included in Table 1 for each metabolite are instrumental limits of detection
680 (estimated for a signal-to-noise ratio of 3) and correlation coefficients (R^2) of the
681 calibration curves determined by linear regression. Note that these values apply only to
682 the aqueous metabolite standard mix (> 95% Milli-Q) and may differ in the presence of a
683 complex chemical matrix such as seawater. Matrix effects may alter the instrument
684 response through ion suppression or enhancement of a given metabolite. As a result, we
685 would expect to see corresponding changes to calibration curve slopes and limits of
686 detection. To consider variability in standards, we calculate a Relative Standard
687 Deviation (RSD) for peaks found in three replicate injections. The RSD is defined as the
688 standard deviation in peak height divided by the mean peak height. Figure 5 gives the
689 %RSD for the metabolite standard mix injected several times at the beginning and end
690 of a sample set, and for a pooled sample injected at multiple points throughout an
691 analytical run. The values in Figure 5 represent the range of %RSD for the metabolites
692 detected in each type of sample. The median %RSD for the metabolite standard mix is
693 28.3% and for the pooled sample is 23.1%. As with the variability assessment for the

untargeted method shown in Figure 1, these results emphasize the need for replicate samples to enable statistical evaluation of changes in the level of a given metabolite.

Several of the metabolite standard stock solutions are made up in 50:50 methanol:water. As a result, the standard solutions used for the calibration curve contain a small percentage (< 5%) of methanol following dilution of the stock solutions with water. By contrast, the metabolite extracts from laboratory or field samples are dried down and brought up in 95:5 water:acetonitrile. We tested the effect of the organic component identity in the injection solvent by drying down an aliquot of the full metabolite standard mix and reconstituting in a 95:5 water:acetonitrile solution. We did not notice a difference in retention time or peak shape or quality for the majority of the standards, likely because of the similarly high aqueous nature of the diluted standard solutions in the presence of acetonitrile or methanol. Interestingly, for three of the metabolite standards (NAD, 5'-AMP, and s-adenosyl-L-homocysteine), there was a pronounced effect on peak shape. With methanol in the injection solvent, the SRM chromatogram contained two peaks at closely spaced retention times, while with acetonitrile the peaks coalesced to a single peak with retention time intermediate to that of the two peaks. For improved peak quality for these metabolites, we recommend matching the organic composition of the standard solutions to that of the mobile phase starting conditions (here, 5% acetonitrile).

The finite scan rate of the triple quadrupole MS is another issue to consider, as it limits the number of compounds that can be measured well in a single LC run. When scanning over a large number of SRM events, sensitivity decreases when the scan time for any individual SRM transition is too short. To mitigate this effect, we schedule SRM transitions in the method such that they are scanned only when a given compound is eluting. The actual number of SRM events in the method extends beyond the 92 standards in Table 1 because we may monitor a particular analyte in positive and negative ion modes. In addition, we monitor two SRM transitions per parent ion. One transition is used for quantification, chosen to maximize signal intensity and minimize matrix interference; the second transition is used for metabolite confirmation. This strategy adds specificity, which is particularly valuable in a complex organic matrix, and reduces the potential for false positive identification. Figure S5 shows an example of the MS/MS spectrum of non-deuterated biotin obtained by directly infusing the standard into the TSQ-MS for optimization of SRM parameters. The two fragment ions chosen to monitor for quantification and confirmation are highlighted with green and orange circles, respectively.

We have used open-source software such as MAVEN (Clasquin et al., 2002; Melamud et al., 2010) as well as the vendor-specific XCalibur, to process the targeted metabolomics data, both of which contain tools for peak detection and quantification. In either program, peak areas are calculated from extracted ion chromatograms (EIC), and

this measure of instrument response is related to compound concentration to generate a calibration curve for each analyte. Representative LC/TSQ-MS results (EICs and standard curves) for compounds from the metabolite standard mix are shown in Figure 6. One important requirement of these initial data analysis steps is an assessment of peak quality. MAVEN utilizes a machine-learning approach to provide a peak quality score. Those with low scores warrant a manual review of the raw data. XCalibur does not, however, provide such data quality estimates and so requires manual curation of standard curves based on user-defined figures of merit (e.g. EIC peak quality, degree and range of linear response). Quality-checked calibration curves for each analyte are used to determine metabolite concentrations. Figure 7 shows an example of targeted metabolomics data for several early-eluting compounds (retention time < 2 minutes) measured in intracellular extracts from a heterotrophic bacterium grown in seawater (Lab experiment E, Table S1). For comparison, we show the peaks from the standard mix. We use concentration values to assess relative changes (i.e. fold changes) in metabolite abundance across the sample set.

As previously discussed, we expect to observe matrix effects at early LC retention times when many metabolite species co-elute. We also anticipate similar matrix effects arising from interference with the chemically complex background of dissolved organic matter in seawater. Figure S6 illustrates the influence of these matrix effects on the ionization efficiency of deuterated biotin. Untargeted metabolomics results from

intracellular and extracellular extracts from Atlantic Ocean deep water are shown in Figure S6A and S6C, respectively. DOM, which is extracted from the sample filtrate along with extracellular metabolites of interest, appears as a broad, unresolved feature centered at ~10 min in Figure S6C. We find that high levels of DOM in the extracellular extract can have a detrimental effect on the ionization of the d2-biotin standard, which co-elutes with the organic matter background. The standard appears as a peak at 9.17 min in the intracellular extract (Figure S6B), but in this case is entirely absent from the extracellular extract (Figure S6D). Matrix effects are a potential concern when calibration solutions made in solvent are applied to the quantitation of analytes in a seawater or other complex matrix (Griffith et al., 2014). As noted above, we have investigated the significance of matrix effects in our targeted metabolomics method but full discussion of these results are beyond the scope of this paper (Johnson, Kido Soule, & Kujawinski, unpublished).

4. SUMMARY

Marine microbial samples pose a particular challenge for metabolomics analyses due to the chemical complexity of the environment and high levels of salt relative to organic matter. We have developed metabolomics methods for the analysis of low molecular weight metabolites from marine microbes. These methods include sampling and

extraction protocols for intracellular and extracellular metabolites, liquid chromatographic conditions that balance metabolite coverage and sample matrix issues, and mass spectrometry platforms for profiling (untargeted metabolomics) and relative quantification (targeted metabolomics) of the metabolome. Data from the untargeted method can provide opportunities for the exploration of microbial metabolic pathways and the discovery of new compounds. Results from the targeted method may provide important quantitative limits on metabolic reactions in the ocean. Going forward, we plan to incorporate alternate metabolite separation strategies such as UPLC, where superior chromatographic resolution may lead to enhanced metabolome coverage and better quantification. We hope that the analytical methods presented here, along with continuing advances in the field of environmental metabolomics, can provide new insights into microbe-DOM interactions.

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Figure Captions

Figure 1. Boxplots presenting the percent of peaks shared across three replicates analyzed with the untargeted method as a measure of technical and biological variability. Table S1 provides the details on Lab experiment B and Field samples A. For the metabolite standard mix ('standard mix'), the three replicates are replicate injections of the same standard mix at different times during the LC run (technical variability). Field samples A includes three samples processed per day on five different sampling days (biological variability). Lab experiment B includes individual samples from three replicate growth flasks at multiple times along a growth curve (biological variability).

Figure 2. Data from extraction protocols tested using cultures of *Ruegeria pomeroyi*, one method described by Rabinowitz and Kimball (2007) and a second method described by Winder et al. (2008). The data are from Lab experiment A described in Table S1. The extracts were processed immediately following the onset of the experiments at day 0, with subsequent extractions at days 2, 3, and 5. Note that no samples were processed for day zero with the Winder et al. protocol. The y-axis is the percent increase in the features in the treatments with *R. pomeroyi* compared to the features observed in the cell-free controls.

Figure 3. Overview of putative identification scheme. Representative LC/FT-MS chromatograms from the analysis of *T. pseudonana* intracellular metabolite extracts by untargeted metabolomics for (A) a sample with cells, and (B) a cell-free control. (C) is

the MS/MS spectrum collected for the peak indicated with an arrow in (A). (D) shows potential matches between the measured MS/MS fragments and *in silico* fragments calculated in MetFrag (Wolf et al., 2010). The compound was putatively identified as a sulfoquinovosyl monoacylglycerol with a C16:0 fatty acid (Longnecker and Kujawinski, unpublished).

Figure 4. EIC peak areas for non-deuterated biotin detected as $[M+H]^+$ and $[M+Na]^+$ in three sample sets: two laboratory experiments (Lab experiment C (diamonds) and D (squares), see Table S1) and one set of water samples from the Atlantic Ocean (Field samples B (stars), see Table S1). The data are plotted on a log scale due to the large range of EIC peak areas and a 1:1 line is provided.

Figure 5. Peak area reproducibility for the targeted method, %RSD, calculated from replicate injections of the metabolite standard mix ($n = 90$) and of a pooled sample ($n = 49$).

Figure 6. Representative LC/TSQ-MS results for selected compounds from the metabolite standard mix analyzed with the targeted metabolomics method. Extracted ion chromatograms (EIC) and calibration curves used for quantification are shown for: betaine, s-adenosyl-L-homocysteine, phenylalanine, 4-hydroxybenzoic acid, and riboflavin. Insets provide metabolite structures.

Figure 7. Early eluting intracellular metabolites (retention time < 2 minutes) from a standard mix (top row), and from an intracellular extract of a heterotrophic marine

1043 bacterium (Samples 1 and 2, in rows 2 and 3, respectively) and analyzed with the
1044 targeted metabolomics method (Lab experiment E, Table S1). The normalization levels
1045 (NL) for each peak represent the instrument response of that metabolite in pure solvent
1046 (Row 1) or in a sample matrix (Rows 2 and 3).

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Table 1. Compounds measured by the targeted LC-MS method in order of retention time^a.

Metabolite	RT (min)	Parent (m/z)	Product ^b (m/z)	Polarity	S-lens (V)	CE ^c (V)	Correlation coefficient (R ²)	LOD ^d (ng ml ⁻¹)
putrescine	1.20	89	72/30	+	30	5/24	0.9912	100
spermidine	1.20	146	72/112	+	46	14/12	0.9415	100
ornithine	1.28	133	70/116	+	41	15/5	0.9981	10
s-adenosyl-L-methionine	1.37	399	250/136	+	94	14/29	0.9823	5
thiamine	1.37	265	122/144	+	61	14/13	0.9795	10
arginine	1.38	175	70/130	+	63	21/12	0.9971	5
cytosine	1.45	112	95/40	+	54	14/47	0.9979	5
D-glucosamine 6-phosphate	1.49	258	79/97	-	65	41/19	0.9944	1
thiamine monophosphate	1.49	345	122/126	+	84	17/28	0.9859	5
serine	1.52	106	60/42	+	36	10/21	0.9868	5
GABA (γ -aminobutyric acid)	1.53	104	45/87	+	40	26/11	0.9979	5
glutamic acid	1.54	146	128/102	-	46	12/15	0.9988	5
choline	1.55	104	60/58	+	70	15/31	0.9851	1
2-aminoethylphosphonic acid	1.55	126	109/81	+	58	14/21	0.9884	5
glutamine	1.55	147	84/56	+	43	16/29	0.9931	5
threonine	1.55	120	74/56	+	45	10/15	0.9940	10
aspartic acid	1.59	134	74/70	+	43	13/14	0.9825	10
citrulline	1.59	176	70/159	+	53	20/7	0.9944	5
dimethylsulfoniopropionate (DMSP)	1.59	135	63/45	+	54	13/24	0.9954	1
sarcosine	1.59	90	44/30	+	34	11/59	0.9894	100
muramic acid	1.60	250	89/43	-	49	14/37	0.9890	10
taurine	1.62	124	80/107	-	62	22/15	0.9991	1
cysteine	1.64	122	59/76	+	35	20/14	0.9906	25
adenine	1.70	136	65/94	+	85	37/20	0.9819	1
ectoine	1.70	143	68/97	+	57	25/16	0.9940	1
chitobiose	1.72	425	126/84	+	67	23/36	0.9729	5
n-acetyl glucosamine	1.72	222	138/96	+	39	15/28	0.9955	25
proline	1.72	116	70/68	+	44	14/29	0.9955	5
chitotriose	1.76	628	204/138	+	113	20/36	0.9913	5
pyridoxine	1.76	170	134/152	+	54	20/12	0.9912	0.5
betaine	1.81	118	58/42	+	59	24/51	0.9949	10
guanine	1.81	152	135/110	+	82	18/20	0.9964	5
glyphosate	1.82	168	79/150	-	57	44/12	0.9987	10
5'-AMP	1.89	348	136/119	+	94	19/51	0.9990	1
glutathione reduced	1.89	308	76/84	+	75	23/33	0.9990	25
methionine	1.90	150	56/61	+	47	17/22	0.9997	5

glucose 6-phosphate	1.91	259	97/79	-	75	17/49	0.9942	1
malic acid	1.91	133	115/71	-	42	12/15	0.9762	10
D-ribose 5-phosphate	1.93	229	97/79	-	58	15/43	0.9898	1
6-phosphogluconic acid	1.95	275	79/97	-	66	44/17	0.9964	10
dihydroxyacetone phosphate	1.95	169	79/97	-	34	41/12	0.9964	5
sn-glycerol 3-phosphate	1.95	171	79/97	-	53	41/18	0.9986	10
2,3-dihydroxypropane-1-sulfonate	1.99	155	80/95	-	51	33/18	0.9976	1
D(-)3-phosphoglyceric acid	2.01	185	79/97	-	51	40/16	0.9968	10
1-deoxy-D-xylulose-5-phosphate	2.04	213	79/97	-	49	43/13	0.9939	1
phosphoenolpyruvate	2.08	167	79/63	-	41	36/72	0.9852	25
phosphomycin	2.08	137	79/63	-	52	40/16	0.9995	1
s-(5'-adenosyl)-L-homocysteine	2.30	385	136/88	+	87	18/36	0.9993	10
citric acid	2.31	191	111/87	-	47	13/17	0.9653	10
glutathione oxidized	2.31	613	355/231	+	126	20/33	0.9753	25
isoleucine	2.31	132	69/56	+	46	16/32	0.9811	25
leucine	2.31	132	41/39	+	42	30/39	0.9939	5
NAD	2.31	664	136/428	+	113	40/25	0.9884	5
s-(1,2-dicarboxyethyl)glutathione	2.31	424	259/214	+	94	19/26	0.9944	5
uracil	2.31	113	96/70	+	57	15/15	0.9799	25
uridine 5'-monophosphate	2.31	323	79/96	-	100	43/22	0.9930	1
NADP	2.32	742	620/407	-	107	17/32	0.9955	5
acetyltaurine	2.34	166	80/107	-	57	35/18	0.9994	0.5
α -ketoglutaric acid	2.34	145	101/56	-	37	8/12	0.9883	50
tyrosine	2.35	182	91/136	+	52	29/13	0.9864	1
adenosine	2.38	268	136/119	+	74	17/43	0.9993	0.5
orotic acid	2.39	155	111/42	-	36	12/22	0.9985	10
(6R)-5,6,7,8-tetrahydrobiopterin	2.40	240	196/165	+	80	13/21	0.9988	5
inosine 5'-monophosphate	2.42	349	137/119	+	72	14/46	0.9986	5
succinic acid	2.52	117	73/99	-	42	13/12	0.9955	25
n-acetyl muramic acid	2.53	294	138/96	+	51	19/36	0.9985	5
xanthine	2.60	153	136/110	+	60	14/16	0.9969	5
fumaric acid	2.75	115	71/27	-	36	6/12	0.9826	25
inosine	2.83	267	135/108	-	98	23/42	0.9994	5
guanosine	2.84	284	152/135	+	58	14/37	0.9997	0.5
phenylalanine	3.42	166	77/103	+	56	35/27	1.0000	1
kynurenine	3.55	209	146/91	+	51	20/37	0.9999	0.5
xanthosine	3.58	285	153/136	+	50	11/31	0.9993	0.5
thymidine	4.15	241	42/138	-	76	19/15	0.9987	10

pantothenic acid	4.37	220	184/202	+	58	11/10	0.9998	5
4-aminobenzoic acid	5.84	138	77/65	+	71	19/25	1.0000	0.5
tryptamine	6.03	144	117/77	+	97	21/36	0.9991	0.5
tryptophan	6.13	205	146/118	+	51	16/25	1.0000	5
5'-deoxy- 5'(methylthio)adenosine	6.44	298	136/119	+	77	16/46	1.0000	0.5
4-hydroxybenzoic acid	8.07	137	93/65	-	48	17/29	0.9997	10
folic acid	8.19	442	295/176	+	75	14/36	0.9942	0.5
caffeine	8.57	195	138/110	+	91	18/22	1.0000	1
2,3-dihydroxybenzoic acid	8.92	153	109/108	-	59	17/25	0.9925	5
biotin	9.02	245	227/97	+	63	14/32	0.9999	0.5
riboflavin	9.15	377	243/172	+	112	23/34	0.9992	0.5
cyanocobalamin	9.30	678	147/359	+	137	34/20	0.9996	0.5
syringic acid	9.31	197	182/123	-	67	15/25	0.9963	25
desthiobiotin	10.11	215	197/179	+	63	12/17	0.9995	0.5
3-mercapto propionate	10.13	209	59/65	-	41	20/46	0.9959	5
indole 3-acetic acid	12.11	176	130/77	+	56	16/39	0.9964	0.5
sodium taurocholate	17.35	514	124/107	-	131	56/56	0.9977	0.5

^a Results shown for retention time (RT), correlation coefficient (R²), and limit of detection (LOD) apply to metabolite standard mixtures in solvent.

^b Two product ions = quantification/confirmation.

^c Optimized collision energies (CE) for quantification/confirmation ions.

^d LOD is defined as the lowest concentration at which the signal-to-noise is at least 3.

1076 **Table 2.** Percentage of deuterated standards recovered during filter extraction of intracellular
1077 metabolites. The mean and one standard deviation of triplicate analyses are reported.

Deuterated Standard	% Retained
D3-glutamic acid	103 ± 13.0
D4-4-hydroxybenzoic acid	93 ± 1.4
D5-taurocholic acid	93 ± 1.0

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