Marine metabolomics: Measurement of metabolites in seawater by gas-chromatography mass spectrometry

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Abstract

All life exchanges molecules with its environment. While these metabolites are commonly measured in terrestrial and limnic ecosystems, the presence of salt in marine habitats has hampered quantitative analyses of the ocean metabolome. To overcome these limitations, we developed SeaMet, a gas chromatography-mass spectrometry (GC-MS) method that detects hundreds of metabolites down to nano-molar concentrations in less than one milliliter of seawater. Using a set of metabolites dissolved in artificial seawater to benchmark our method, we show metabolite signal detection increased on average across ions by 324 fold in comparison to standard GC-MS methods. Our observed signal improvement occurred across tested metabolite classes and provides reproducible and quantifiable results. To showcase the capabilities of our method, we used SeaMet to explore the production and consumption of metabolites during culture of a heterotrophic bacteria that is widespread in the North Sea. Our

approach revealed successional uptake of amino acids, while sugars were not consumed, and highlight the power of metabolomics in providing insights into nutrient uptake and energy conservation in marine microorganisms. We also applied SeaMet to explore the in situ metabolome of coral reef and mangrove sediment porewaters. Despite the fact that these ecosystems occur in nutrient-poor waters, we uncovered a remarkable diversity and abundance of sugars and fatty acids, compounds predicted to be rapidly consumed by marine microorganisms. Our method advances marine metabolomics by enabling the unbiased, and quantitative analysis of marine metabolites, and will help provide new insights into carbon cycle dynamics and ocean biogeochemistry.

Gas chromatography-mass spectrometry (GC-MS) analysis enables the detection of primary metabolites, small molecules that occur in central metabolic pathways across biological systems **(1,2)**. High reproducibility coupled to the widespread availability of annotation resources make GC-MS the "workhorse" of analytical chemistry facilities. GC-MS has allowed the identification of metabolites associated with human disease **(3)**, detection of compounds that serve as environmental cues in foraging **(4)**, and description of metabolic fluxes within and between cells **(5)**. GC-MS is also widely used for environmental profiling of soils and microbial activity on land **(6,7)**.

In contrast to terrestrial or limnic systems, the defining characteristic of marine habitats - high salt concentration - inhibits MS-based metabolomic analysis. Consequently, our knowledge of metabolite composition of the oceans is severely limited. This, in turn, restricts our understanding of global biogeochemical cycles, as the oceans contain the largest organic carbon pool on Earth **(8)**. While metagenomic and metatranscriptomic studies of the ocean, driven by low sequencing costs and projects like Tara Oceans **(9)**, have deepened our knowledge of the identity and activity of marine microbes, current metabolomic techniques do not permit equivalent surveys of the ocean metabolome.

The ocean metabolome remains largely undefined, despite a growing field of research exploring the molecular composition of dissolved organic matter **(8, 10-12)**. Current efforts rely on solid phase extraction (SPE) methods to remove salt prior to MS analyses **(10,11)**. SPE's successful removal of salt from marine samples is accopanied by co-removal of small polar compounds, which are the primary components of the liable organic matter pool **(13)**. Consequently, while SPE-based studies can measure up to 50% of the organic carbon from the ocean, they fail to detect the majority of compounds involved in the central metabolism of cells. Furthermore, current DOM analytical approaches remain largely inaccessible for the majority of research institutions and projects. This is largely due to high instrumentation costs, large sample volume requirements, and the long amount of time needed for metabolome analysis. To more efficiently decipher ocean metabolism, it is necessary to develop cost-effective, high-throughput, and untargeted workflows that can readily identify and quantify molecules

from high salinity environments. Here, we present SeaMet, a marine metabolomics method that uses sample derivatization to enable metabolite detection in seawater using GC-MS.

Results and Discussion

SeaMet modifies the well-established two-step metabolite derivatization procedure, which permits the detection of non-volatile compounds using GC-MS, and involves methoximation followed by trimethylsilylation **(14)**. Like other GC-MS sample preparation techniques **(15,16)**, SeaMet removes liquid through vacuum drying prior to derivatization - a process that results in a salt pellet when working with marine samples, which restricts MS analysis. Our initial results suggested that water locked within the dried salt crystals hindered the chemical reactions needed for GC-MS (**Figure S1A, B**). Our method overcomes this limitation by first eliminating residual water within the salt crystals and then extracting small compounds into the derivatization reagents (**Figure 1A**).

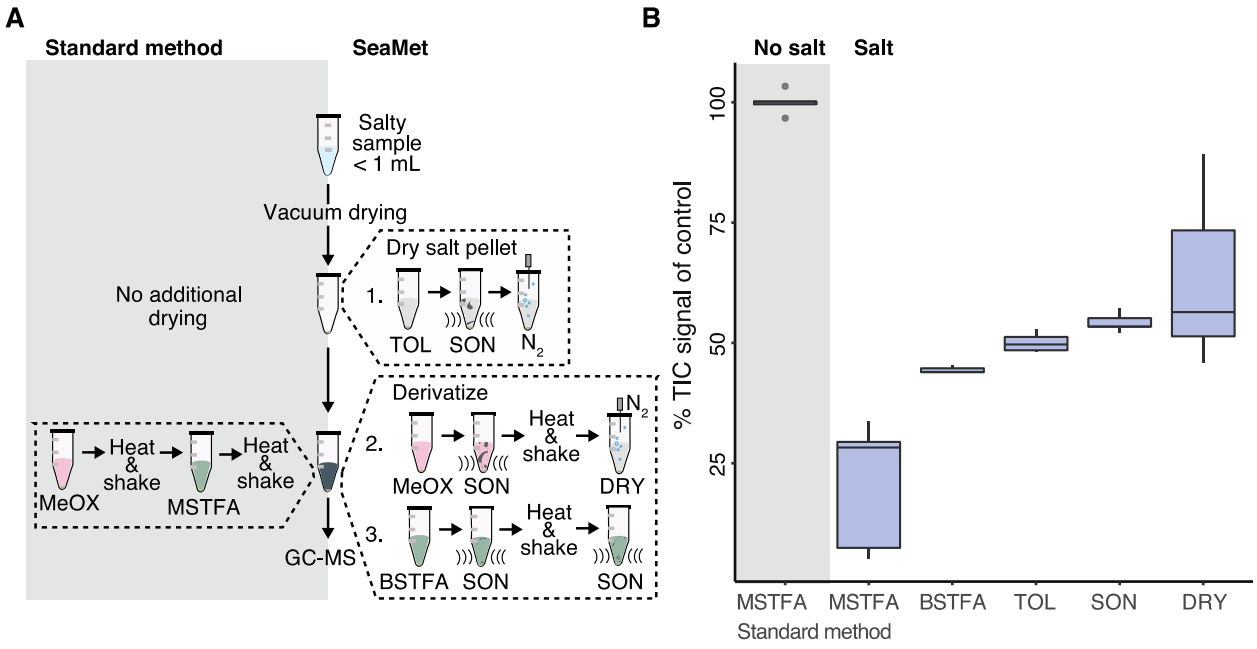

Figure 1. How SeaMet works. A, Modifications to the standard two-step methoximation (MeOX)-trimethylsilylation (TMS) derivatization protocol include key steps that enhance metabolite detection in seawater as shown in **b.** Steps modified from the standard method include a switch in derivatization reagents from MSTFA to BSTFA, further drying of the salt pellet using toluene (TOL) to remove water azeotropically, ultrasonication (SON) after the addition of TOL, MeOX, BSTFA, and after the BSTFA reaction, and drying (DRY) of the pyridine after

the MeOX reaction prior to BSTFA addition. **B,** Boxplots showing the total detected signals from each chromatogram after GC-MS from a synthetic mixture of 45 metabolites (**Table S1**) dissolved in 0.5 mL of seawater (n = 5) relative to the no salt control average.

A promising GC-MS method to advance marine metabolomics research

We used a synthetic mixture of 45 metabolites (**Table S1**) dissolved in artificial seawater to document the performance in metabolite detection of our method. Overall, SeaMet increased total signal intensity on average by 42% and up to 89% for high salinity samples in comparison to the standard GC-MS sample preparation (**Figure 1B**). We first replaced the most commonly used trimethylsilylation reagent, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)**(16)**, with one that is less susceptible to inhibition by water, N, O-Bistrifluoroacetamide (BSTFA), which resulted in higher metabolite signals (**Figure S1B**). To eliminate water from the samples, we increased the speed-vacuum drying time from four to eight hours, and integrated a toluene drying step that is used in urine-based metabolomic analyses **(15)**. We further enhanced metabolite signals by treating the salt pellet to a combination of ultrasonication and vortexing after the addition of toluene and both derivatization reagents, and following completion of the trimethylsilylation reaction. These steps break apart the salt crystals and release water into the toluene to enhance salt drying and metabolite extraction. Finally, following a recently described method for improving metabolite detection regardless of sample type **(17)**, we dried the samples between the methoximation and trimethylsilylation derivatization reactions under N_2 gas (see **Figure 1B** for signal improvements of each step).

Overall, SeaMet allowed us to detect significant increases in metabolite abundances across molecular classes when compared to the standard method (adjusted p-value < 0.05; mean fold change across all ions = 323; **Figure 2A**). This included measurement of organic acids, amino acids, and fatty acids, as well as sugars (and their stereoisomers), sugar alcohols, and sterols (**Table S1**). Our detection limits in the nano-molar range are comparable to those of targeted techniques for marine ecosystems that were developed to quantify single compounds from specific molecular classes (**Tables S2 and S3**). Moreover, our workflow only requires 0.5 mL to 1 mL of seawater for metabolite detection, at least an

order of magnitude less sample volumes than previously published techniques **(13,18)**. To date, we have successfully measured 107 metabolite standards dissolved in artificial seawater, representing major metabolite groups involved in primary metabolic pathways (**Table S4**), highlighting that SeaMet can be used for both targeted and untargeted applications. Our method provides reproducible quantification and gives similar linearity, dynamic range, and coefficient of variation values as salt-free samples prepared with the standard GC-MS derivatization procedure (**Table S2**; **Figure S2A-H**).

SeaMet can monitor cell culture physiology

To demonstrate that our method captures compounds commonly missed by SPE-based metabolomic approaches, we compared GC-MS profiles using SeaMet before and after SPE sample treatment. Our method detected small polar compounds that are co-removed with salt during SPE preparation, such as sugars, sugar alcohols, amino acids, and organic acids (**Figure 2B**). Given our results, SeaMet is complementary to current marine DOM characterization approaches that require SPE preparation and high-resolution MS for metabolite detection. Thus, SeaMet expands the possibilities for metabolite analysis in the ocean and advances marine metabolomics by recovering compounds that previously could not be analyzed by current platforms in a single analysis.

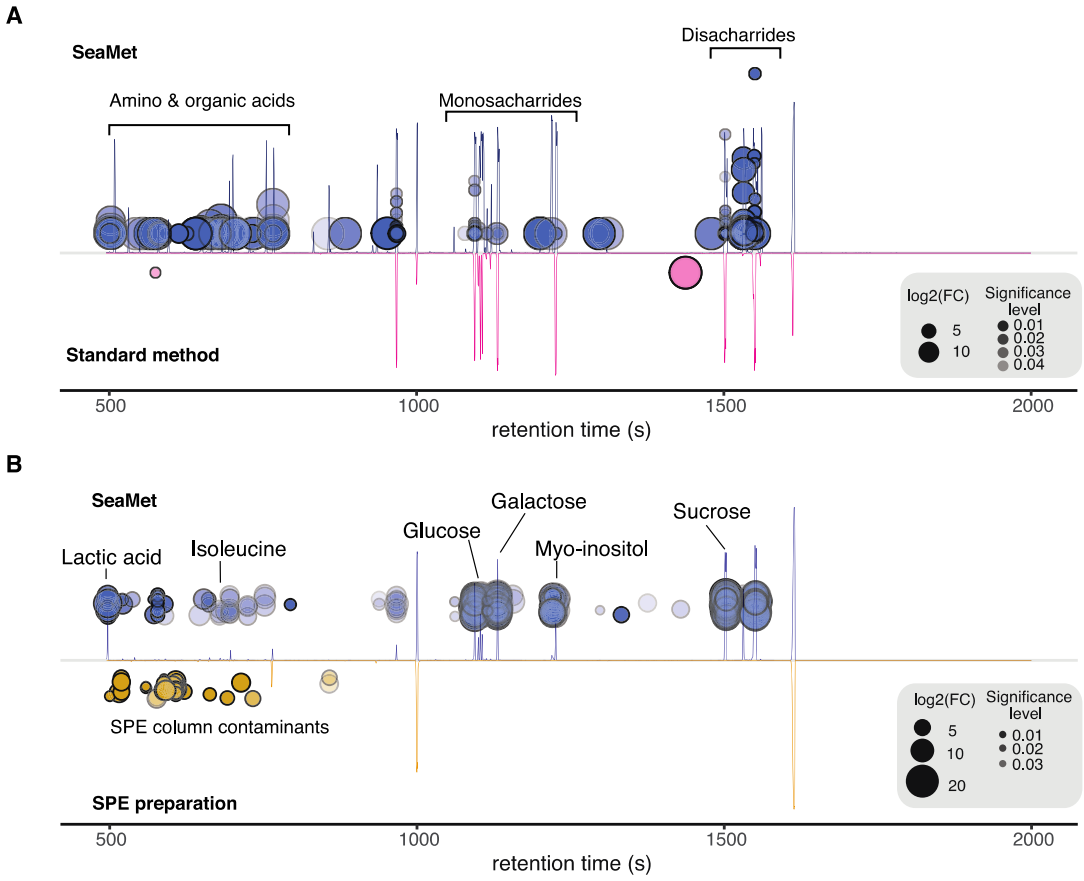

Figure 2. SeaMet enhances the detection of metabolites in marine samples. A, B Total ion chromatogram cloud plots from metabolite mixture samples indicate significant differences (B.H. $p < 0.05$) between ion abundances when comparing **A,** SeaMet (top) to the standard (bottom) protocol and **B,** chromatograms using SeaMet before (top) and after (bottom) solid phase extraction (SPE). Larger bubbles indicate higher log2(fold changes) between groups and more intense colors represent lower t-test p-values when comparing individual feature (*m/z* ions) intensities. Samples prepared with SeaMet had high abundances of organic acids (lactic acid, succinic acid, and fumarate), amino acids (isoleucine, leucine, threonine and valine), sugar alcohols (myo-inositol and mannitol), and sugars (fructose, glucose, cellobiose, maltose, ribose, galactose, and sucrose) in comparison to SPE-based sample preparation. Representatives of each class are indicated in **B**.

To demonstrate the power of our approach in characterizing metabolite profiles of marine bacteria, we monitored changes in the exo-cellular metabolome during growth of a heterotrophic Proteobacterium, *Marinobacter adhaerens*. Using SeaMet, we observed significant changes in the metabolite composition of marine culture medium during the bacteria's initial growth phase (adjusted *p*-value < 0.05; **Figure 3A; Figure S3A, B).** The bacteria took up different carbon and nitrogen resources in a cascade-like fashion**,** and later in growth, began excretion of an undescribed compound (**Figure 3A, B; Figure S4**). Successional dynamics in substrate use is a common energy conservation mechanism in

bacteria **(19)** and affects central carbon and nitrogen dynamics during growth. With SeaMet, we show that like many bacteria, *M. adhaerens* participates in the release of organic carbon, which can be metabolized by other microorganisms or will contribute to the complexity of refractory DOM. By identifying and quantifying metabolites that are consumed and excreted in cultivable marine bacteria, our method expands our understanding of key primary compounds involved in the transformation of organic matter in the ocean.

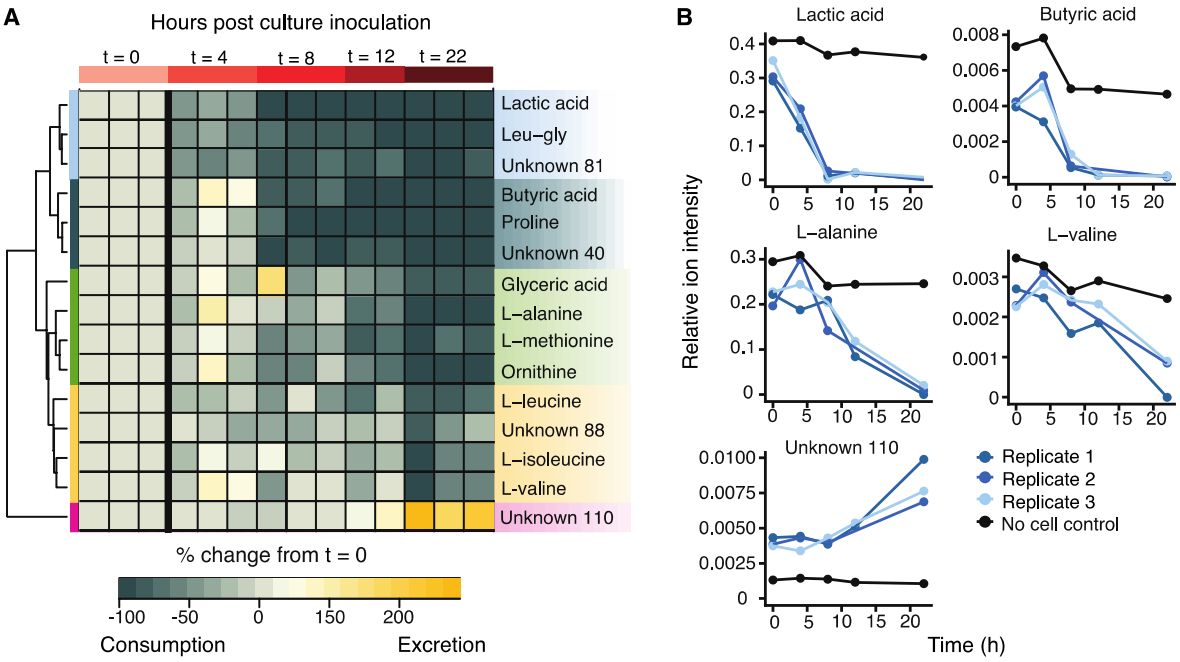

Figure 3. Metabolite consumption and excretion during culture of a marine bacterium. A, A heatmap of metabolite abundances relative to starting conditions during the culture of the heterotroph *Marinobacter adhaerens* in marine broth indicates some compounds, like the dipeptide leucine-glycine (leu-gly), and lactic acid are taken up before others, such as branch chain amino acids. After 12–22 hours of growth, the bacterial culture excretes an unknown compound (Unknown 110). Hierarchical clustering shows groups of metabolites that changed significantly during the experiment (left hand colored bars, B.H. adjusted p-value < 0.05; fold change > 2). These metabolite groups represent successive stages in *M. adhaerens* consumption and production of marine broth components. **B,** Relative ion abundances over time for select metabolites from each cluster group depicted in **A.** The blue lines represent biological replicate cultures while the black line shows results from a control sample with no cell addition. Low variation among biological replicates highlights the reproducibility of our marine metabolomics method.

SeaMet is a powerful tool for exploring ocean chemistry

To test the ability of our workflow to assess complex environmental metabolomes, we applied SeaMet to samples from coralline and mangrove sediment porewaters. Coral reefs and mangroves, two globally important coastal ecosystems, contain many biological compounds that remain undescribed. It is essential to characterize the metabolome of these habitats to understand the role of these ecosystems in biogeochemical cycling. Our approach detected 295 and 428 metabolite peaks from coralline and mangrove sediment profiles (**Figure 4 A, B**), including sugars and amino acids, organic acids, fatty acids, and background signals. Diverse and abundant sugars from coralline sediments as well as fatty acids from mangroves drove the observed significant differences between habitats (ADONIS p-value < 0.001, R^2 = 0.514; **Figure S5 A, B and Table S5**). Considering corals and mangroves thrive in oligotrophic waters and their associated sediments harbor diverse and abundant microorganisms, we were surprised to measure high abundances of energy-rich metabolites that are typically consumed in primary metabolism. These data call for a reexamination of carbon sequestration in coastal sediments using techniques that can identify and quantify the accumulation of liable metabolites.

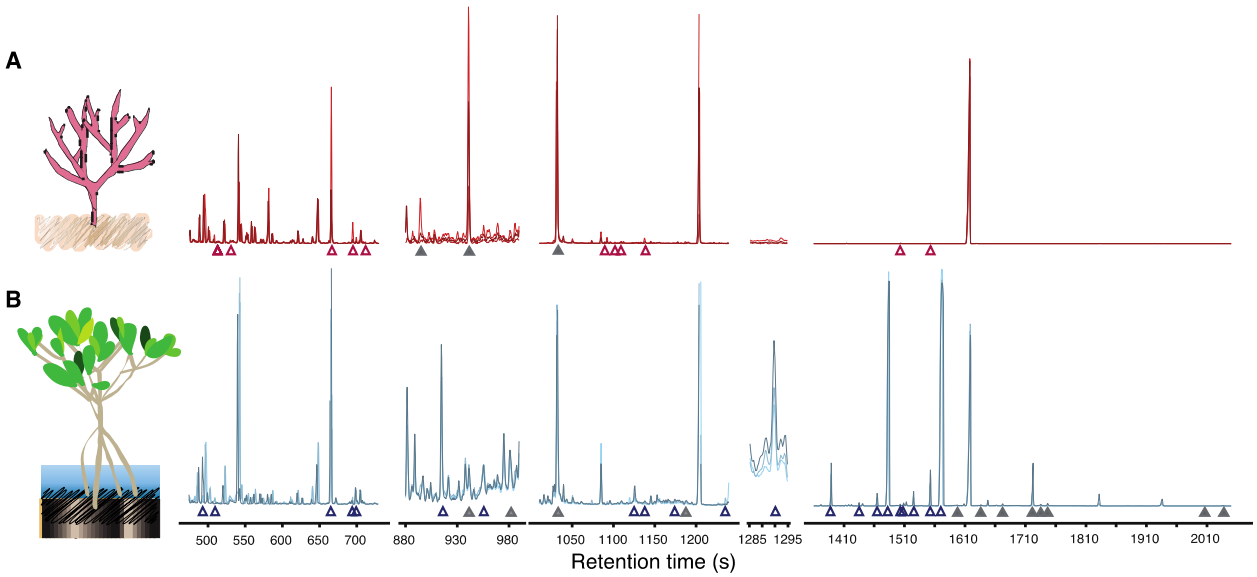

Figure 4. Metabolite profiles from marine habitats. SeaMet GC-MS metabolomic profiles from **A,** coralline and **B,** mangrove sediment porewaters show high concentrations of identified (open triangles) fatty acids and sugars that are driving multivariate differences in composition (***SI Appendix* Fig. S5A**). Profiles also reveal unknown peaks (filled triangles) for which no matches were found in public databases (**Table S5**).

Due to the technical difficulties of detecting metabolites in seawater, a large portion of ocean chemistry remains unannotated, reflecting one of the central challenges in metabolomics research **(20)**. By providing a new method to measure a broad scope of the marine metabolome, we offer an avenue to identify molecules from marine environments and expand existing mass spectrometry databases that aim to characterize chemical space across ecosystems. As an example, our samples from sediment porewaters of mangroves and coral reefs revealed 11 metabolites driving variation between habitats that did not match public database entries (**Table S5)(21,22)**.

Conclusions

We have presented SeaMet, a marine metabolomics workflow that enables the analysis of primary metabolism in the oceans. It is time efficient, allows the detection of diverse metabolite classes in a single run, and expands the analytical window for molecules that can be detected within marine samples. This advance enables untargeted metabolomics for marine ecosystems using a low-cost, easy to use GC-MS platform. Moreover, SeaMet is independent of MS instrumentation, allowing it to be combined with time-of-flight or Orbitrap MS detectors to provide faster analysis time and higher mass resolving power to improve metabolite identification. We expect our marine metabolomics workflow will enable the exploratory analysis of metabolites occurring in seawater and thereby advance our understanding of the ocean's vast and largely unexplored metabolome.

Materials and Methods

Data availability. All metabolite profile data will be made publicly available at Metabolights (https://www.ebi.ac.uk/metabolights/) under identification numbers MTBLS826, MTBLS839, MTBLS843, MTBLS844, MTBLS848, and MTBLS849 (currently IN REVIEW) or by contact with the authors. Reviewer links:

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Reagents and experimental sample preparation. The derivatization chemicals, trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from CS-Chromatographie Service and pyridine from Sigma-Aldrich at >99.98% purity. Methoxyamine hydrochloride (MeOX; Sigma-Aldrich) aliquots were further dried at 60 °C in a drying oven for 1 h to remove residual moisture. Artificial seawater (ASW) was prepared within the range of natural salinity (36‰) by dissolving (per L of water) 26.37 g sodium chloride, 6.8 g magnesium sulfate heptahydrate, 5.67 g magnesium chloride hexahydrate, 1.47 g calcium chloride, 0.6 g potassium chloride, and 0.09 g potassium bromide. Following autoclave sterilization, pH was adjusted to 7.7 using sodium hydroxide. 1 mL of the following supplements and solutions were added: 150 mM monopotassium phosphate, 500 mM ammonium chloride pH 7.5, trace element solution, selenite-tungstate solution, vitamin solution, thiamine solution, B12 solution and 0.21 g sodium bicarbonate **(23)**. Ultra-pure water (MQ) was prepared by purifying deionized water with an Astacus membraPure system (Astacus membraPure, 18.3 mΩ × cm 25 °C).

Metabolite standards were obtained from commercial sources (**Table S4**) and combined into mixtures in which each compound had a final concentration of 0.4 mM. Metabolite mixtures were prepared to (a) test the effect of salt and water on metabolite detection, (b) develop SeaMet, our marine metabolomics workflow, (c) compare metabolite detection before and after solid phase extraction (SPE) based sample preparation, and (d) to quantify the detection limits of specific compound classes (**Table S6**). Finally, multiple mixtures were prepared to document the retention times of 107 standards dissolved in ASW using SeaMet (**Table S4**). Sample aliquots for the above mentioned experiments were prepared by drying down 200 µL of the mixture in a speed vacuum concentrator (Eppendorf Concentrator Plus$^{(R)}$, 2.5 h, 45°C, V-AQ) for all experiments except SPE comparison and quantification of detection limits. For the SPE comparison experiment, 400 µL of the mix were dried down. For the quantification of metabolite classes, a serial dilution of the mix was prepared to obtain concentrations between 0.5 nmol and 80 nmol of each compound. All dried mixture samples were stored at 4 °C.

SeaMet metabolite derivatization. To prepare marine samples for gas chromatography-mass spectrometry (GC-MS) analysis, 0.5 to 1 mL of a saltwater sample or experimental mixture dissolved in ASW was dried in a speed vacuum concentrator for 8 hours (Eppendorf Concentrator Plus$^{(R)}$, 45°C, V-AQ). To further remove residual water locked within the salt pellet, 250 µL of toluene (99.8%, < 0.2 % water) was added to each sample and the mixture was ultrasonicated for 10 min at maximum intensity. The toluene was subsequently removed under a gentle flow of N_2 gas. Metabolite derivatization was performed by adding 80 µL of MeOX dissolved in pyridine (20 mg × mL^{-1}) to the dried pellet. The mixture was ultrasonicated (EMag Emmi-12HC®) for 10 min at maximum intensity, briefly vortexed to dissolve the pellet into solution, and subsequently incubated for 90 min at 37 °C using a thermal rotating incubator under constant rotation at 1350 rpm. The pyridine was removed from the sample at room temperature under a gentle flow of N_2 gas (approximately 1 hour). Following the addition of 100 µL of BSTFA, the mixture was ultrasonicated for 10 min at maximum intensity, vortexed, and incubated for 30 min at 37 °C using a thermal rotating incubator under constant rotation at 1350 rpm. The derivatized

mixture was ultrasonicated for 10 min at maximum intensity. Remaining salt in each sample was pelleted through centrifugation at 21.1 *g* for 2 min at 4 °C. 100 µL was transferred to a GC-MS vial for analysis. The full proposed method is publicly available at dx.doi.org/10.17504/protocols.io.nyxdfxn.

GC-MS data acquisition. All derivatized samples were analyzed on an Agilent 7890B GC coupled to an Agilent 5977A single quadrupole mass selective detector. Using an Agilent 7693 autosampler, 1 µL was injected in splitless mode through a GC inlet liner (ultra inert, splitless, single taper, glass wool, Agilent) onto a DB-5MS column (30 m × 0.25 mm, film thickness 0.25 µm; including 10 m DuraGuard column, Agilent). The inlet liner was changed every 50 samples to avoid damage to the GC column and associated shifts in retention times. The injector temperature was set at 290 °C. Chromatography was achieved with an initial column oven temperature set at 60 °C followed by a ramp of 20 °C min^{-1} until 325 °C, then held for 2 mins. Helium carrier gas was used at a constant flow rate of 1 mL min^{-1}. Mass spectra were acquired in electron ionization mode at 70 eV across the mass range of 50–600 m/z and a scan rate of 2 scans s^{-1}. The retention time for the method locked using standard mixture of fatty acid methyl esters (Sigma Aldrich).

Data processing and analysis. Raw Agilent data files were converted to mzXML files using Msconvert **(24)** and imported into XCMS (v. 2.99.6)**(25)** within the R software environment (v. 3.4.2) for data processing and analysis. Total ion chromatograms (TIC) were obtained using the xcmsRaw function. TICs comparing sample preparation steps were expressed as a percentage of the MQ control. For environmental and cell culture GC-MS profiles, peaks were picked using the matchedFilter algorithm in XCMS with a full width at half maximum set to 8.4, signal to noise threshold at 1, *m*/*z* width of 0.25 (step parameter), and *m*/*z* difference between overlapping peaks at 1 (***SI Appendix* Text 1**). Resulting peaks were grouped, retention times corrected and regrouped using the density (bandwidth parameter set to 2) and obiwarp methods. Following peak filling, the CAMERA (v.1.32.0)**(26)** package was used to place *m*/*z* peaks into pseudo-spectra by grouping similar peaks with the groupFWHM function. Masses below

150 *m*/*z* were removed from the resulting peak table and all profiles were normalized to the ribitol internal standard. Peaks occurring in run blanks and those with higher relative standard deviation scores (%RSD > 25) in quality control samples (cell culture experiment only) were removed from the dataset. To determine differences in metabolite abundances between sediment habitats, metabolite peak data were analyzed using a Bray-Curtis informed non-metric multidimensional scaling analysis followed by an analysis of variance using distance matrices (ADONIS) to test if there are significant differences in metabolite composition between sites. To identify individual peaks that differed significantly between sediment habitats and between cell culture sampling time points, resulting peaks tables were also log transformed and compared using a one-way analysis of variance. All p-values were adjusted using the Benjamini-Hochberg method to control for false positives **(27)**. Significant variables exhibiting large fold-change differences between starting and ending conditions were further investigated. CAMERA grouped peaks from the environmental survey, and those important to shifts in the cell culture experiment were identified using AMDIS **(28)**. Peaks with NIST hits below 800 were compared to online BinVestigate **(29)** and Golm **(21)** data repositories. If no hit was provided, these were considered unknowns.

The effect of salt and water on metabolite detection. To test the effect of salt on metabolite derivatization, metabolite mix aliquots were resuspended in 1 mL of ASW ranging in salinity from 0 to 34‰ and dried as described above. Methoxamine-trimethylsilylation (TMS) two step derivatization was performed by resuspending each sample in 80 µL of MeOX in pyridine (20 mg mL^{-1}) and incubating for 90 min at 37 °C using a thermal rotating incubator under constant rotation at 1350 rpm. MSTFA was subsequently added to the mixture, and the mixture incubated under the same conditions for 90 min **(14)**. Derivatized samples were centrifuged to pellet salt and the supernatant was transferred to a GC-MS vial for analysis. To test the independent effect of water on metabolite derivatization reactions, MQ was added to dried mixture aliquots in steps of 1 µL from 0 to 10 µL. Replicate water gradient samples were

subsequently derivatized as before using MeOX and MSTFA or by replacing the MSTFA reagent with BSTFA.

Marine metabolomics method development. To show how each method development step increased signal intensity and reduced variation in metabolite detection, replicate mixture aliquots (n = 5) were resuspended in 0.5 mL of ASW. Mixture aliquots (n = 5) were also resuspended in MQ as a no-salt control to highlight the effects of saltwater on metabolite derivatization. 40 µL ribitol (0.2 mM) and 100 µL cholestane (1 mM) were added to each aliquot as internal standards. MQ and ASW samples were first derivatized following the (i) two-step methoxamine-trimethylsilylation (TMS) previously described. Successive steps in the proposed protocol were then applied to ASW samples to demonstrate the combined effects on metabolite detection: (ii) exchange of MSTFA for BSTFA, (iii) removal of residual water from the salt pellet by increasing the speed vacuum drying time and by introducing a toluene drying step to help extract water from the salt pellet, (iv) ultrasonication of the samples after the steps involving addition of toluene, MeOX, BSTFA and following the last derivatization step, and (v) drying the MeOX in pyridine reagent between derivatization reactions. Resulting GC-MS profiles were used to show increases in total signals detected with successive changes in the proposed protocol. Additionally, a cloud plot (using processed peak integration data) was generated to compare compounds dissolved in seawater and to show which metabolite ions exhibited significant (B.H. adjusted $p < 0.05$) and large fold changes ($\log2(FC) > 2$) between the standard and the SeaMet method.

Solid phase extraction. Replicate metabolite mix aliquots (n = 6) were resuspended in 2 mL of artificial seawater. 0.5 mL was reserved from each sample to compare GC-MS profiles before and after SPE sample concentration. Inorganic salts were eluted and metabolites extracted from the remaining 1.5 mL mixture following a SPE based technique using Bond Elut styrene-divinylbenzene (PPL) columns (**13**). The internal standards ribitol and cholestane were added to both, the reserved sample (before SPE) and the resulting SPE-concentrated sample (after SPE). All samples were prepared for GC-MS analysis

following the proposed marine metabolomics method. Resulting profiles were compared using a cloud plot to show which metabolite ions exhibited significant (B.H. adjusted $p < 0.05$) and large fold changes (log2(FC) > 2) between the pre- and post- SPE treatments.

Environmental sampling. Replicate porewater profiles were collected from coralline ($n = 4$) and mangrove ($n = 6$) sediments from Carrie Bow Cay (N 16° 04' 59", W 88° 04' 55") and Twin Cayes, Belize (N 16° 50' 3", W 88° 6' 23") using a 1 m steel lance with a 2 µm inner diameter covered by 0.063 mm steel mesh. Samples (2 mL water) were collected every 5 cm from the sediment surface to 15 cm depth. Samples were immediately frozen at -20 °C until further analysis. Directly before preparation for GC-MS, the internal standards ribitol and cholestane were added to 0.5 mL of each environmental sample. The mixture was subsequently prepared for GC-MS analysis using the SeaMet method described above.

Cell culture sampling. Replicate cultures ($n = 3$) *of Marinobacter adhaerens* HP15 DsRed were cultivated in Marine Broth media at 18 °C and 240 rpm as previously described **(30)**. Media samples from the cell cultures and a no-bacteria control media were collected at 0, 4, 8, 12, and 22 h post culture inoculation. Cell counts were monitored at each time point by measuring the optical density at 600 nm (OD_{600}). Sampling was carried out by collecting 2 mL of each culture and pelleting the cells through centrifugation for 10 min, at 21.1 *g* and 4 °C. The supernatant was immediately stored at -20 °C until preparation for GC-MS analysis. Prior to sample derivatization using SeaMet, ribitol (0.2 mM; 40 µL) and cholestane (100 mM; 100 µL) were added to 0.5 mL of each experimental sample and subsequently dried down in a speed vacuum concentrator (8 hr, 45 °C, VA-Q). To control for technical variation, quality control (QC) samples ($n = 3$) were prepared by combining 0.25 µL of each culture supernatant and an extraction blank generated by drying down 0.5 mL of MQ.

Supporting information

Supporting information includes supporting tables, figures and references

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