Journal of proteome research

pubs.acs.org/jpr

Article

Benzoyl Chloride Derivatization Advances the Quantification of Dissolved Polar Metabolites on Coral Reefs

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Cite This: *J. Proteome Res.* 2024, 23, 2041−2053

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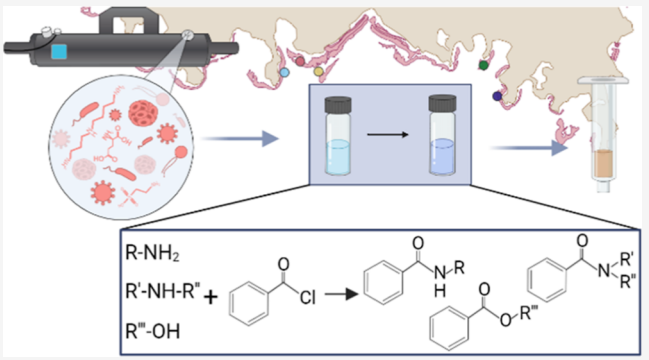
ABSTRACT: Extracellular chemical cues constitute much of the language of life among marine organisms, from microbes to mammals. Changes in this chemical pool serve as invisible signals of overall ecosystem health and disruption to this finely tuned equilibrium. In coral reefs, the scope and magnitude of the chemicals involved in maintaining reef equilibria are largely unknown. Processes involving small, polar molecules, which form the majority components of labile dissolved organic carbon, are often poorly captured using traditional techniques. We employed chemical derivatization with mass spectrometry-based targeted exometabolomics to quantify polar dissolved phase metabolites on five coral reefs in the U.S. Virgin Islands. We quantified 45 polar exometabolites, demonstrated their spatial variability, and contextualized these findings in terms of geographic and benthic cover differences. By comparing our results to previously published coral reef exometabolomes, we show the novel quantification of 23 metabolites, including central carbon metabolism compounds (e.g., glutamate) and novel metabolites such as homoserine betaine. We highlight the immense potential of chemical derivatization-based exometabolomics for quantifying labile chemical cues on coral reefs and measuring molecular level responses to environmental stressors. Overall, improving our understanding of the composition and dynamics of reef exometabolites is vital for effective ecosystem monitoring and management strategies.



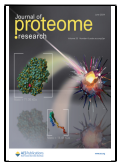
KEYWORDS: *reef diagnostics, chemical derivatization, targeted metabolomics, U.S. Virgin Islands, dissolved organic matter, nitrogen pollution, environmental stressors, exometabolomics, coral reefs, mass spectrometry*

INTRODUCTION

The immense diversity of small organic molecules released and transformed by organisms in aquatic environments has long been a barrier to understanding organismal interactions and ecosystem processes.[1] Recent methodological and technological advances in chemistry (e.g., better separation methodologies, sensitivity, accuracy, and resolving power) have made the detection and quantification of low-concentration and high-flux compounds more tractable.[2,3] Because of its unmatched sensitivity, mass spectrometry (MS) has become the dominant and most widely used technology for the chemical characterization of marine dissolved organic matter (DOM). However, directly analyzing low-concentration organic compounds in seawater containing high concentrations of salt is extremely challenging. For example, sulfate and chloride ions comprise tens to hundreds of millimolar (mM) concentrations in seawater, respectively. Comparatively, steady-state concentrations of bulk dissolved organic carbon occur in the micromolar (μM) range, with individual compounds present at nanomolar (nM) to picomolar (pM) concentrations.[4]

Given the limitations of direct analysis, various methods have been developed to concentrate and isolate marine DOM, including tangential flow filtration (TFF),[5] reverse osmosis/electrodialysis (RO/ED),[6] and solid-phase extraction (SPE).[7] However, these approaches result in significant biases in the chemical species retained for analysis. In particular, small and polar metabolites (characteristics of many labile biomolecules and majority drivers of carbon flux) are lost during TFF or RO/ED and are not well retained [extraction efficiencies (EE) < 50%] on SPE resins such as the commonly used styrene−divinylbenzene polymer (PPL) resin.[8] While a subset of these polar metabolites have been detected using SPE,[8] metabolites with low standing stock concentrations, quick turnover times, or highly polar moieties are largely overlooked. To overcome some

Received: January 22, 2024
Revised: April 23, 2024
Accepted: May 13, 2024
Published: May 23, 2024



https://doi.org/10.1021/acs.jproteome.4c00049
J. Proteome Res. 2024, 23, 2041−2053

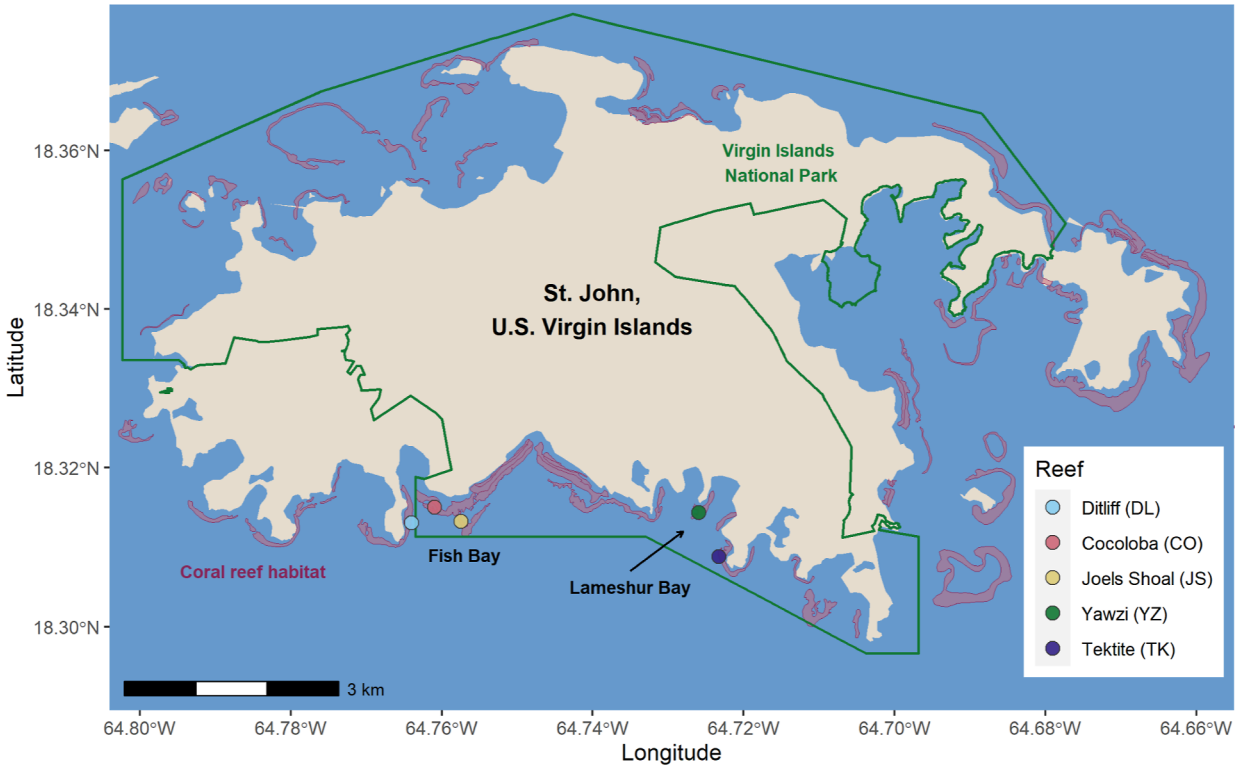


Figure 1. Map of St. John, USVI (tan) and the Virgin Islands National Park (green boundary). Coral reefs are depicted around the island (pink areas). Coral reefs where reef water was collected are shown using circles and labeled by reef name and abbreviation in the legend. Two bays where reefs were located are shown labeled on the map. Map shape files used to generate the figure are open access courtesy of Stanford EarthWorks (St. John, USVI boundary), the National Park Service land resources division (National Park boundary), and NOAA National Centers for Coastal Ocean Science (USVI benthic habitat map).

of these limitations, alternative sample preparation approaches have been developed, allowing for specific chemical species to be retained, concentrated, and analyzed. Specifically, alternative SPE resins (i.e., cation exchange[9]) and chemical derivatization[10−12] have recently been shown to improve the analysis of otherwise elusive polar compounds. While the development of these methodologies has been described, their widespread application to various marine ecosystems remains limited. Further employment of these methodological advancements will allow for the identification and quantification of a wider range of the labile dissolved molecules that are rapidly consumed by heterotrophic bacteria and other organisms, thus enhancing our baseline understanding of these systems.[4] By expanding our view of the chemical "players", we can begin to decipher the complex relationships between sources and sinks of metabolites and how they change over time (flux), under stress (e.g., incidents of disease), and with global climate changes.

One such environment that would benefit from these innovative methods is coral reefs. Coral reefs are one of the most productive, biologically diverse, and economically valuable ecosystems worldwide.[13] Yet, our understanding of the chemistry occurring within reef systems remains limited, with large knowledge gaps in chemical composition, biogeochemical cycles, and chemical diversity (regionally and in the face of global changes/stressors). Previous studies have shown that extracellular DOM released in a species-specific manner by benthic primary producers plays a key role in fueling the reef food web and regulating ecosystem function.[4,14,15] Unfortunately, coral ecosystems have undergone significant decline due to climate change, natural disturbances, disease, and anthropogenic factors, resulting in changes in benthic and microbial composition.[16,17] DOM released by altered communities of benthic primary producers may thereby negatively impact coral health by changing microbial community structure, perhaps favoring coral pathogens, and/or increasing microbial respiration, causing localized hypoxia.[14,18] Such findings underscore the need to transition from bulk measures of DOM to analyses of individual molecules in order to better understand changes in the composition of reef DOM.

Metabolomics studies using untargeted approaches have been more widely, yet still modestly, used to generate a molecularly resolved view of the total DOM pool in reef seawater and tissue homogenate. However, targeted approaches employing matched internal standards are required for absolute quantification and confident metabolite identification. Metabolomic measurements of dissolved exudates from coral reefs using the conventional PPL-SPE approach (both targeted and untargeted) have investigated a range of coral reef phenomena, including metabolites associated with pristine reef systems,[19,20] nonself interactions,[20] bleaching,[21] disease,[22] and benthic-pelagic coupling.[23] However, our knowledge on these topics is still limited because these datasets lack detection of some of the most polar compounds, which are likely central components of nutrient and energy cycling on oligotrophic coral reefs.

Herein, we identify and quantify the exometabolome of five coral reefs in St. John, U.S. Virgin Islands (USVI), located within the Virgin Islands National Park, using the pre-extraction benzoyl chloride (BC) derivatization method developed by Widner et al. 2021[10] and targeted liquid chromatography−tandem MS. We compare our findings to previously quantified

https://doi.org/10.1021/acs.jproteome.4c00049
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reef exometabolomes and describe 23 newly quantified compounds on coral reefs. Additionally, we compare the polar exometabolomes over five geographically separated reefs and describe trends related to benthic composition and potential anthropogenic influences. This dataset significantly expands our lexicon of polar, labile metabolites present on coral reefs.

METHODS

Sample Collection

Samples were collected in January 2021 in St. John, USVI, within the Virgin Islands National Park (Figure 1 and Table S1). Seawater was collected at five coral reefs, two located in Lameshur Bay: Tektite (TK, n = 3, 18.309°N, 64.723°W, 8.3 m reef depth) and Yawzi (YZ, n = 3, 18.314°N, 64.726°W, 8.4 m), and three in Fish Bay: Ditliff (DL, n = 4, 18.313°N, 64.764°W, 6.10 m), Cocoloba (CO, n = 4, 18.315°N, 64.761°W, 7.01 m), and Joel's Shoal (JS, n = 3, 18.313°N, 64.757°W, 8.8 m). All reefs fell within the Virgin Islands National Park except for DL, which is on the eastern edge (Figure 1).

Scuba divers collected reef depth seawater (within 0.25 m of the benthos) in acid-washed Niskin bottles (General Oceanics, Miami, Florida, United States). Specifically, divers descended with Niskin bottles in the open position, thoroughly rinsed the Niskins with reef depth seawater, located three or four areas of the reef that were topographically complex, and triggered each Niskin to close, capturing reef depth seawater within the Niskin chamber at three or four distinct locations on each reef. After ascent, the Niskins were drained into individual polycarbonate bottles, placed on ice, and processed within 2 h of collection. Each individual Niskin bottle was considered a representative sample of each reef (herein defined as a replicate), with a total of 3−4 replicates collected per reef (Table S1).

Reef and Microbial Descriptions

Benthic Survey. Point intercept benthic surveys using 10 m transects were conducted at four random locations at each reef during January 2021. Every 10 cm, the underlying biological reef organisms or substrates were recorded with the following categories: hard coral, macroalgae, cyanobacterial mats (CYAN), crustose coralline algae (CCA), diseased coral, the invasive CCA Ramicrusta, sponge, soft coral, turf algae, substrate (nonbiological), and others. The "other" category included other invertebrates, Millepora, dead coral, and eelgrass. Counts of individual categories were converted to relative abundance. The benthic cover across reefs was compared with a principal component analysis (PCA) using the PCA function from the FactoMineR (v2.6) R package,[24] with each value scaled to unit variance, followed by visualization with the *fviz_pca* function from the factoextra (v1.0.7) R package.

Total Organic Carbon (TOC) and Microbial Abundances. From each Niskin water collection described above, 40 mL of reef depth seawater was processed and analyzed for nonpurgeable organic carbon (NPOC) and total nitrogen (TN), and 1.4 mL was collected, processed, and analyzed for the enumeration of *Prochlorococcus*, *Synechococcus*, picoeukaryotic cells, and unpigmented cells (heterotrophic bacteria and archaea), following the methods described in the Supporting Information, Methods and Weber et al. 2022,[23] respectively.

METABOLITE PROCESSING

Metabolomics

For metabolomic analysis, reef depth seawater collected within the individual Niskin bottles was transferred into polycarbonate bottles using acid-washed thermoplastic elastomer (TPE) tubing (PharMedBPT MasterflexTM, Cole-Parmer, Vernon Hills, IL, United States) for filtration. Seawater was filtered through polytetrafluoroethylene (PTFE) 0.2 μm, 47 mm filters (Omnipore, EMD Millipore Corporation, Billerica, MA, United States) using peristalsis (MasterFlex L/S pump and pump heads, Cole-Parmer, Vernon Hills, IL, United States). TPE tubing and acid-washed fluorinated ethylene propylene tubing (890 Tubing, Nalgene, Thermo Scientific, Waltham, MA, United States) were used to pump seawater through the filter membrane and into acid-washed polycarbonate collection bottles. 40 mL of filtrate for each sample was collected into combusted 40 mL amber vials for BC derivatization. Samples were stored and transported at −20 °C until sample processing, and derivatization were conducted.

Benzoyl Chloride Metabolite Derivatization and Extraction

Filtered, frozen seawater samples were thawed, and 25 of the 40 mL collected from each sample was transferred into a fresh combusted 40 mL amber glass vial for ^{12}C-benzoyl chloride (^{12}C-BC) derivatization conducted according to Widner et al. 2021[10,25] A nine-point standard curve was derivatized (^{12}C-BC) in parallel with samples, using off-reef seawater as the matrix. The use of off-reef seawater is multipurpose. Off-reef seawater generates the standard curve in a matrix that closely matches that of the experimental samples, allowing for consistent derivatization across standards and samples and, therefore, accurate quantification. Additionally, off-reef seawater serves to provide context for which metabolites are uniquely associated with the reef benthos or overlap with the off-reef seawater blank and generates quantitative data on those differences. The standard curve spanned a concentration range of 0−25 nanograms (ng) added of each standard and included the values: 0, 0.125, 0.25, 0.75, 1.75, 2.5, 7.5, 17.5, and 25 ng-added. ^{13}C-labeled stable isotopically labeled−internal standards (SIL-IS) were derivatized in parallel using ^{13}C-BC and spiked into all experimental and standard curve samples. Derivatization methods are described in detail in the Supporting Information, Methods. Derivatized samples were then extracted by SPE using 6 mL, 1 g Bond Elut PPL cartridges (Agilent, Santa Clara, CA, USA), and the final eluent was dried via vacufuge to near-dryness at 30 °C. Dried samples were stored at −20 °C until LC−MS analysis, at which time each sample was reconstituted in 5% acetonitrile (MeCN), transferred to a 2 mL LC vial with a small volume insert, and topped with 5 μL of 100% MeCN. Samples were stored at 4 °C until instrumental analysis.

Targeted Metabolomics UHPLC-MS/MS Data Collection. Reverse phase chromatography was performed according to Widner et al. 2021[10] Using an ultrahigh performance liquid chromatography system (UHPLC; Vanquish, Thermo Scientific, Waltham, MA, USA), separation was achieved on a Waters Acquity HSS T3 column (100 Å, 1.8 μm particle size, 2.1 mm × 100 mm) equipped with an Acquity HSS T3 VanGuard precolumn (100 Å, 1.8 μm particle size, 2.1 mm × 5 mm) coupled to a heated electrospray ionization source (H-ESI) and an Orbitrap mass spectrometer (Orbitrap Fusion Lumos, Thermo Scientific). Chromatography gradient, mass spectrometer settings, and quality assurance and control (QA/QC) are described in detail in the Supporting Information, Methods. A

https://doi.org/10.1021/acs.jproteome.4c00049
J. Proteome Res. 2024, 23, 2041−2053

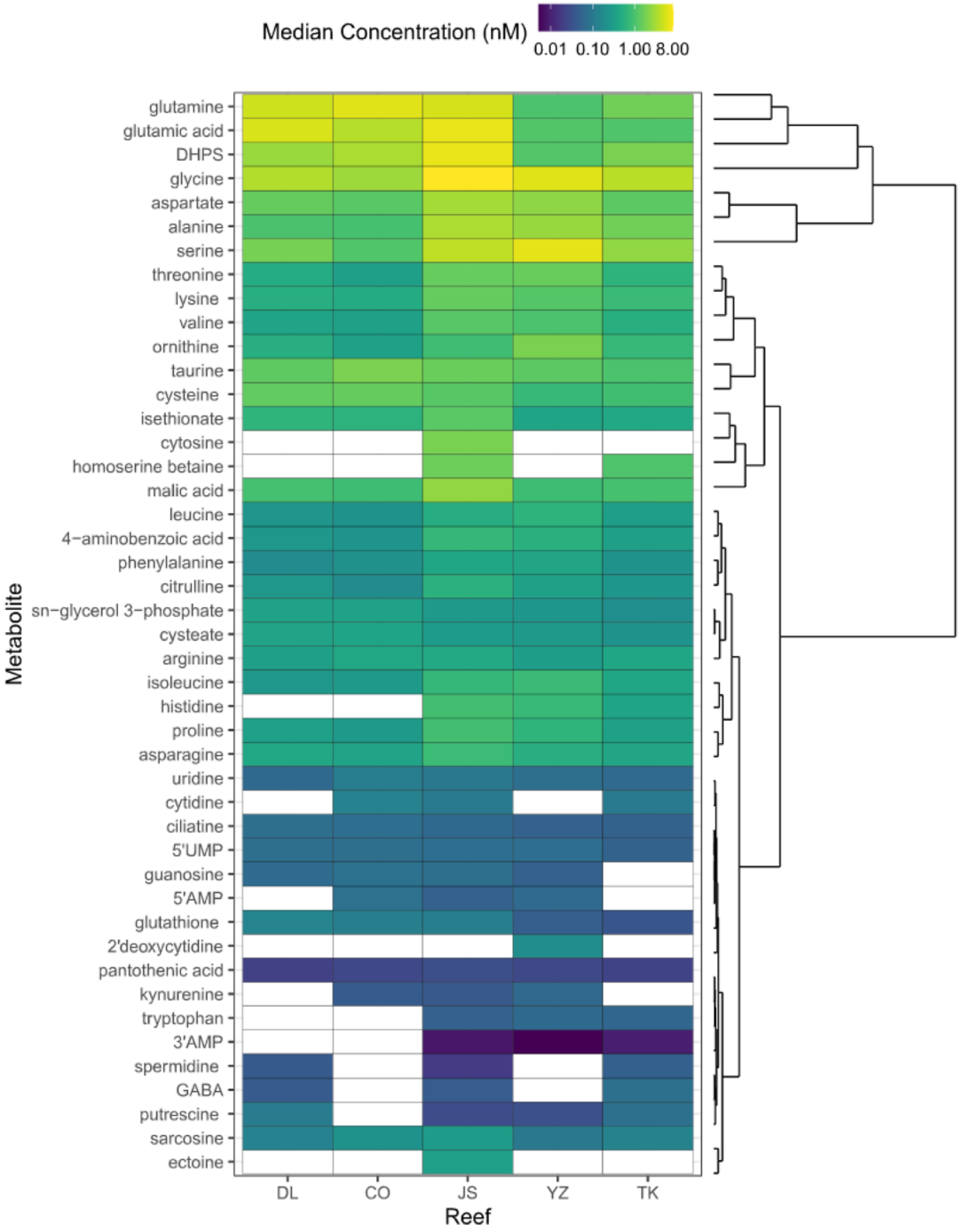

Figure 2. Metabolites were common across five coral reefs. Heatmap of log-transformed dissolved metabolite concentrations (nM). Values shown are the median concentration across replicates (n = 3−4). Warm colors reflect higher concentrations, whereas cooler colors represent lower concentrations. Concentrations that fell below the LOD in all replicates are depicted using a white fill. X-axis labels reflect the five coral reefs (DL-Ditliff, CO-Cocoloba, JS-Joel's Shoal, YZ-Yawzi, and TK-Tektite) and y-axis labels indicate the metabolite name. Metabolites were clustered based on metabolite concentrations and ordered accordingly. Hierarchical clustering is shown as a dendrogram adjacent to the y-axis.

list of targeted standards and their corresponding coefficients of determination (R^2) and limits of detection (LOD) and quantification (LOQ) can be found in Table S2.

Metabolomics Data Processing. Thermo.raw files were processed using Skyline (v. 21.2.0.568).[26,27] Peaks corresponding to detected metabolites were integrated based on accurate mass (±10 ppm), retention time, and MS/MS fragment ion confirmation. Calibration curves for each compound were constructed based on the amount of metabolite standard added (ng-added) versus the integrated light-to-heavy peak area ratios. Exported quantification tables from Skyline containing peak areas for each metabolite were imported into MATLAB (v. R2022a). An in-house MATLAB script (considerSkyline.m) was used to generate linear regression calibration curves, quantify each metabolite, calculate LOD and LOQ, and merge positive and negative polarity mode data (further described in Supporting Information, Methods and Table S2). All MATLAB scripts used for processing the Skyline outputs are available at

https://doi.org/10.1021/acs.jproteome.4c00049
J. Proteome Res. 2024, 23, 2041−2053

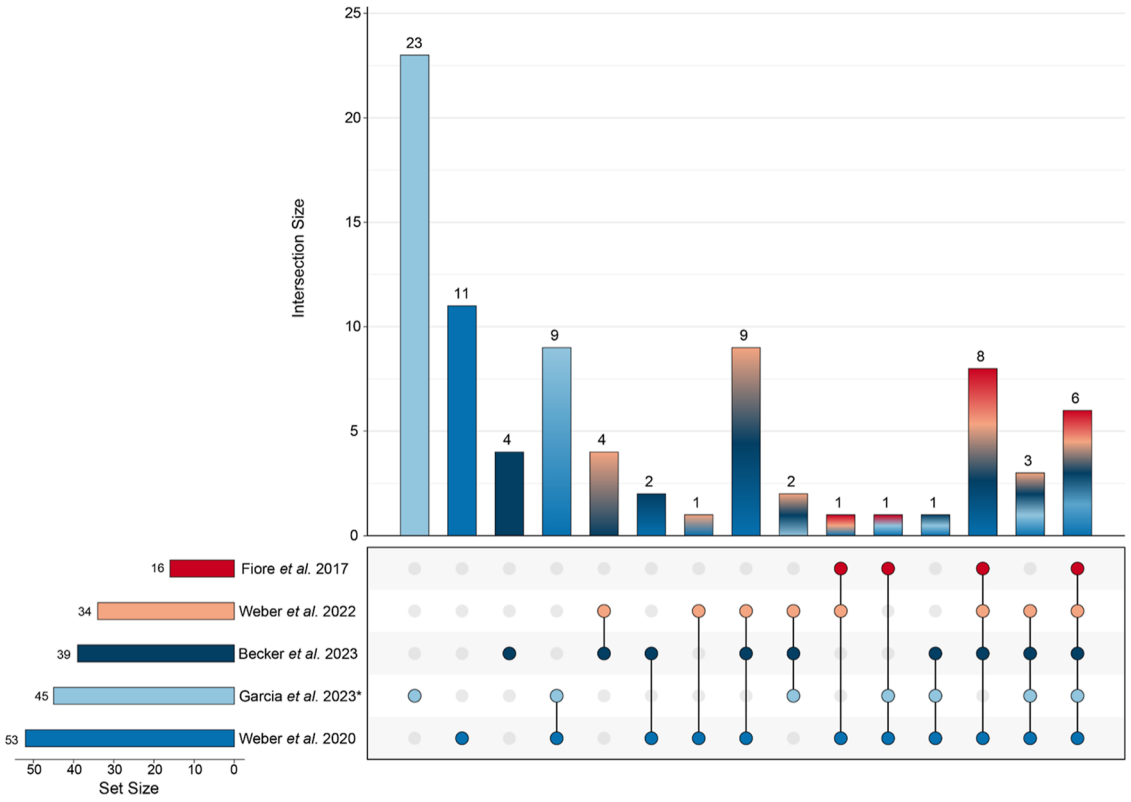

Figure 3. Comparison of coral reef studies shows that 23 metabolites were uniquely captured with the BC derivatization method. Upset plot of metabolite overlap between five targeted benthic exometabolome studies: Fiore et al. 2017 (red), Weber et al. 2022 (orange), Becker et al. 2023 (dark blue), Garcia et al. 2023-current study (light blue), and Weber et al. 2020 (blue). Horizontal bar plots show the set size (sum of metabolites identified for a single study). Vertical bar plots show the intersection size (sum of metabolites unique within or overlapping across studies). Symbol * indicates a study utilizes BC derivatization method. All other studies utilize a standard PPL-SPE (nonderivatization) approach.

https://github.com/KujawinskiLaboratory/SkyMat. The resulting merged quantification table (Table S3) was further filtered in MATLAB based on the QA/QC metrics described in the Supporting Information, Methods.

Metabolite Comparison across Studies. Comparisons between the metabolites observed in this study using the BC derivatization method and four independently collected, targeted benthic exometabolomics datasets that did not employ BC derivatization were investigated. Studies were selected due to their quantitative measurements of exometabolites on coral reefs, allowing for valid comparisons, and included reef seawater samples collected in Cuba[19] and along the coast of Florida,[28] samples from a USVI coral reef seawater incubation experiment,[23] and Florida field-collected sponge inhalant and exhalant seawater samples.[29] All four studies utilized PPL SPE (without derivatization), the most common methodology employed for characterizing DOM by LC−MS, originally described by Dittmar et al. 2008,[7] and were analyzed on a Thermo Fisher Scientific TSQ Vantage Triple Quadrupole mass spectrometer using selected reaction monitoring (SRM). SRM targeted compound lists were generally consistent across the four studies, excluding two metabolites (5-(aminomethyl)-2-methyl-4-pyrimidinamine and valine) absent from the earliest study, Fiore et al. 2017, and one metabolite (3-hydroxybenzoic acid), which was only targeted in the latest study, Becker et al. 2023. Metabolite names from each study were downloaded from each article's publicly available metadata. Metabolites described as unresolved (i.e., isomers "homoserine and threonine" in Weber et al. 2020) were excluded from the analysis. Metabolite names were converted to InChIKeys using the Chemical Translation Service (Table S4).[30] In instances where no conversion was possible, InChIKeys were obtained manually from the Public Chemical Database (PubChem).[31] The International Chemical Identifier code (InChI) or InChIKey annotates metabolites by their chemical structures, allowing for more standardized comparisons.[32] The collective metabolite names and their corresponding InChIKeys from all five studies were imported into R. Unique InChIKeys were queried across all studies, producing a binary matrix where a value of one indicated the presence of that metabolite in a study and zero indicated the metabolite's absence (Table S4). The UpSetR package *upset* function was used to generate an upset plot depicting the overlap of metabolites across studies.[33]

Statistical Analysis. Statistical analyses were carried out in R Studio (v. 2022.12.0 Build 353) running R (v4.2.2, 2022-10-31).[34,35] All code and data used for recreating figures are publicly available on GitHub (https://github.com/bmgarcia/CINAR_Habitat_Metabolomics). Data was tested for normality visually via histogram plots and mathematically using the Shapiro−Wilk test (*shapiro.test*). The majority of metabolites were not normally distributed ($P < 0.05$), and thus nonparametric tests were used for all downstream analyses. Nonmetric multidimensional scaling (NMDS) using the "vegan"[36] and "ggplot2"[37] packages was used to visualize the Bray−Curtis dissimilarity between metabolomic samples. A Permutational Multivariate Analysis of Variance (PERMANOVA) using Bray−Curtis distance matrices was carried out on metabolite and benthic data using the *adonis2* function in the "vegan" package. A

https://doi.org/10.1021/acs.jproteome.4c00049
J. Proteome Res. 2024, 23, 2041−2053

Kruskal–Wallis test (*kruskal.test*) was used to test for significant metabolites between reefs and bays (significance level $P \leq 0.05$). Pairwise Wilcoxon Rank Sum tests (*pairwise.wilcox.test*) were conducted on significant metabolites from the Kruskal–Wallis test to determine pairwise significance. *P*-values were adjusted to account for multiple comparisons using the Benjamini–Hochberg correction,[38] and metabolites were considered significant with a p_{adj} value ≤ 0.1. Pairwise Spearman correlation coefficients were determined between metabolite concentrations and benthic survey proportions using the *cor* function in the base "stats" R package (v4.2.2.). Statistical results can be found in the Supporting Information (Tables S5–S7).

RESULTS AND DISCUSSION

Metabolites Released from Coral Reefs

Using pre-extraction BC derivatization coupled with UHPLC-MS/MS, we detected 45 dissolved phase metabolites above five coral reefs in the USVI, out of the 77 metabolites in this method. The number of detected metabolites varied by reef; 31 metabolites were present at all five reefs, and 14 fell below the LOD at one or more locations (Figure 2 and Table S1). Exudates from JS had the highest number of detected metabolites (44), followed by TK and YZ (39), and DL and CO (35). Of the 45 metabolites, two were detected and 43 were quantified (Table S8-quantified is defined herein as a metabolite having a measured value greater than the LOQ in at least one sample). Metabolite concentrations ranged from 5 pM to 52 nM, with a median concentration of 390 pM (Figure 2). Of the 45 metabolites that were captured using the BC method, 30 had reported EE of less than 1% using PPL SPE (without derivatization), highlighting the longstanding challenges of detecting these polar metabolites in seawater.[8] For example, DHPS (2,3-dihydroxypropane-1-sulfonate) is poorly retained using PPL SPE (EE < 1%), but was measured across all reefs at relatively high concentrations ranging from 600 pM to 52 nM, with a median concentration of 2 nM. DHPS is an organosulfur molecule hypothesized to play a major role in the flux of sulfur and carbon through marine food webs.[39,40] Significant concentrations of other sulfur-containing metabolites [i.e., dimethylsulfoniopropionate (DMSP) and dimethyl sulfide] have been recorded in coral reef hosts and their symbiotic dinoflagellates, suggesting that coral reefs might play a substantial role in sulfur cycling in largely oligotrophic regions.[41−44] Unlike DMSP, our understanding of the breadth of DHPS producers and their role in sulfur biogeochemistry remains limited. Sulfonates such as DHPS are emerging as important chemical links between marine phytoplankton and bacteria, which together fuel the microbial sulfur cycle in the surface ocean.[45,46] The identification of DHPS on coral reefs in this study requires further investigation into its source(s) (i.e., coral host, *Symbiodinium* spp., or associated microbes) and its role(s) as a potential key player in sulfur cycling in coral reefs. The use of alternative chemical approaches, such as chemical derivatization, is key to increasing our understanding of these important labile biomolecules in the context of marine microbial systems.

BC Derivatization Retains Polar Metabolites Previously Undetected in the Coral Reef Exometabolome

To determine the advantages and complementarity of incorporating pre-extraction BC derivatization into the marine metabolomics workflow, we assessed the overlap of identified metabolites across coral reef benthic communities from four previously published PPL-SPE reef water targeted metabolomic datasets (Fiore et al. 2017,[29] Weber et al. 2020,[19] Weber et al. 2022,[23] and Becker et al. 2023[28]) and the current study (Figure 3). A total of 85 distinct metabolites were identified across the five studies. Of these 85 metabolites, 38 were found to be study-dependent (only identified in a single study), 41 were identified in a minimum of two studies, and six were ubiquitously identified (Figure S1). The largest number of identified metabolites (53) was by Weber et al. 2020 on highly protected and biodiverse Cuban coral reefs, followed by the current study's BC derivatized dataset (45). Variations observed in metabolite overlap between the nonderivatized datasets are unlikely to be platform-dependent since all four datasets were collected on the same MS platform and are more likely dependent on chemical, microbial, and/or environmental factors. For example, the remote and pristine Jardines de la Reina, Cuba reef system studied by Weber et al. 2020 was less exposed to the anthropogenic influences, we might expect to find off the populated coast of Florida (Becker et al. 2023), likely resulting in detectable differences in the exometabolome. Microbial community composition, reef productivity and biodiversity, and environmental inputs (e.g., rain and runoff) are likely contributors to variations observed between the datasets.

Using the BC derivatization method, 23 unique metabolites were captured, the highest proportion of unique metabolites of the five investigated studies. Metabolites solely captured using the BC method consisted of amino acids, amines, pyrimidine nucleosides, and organosulfonic acids; metabolites that have been difficult to capture in the coral exometabolome even with alternative chromatographic and derivatization approaches, such as GC–MS.[11] This approach allowed us to uniquely quantify essential components of central carbon metabolism, carbon fixation in photosynthetic organisms (e.g., aspartate and alanine),[47,48] and nutrient and substrate transport in prokaryotes (e.g., taurine and putrescine).[49] The metabolic pathways these compounds comprise are essential for understanding complex organismal processes such as cell viability, virulence, pathogenicity, organism growth, and survival. Furthermore, this comparison highlights the large fraction of polar metabolites that have thus far been excluded from DOM chemical investigations, limiting our holistic understanding of chemical exchange and microbial metabolism on coral reefs.

Of the 85 distinct metabolites across the five studies, 40 metabolites were not analyzed or detected using the BC derivatization method; 21 of which were intentionally excluded from our targeted list due to (1) successful measurement using PPL-SPE alone, thus negating the need for derivatization or (2) lacking the appropriate moieties (amine or alcohol functional groups) for successful BC derivatization. The 19 metabolites in the BC targeted list that were not detected in this study but were detected in one or more of the SPE studies provide interesting metabolites to query for geographical, ecological, and chemical insights within these reefs. For example, the metabolites adenosine, inosine, tryptamine, tyrosine, and xanthosine were detected in all four SPE datasets but fell below the LOD in the BC derivatized dataset. These metabolites are particularly challenging to detect due to their low standing stock concentrations. All five metabolites were detected in the low fM to pM levels in the SPE-based studies, which utilize much larger extraction volumes (>1 L) in comparison to the 25 mL of seawater used for the derivatization protocol. While reduced sample volume allows for increased experimental throughput and replication, which has a multitude of benefits, the LOD for

https://doi.org/10.1021/acs.jproteome.4c00049
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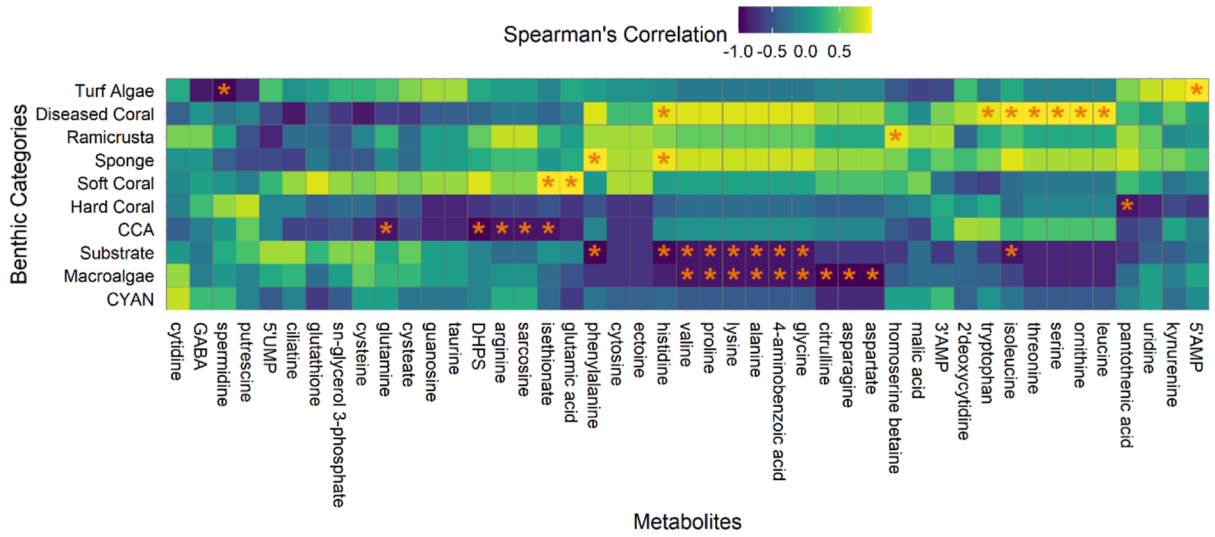

Figure 4. Variations in metabolite concentrations and benthic compositions are strongly related. Spearman correlations between targeted metabolite (x-axis) concentrations and benthic survey data (y-axis). Significant ($p < 0.05$) correlations are indicated with an orange star. Color corresponds to the strength of the correlation with warmer colors indicating a positive correlation and cooler colors indicating a negative correlation. CCA = crustose coralline algae and CYAN = cyanobacterial mats.

these five molecules using the BC method were greater than those determined using PPL-SPE alone. Thus, the set of 19 metabolites undetected using BC derivatization may have been present at our study sites but evaded detection due to their LOD. While no single extraction method can capture the DOM pool in its entirety, this comparison demonstrates the complementarity and benefits of BC derivatization relative to more standard approaches. Future studies incorporating both BC derivatization and nonderivatized SPE will allow for a more holistic view of the coral exometabolome, further expanding our understanding of the chemical composition and variability of marine DOM, essential to our comprehension of coral reef ecology and chemical cycling. Specifically, improving the breadth of quantitative measurements of labile components of DOM through these two approaches opens avenues for asking questions related to metabolic pathways and flux analyses.

Relationship between Reef Benthos and Dissolved Metabolites

Ordination-based approaches were used on both habitat and metabolite composition to better understand how biogeographic changes may influence both data types. We used a PCA on benthic composition and found that reefs were significantly structured by both reef and bay (PERMANOVA, Reef: $p = 0.001$, $R^2 = 0.65$, Bay: $p = 0.022$, $R^2 = 0.14$) (Figure S2a). Vectors representing the variable contributions of the 11 benthic categories were overlaid on the PCA scores plot to demonstrate the benthic composition of the reefs. For example, Tektite reef had the highest amount of hard coral and cyanobacterial mats (CYAN), while Ditliff and Cocoloba harbored the highest substrate (Figure S2a). Joel's Shoal and Yawzi generally had higher soft coral and turf algae (Figure S2a). Compared to the benthic habitat, the composition of metabolites, as examined with NMDS using Bray−Curtis distance, was structured by reef and bay to a lesser extent (PERMANOVA, Reef: $p = 0.145$, $R^2 = 0.33$, Bay: $p = 0.082$, $R^2 = 0.12$) (Figure S2b). Although reef did not significantly structure metabolite composition, it explained 33% of the variance in the metabolite data. Reef habitat components and biogeography are known to influence reef water metabolite composition,[23,28,50] and further sampling across USVI reefs in the future, specifically with increased temporal/spatial resolution and statistical power, is needed to better understand variations in metabolite composition across reefs.

To understand potential benthic−metabolite relationships, Spearman correlations (Figure 4) between metabolite concentrations and benthic proportions were calculated. Correlations illuminated 96 strong (correlation coefficient > 0.7) benthic−metabolite relationships, 48 of which were significant ($p < 0.05$, Table S9). The most prevalent benthic organisms (excluding "other" and substrate) found to have significant correlations to the targeted metabolites include diseased coral, macroalgae, and CCA. This is consistent with previous findings that benthic primary producers, including CCA, sponges, and algae, variably influenced the DOM pool on coral reefs.[15,29,51,52] Previous work by Becker et al. 2023 highlighted significant correlations between stony coral tissue loss disease and the targeted metabolome of reef seawater.[28] Here, diseased coral showed seven significantly positive correlations with exometabolites, all of which were amino acids (Trp, Ile, Thr, Ser, Orn, and Leu). The overexpression of mRNA associated with the production of amino acids and amino acid derivatives has been observed previously in corals infected with black band disease (BBD) in conjunction with changes in bacterial community composition.[53] BBD etiology is associated with a polymicrobial consortium, including cyanobacteria, sulfur-reducing and -oxidizing bacteria, and heterotrophs, generating a toxic anoxic and sulfur-rich microenvironment against the coral tissue.[54] The correlation between coral disease and increased extracellular amino acid concentration observed here could be indicative of a change in microbial community composition and thereby a reduction in the efficiency of metabolite recycling; however, further work is necessary to explore this hypothesis. The use of metabolomic approaches for understanding coral reef disease dynamics and prevention efforts has proved promising but largely excluded polar, labile metabolites.[22,55,56] With the unprecedented increase in coral reef diseases, alternative omics-based approaches aimed at disease diagnostics and prevention are desirable; however, much remains unknown

https://doi.org/10.1021/acs.jproteome.4c00049
J. Proteome Res. 2024, 23, 2041−2053

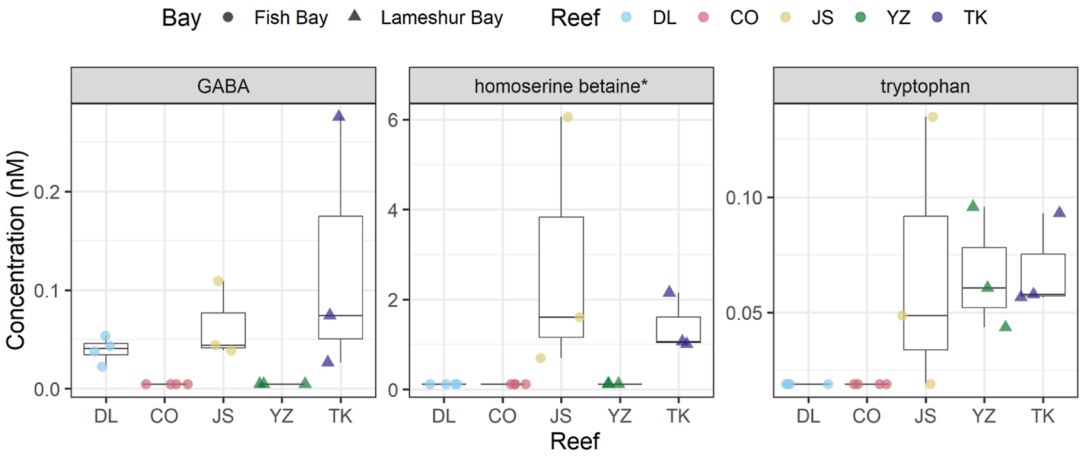

Figure 5. Metabolites GABA, homoserine betaine*, and tryptophan show significant differences in concentration across reefs. Boxplots representing the concentrations of significant metabolites by reef (Kruskal–Wallis and Pairwise Wilcoxon Rank Sum Tests, adjusted p-value ≤ 0.1). Boxplots represent the interquartile range (IQR), or the area between the 25% and 75% quantiles, with the median as the line in the center. Lines extend beyond the box to 1.5× IQR. Points beyond the lines are considered outliers. Color corresponds to each reef and shape indicates the bay. *X*-axis indicates the reef abbreviation and the *y*-axis displays metabolite concentration values (nM). *Y*-axis scale range varies by metabolite. Metabolite names followed by an asterisk (*) indicate metabolites quantified using the BC-derivatization method, but unretained using PPL-SPE alone.

regarding the chemical indicators of disease, specific vectors of various diseases, and the metabolic pathways impacted by infection and disease progression. Factors such as coral species,[57] disease susceptibility, symbiont genera,[58] and geographic location[28] all have been shown to influence disease dynamics and are essential for understanding these complex phenomena.

Metabolites were tested for significant concentration differences between the five reefs and their respective bays. Due to our small sample sizes when comparing differences between reefs (n = 3–4), a p_{adj} value of 0.1 was selected for our significance threshold. Metabolites homoserine betaine, tryptophan, and γ-aminobutyric acid (GABA) were significantly different based on reef (Figure 5). Tryptophan was found at elevated median concentrations ranging between 49 and 61 pM at reefs JS, YZ, and TK and fell below the LOD at DL and CO. Interestingly, tryptophan appears to be a core metabolite in the reef exometabolome, as it was also detected in all the prior studies compared earlier. Though the specific role of tryptophan on coral reefs is unknown, bacteria associated with the surface of the coral species *Pocillopora damicornis* and *Acropora aspera* have been shown to exhibit significant levels of chemotaxis toward amino acids, including tryptophan.[59] There is a need to further investigate the role specific exometabolites play in chemical signaling in coral reef holobiont assemblages.

In contrast to tryptophan, the metabolites GABA and homoserine betaine have largely gone undetected on coral reefs due to their low (<1%) PPL EE, with this study being the first measurement of dissolved homoserine betaine in the marine environment. Homoserine betaine was detected at two reefs (JS and TK) and had a significant positive correlation to the encrusting red-brown algae*Ramicrusta* (Figure 4), an invasive species on coral reefs shown to overgrow corals and threaten coral recruitment.[60,61] Homoserine betaine is a novel metabolite structurally identified by Pade et al. 2016 and previously reported in a green alga *Monostroma nitidium*,[62] shellfish *Callista brevisiphonata*,[63] and a turban shell *Turbo argyrostoma*.[62] Its specific function in reef and nonreef organisms is largely undescribed, excluding Pade et al. 2016, where the novel role of homoserine betaine as a previously unknown, compatible solute produced by the cyanobacterium *Trichodesmium* spp. was elucidated.[64] Using BC derivatization, Widner et al. 2021 quantified nanomolar concentrations of dissolved homoserine betaine in marine bacterium *Ruegeria pomeroyi* cultural exudates.[10] The presence and relatively high median concentrations of homoserine betaine at JS (1.6 nM) and TK (1.1 nM) is an intriguing finding that requires further investigation due to the limited information available regarding the potential functions and sources of homoserine betaine on coral reefs and other marine environments. Previous findings suggest homoserine betaine may be widely distributed among marine organisms, with large influxes into the extracellular space having impacts on our understanding of biogeochemical cycling. BC derivatization provides a path forward for quantifying this formerly elusive dissolved metabolite.

GABA is a nonprotein amino acid and one of the major inhibitory neurotransmitters in the animal central nervous system. In marine environments, GABA has been shown to play a variety of chemical roles, serving as a signaling compound, osmolyte, and carbon substrate.[65−67] However, the identification and specific role of GABA on coral reefs are not well understood. Weber et al. 2020 detected GABA in coral reef exudates in the highly protected Jardines de la Reina, Cuba reefs; however, the concentrations fell below their LOQ.[19] We observed elevated median concentrations of GABA ranging from 41 to 74 pM at reefsDL, JS, and TK; in contrast, GABA was absent or fell below our LOD at CO and YZ. These findings demonstrate the benefits of chemical derivatization for capturing low-abundance metabolites such as GABA that are clearly present within these systems but often evade detection. With the increased replication of our current study, we can confidently quantify GABA on St. John reefs. GABA has been used as an indicator of ecosystem health in snapper,[68] and has been found as a settlement cue of marine invertebrates.[65] Coral larvae are also capable of sensing fine-scale settlement cues when they actively explore the benthos in search of highly inductive settlement surfaces for coral.[69] GABA did not have any significant correlations to the 11 benthic categories (Figure 4); however, the observed metabolite trends were consistent with elevated total coral coverage (Figure S3). These results

https://doi.org/10.1021/acs.jproteome.4c00049
J. Proteome Res. 2024, 23, 2041−2053

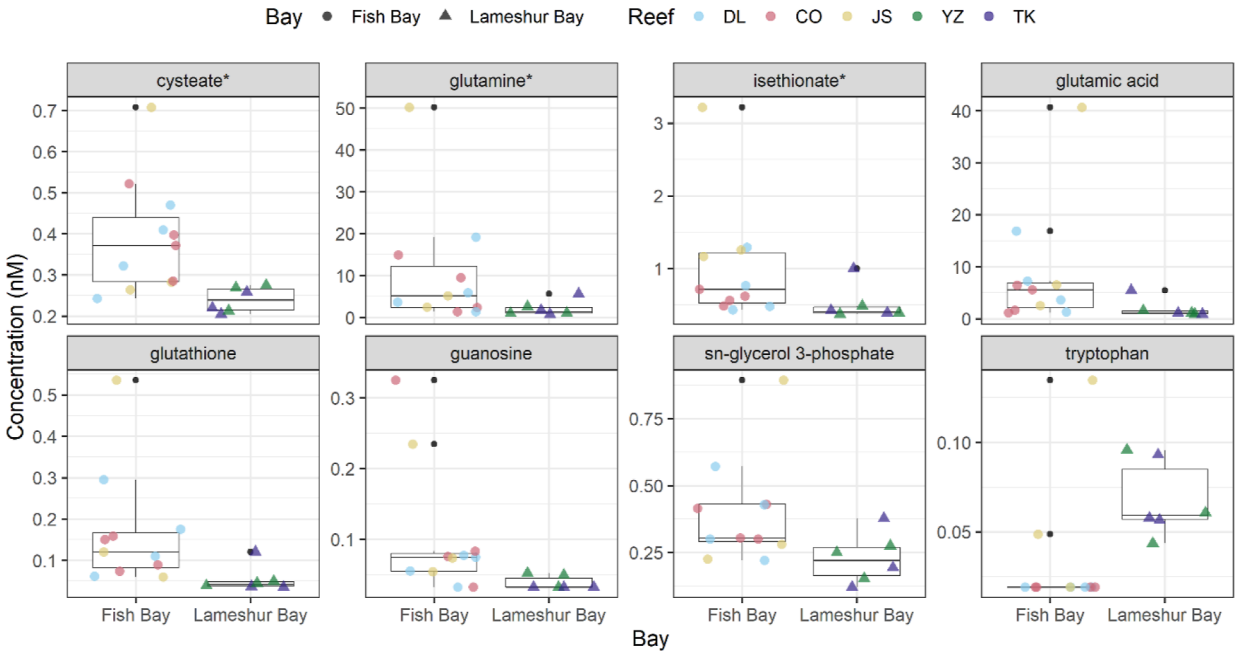

Figure 6. Metabolites significantly elevated at Fish Bay. Boxplots representing the concentrations of significant metabolites on reefs based on bay location (Kruskal–Wallis and Pairwise Wilcoxon Rank Sum Tests, adjusted p-value ≤0.05). Boxplots represent the IQR, or the area between the 25% and 75% quantiles, with the median as the line in the center. Lines extend beyond the box to 1.5× IQR. Points beyond the lines are considered outliers. Color corresponds to each reef, and shapes are indicative of bay. X-axis labels the bay and the y-axis displays metabolite concentration values (nM). Y-axis scale range varies by metabolite. Metabolite names followed by an asterisk (*) indicate metabolites quantified using the BC-derivatization method, but unretained using PPL-SPE alone.

suggest GABA could act as a chemical cue associated with increased coral coverage; however, additional experiments are needed to address this hypothesis.

While only three metabolites showed significant differences between reefs, the metabolites observed reproducibly across reefs are also essential to further our understanding of marine DOM. For example, DHPS was detected at high concentrations and consistently across reefs. Additionally, DHPS was significantly negatively correlated (Figure 4) with CCA. While this metabolite and others were statistically insignificant between reefs, their quantification allows for additional hypotheses to be formed and experiments to be conducted investigating the ecological significance of coral exometabolites in relation to varying benthic organisms, and more largely, in the context of marine biogeochemical cycling.

In addition to assessing metabolite variation by reef, we grouped reefs according to location in nearby bays (Fish Bay compared to Lameshur Bay) to evaluate whether coastal hydrodynamics or anthropogenic factors impacted DOM composition. Differences between these two bays that may impact metabolite variation are described in detail in the Supporting Information, Results. Using a significance threshold of $p_{adj} \leq 0.05$, eight metabolites were significantly different between the two bays, including cysteate, glutamate, glutamine, glutathione, guanosine, isethionate, *sn*-glycerol-3-phosphate, and tryptophan (Figure 6). Of these, cysteate, isethionate, and *sn*-glycerol-3-phosphate have only now been captured in the coral exometabolome using BC derivatization, further highlighting the importance of capturing the polar component of marine DOM. Interestingly, all metabolites, excluding tryptophan, were significantly elevated at Fish Bay. These nitrogen-, sulfur-, and phosphorus-rich metabolites are compositionally important in oligotrophic waters where nutrients are limited. However, large influxes or reservoirs of these elements could be a sign of pollution and eutrophication. Specifically, nitrogen enrichment has been shown to have adverse effects on coral growth and calcification,[70] coral heat resistance,[71] individual species resilience,[72] and the incidence and severity of coral disease.[73] TN (inorganic + organic) concentrations were significantly increased ($p_{adj} \leq 0.05$) at Fish Bay ($[TN]_{median}$ = 9.15 μM) compared to Lameshur Bay ($[TN]_{median}$ = 6.85 μM), consistent with current hypotheses that increased coastal development may significantly contribute to nitrogen loading (Figure S4). Becker et al. 2020 reported inorganic nitrogen and ammonium concentrations from coral reefs (TK, YZ, DL, and CO) located within these two bays, with median concentrations of ∼0.15 μM, suggesting the majority of the TN measurements may be due to organic nitrogen contributions.[74] Of the 45 identified metabolites, glutamine and glutamate were found at the highest concentrations across reefs, ranging between 744 pM to 50 nM with a median concentration of 2.5 nM (Figure 2). The majority of ammonium in coral reefs is assimilated by the dinoflagellate symbiont via the glutamine synthetase/glutamine:2-oxoglutarate aminotransferase (GS/GOGAT) cycle or by the coral host via GS and/or glutamate dehydrogenase.[75] Thus, the abundance of glutamine and glutamate could be an indicator of nitrogen assimilation,[75] with release suggesting symbiont production exceeding host demand, although other organismal uses are also possible. Incorporating exometabolomic approaches into reef monitoring workflows in conjunction with global measurements of TN and TOC enables us to identify which organic compounds are contributing to this bulk pool of essential elements and to what degree, furthering our understanding of these complex, rapidly changing marine systems.

https://doi.org/10.1021/acs.jproteome.4c00049
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CONCLUSIONS

Here, we show that BC derivatization successfully captured 45 polar metabolites, 23 of which were previously undetectable on coral reefs, and that these metabolites were significantly different between individual reefs and across bays. While additional targeted experiments are needed to relate these observations to their specific source (i.e., runoff, increased biomass, and host/symbiont excretion), this data showcases our ability to measure and quantify low abundance, high flux, and ecologically relevant metabolites in low volumes of seawater that have previously gone undetected due to methodological limitations. As anthropogenic stressors increase, there is a pressing need to revolutionize our approaches and mindset regarding monitoring strategies. Exometabolomics has the potential to elucidate previously overlooked chemical compounds, and novel sample preparation methods, such as chemical derivatization, will expand the suite of detectable molecules. As implementation of these methods continues to increase, we will begin to uncover the dynamics of baseline biogeochemical cycles in complex marine environments and harness that information to monitor fragile ecosystems for signs of change related to local and global stressors. Incorporating this method into the standard systems biology profiling approach (i.e., genomic sequencing, water chemistry, and flow cytometry) will allow for additional points of comparison across geographic regions and reef holobiont structure to advance our understanding of the role of labile DOM within complex coral reef ecosystems, and provide novel targets to investigate their ecological role, sources, sinks, and biomarker potential.

ASSOCIATED CONTENT

Data Availability Statement

All MS data are under review at MetaboLights[76] and will be available under accession no. MTBLS9008 (https://www.ebi.ac.uk/metabolights/MTBLS9008). A step-by-step BC derivatization protocol is publicly available on protocols.io (dx.doi.org/10.17504/protocols.io.biukkeuw).[25] All MATLAB scripts used for processing the Skyline outputs are available online at https://github.com/KujawinskiLaboratory/SkyMat. R scripts for recreating all figures within the manuscript can be found at https://github.com/bmgarcia/CINAR_Habitat_Metabolomics.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.4c00049.

Additional detailed information on the chemicals and standards used; TOC and microbial abundance measurements; BC derivatization method, targeted metabolomics UHPLC-MS/MS gradient, mass spectrometer settings, LC−MS QA/QC, metabolite quantification and LOD calculations, and data processing QA/QC; additional analysis results, including differences between Fish bay and Lameshur bay, metabolite comparison, and relationship between reef benthic composition and dissolved metabolites; sampling site details and number of metabolites detected/quantified; Bubbleplot of BC and SPE metabolite comparison; NMDS and PCA ordinations of benthos and metabolome; proportion of total coral coverage across reefs; flow cytometry and inorganic nutrient measurements across reefs; benthic proportions of each coral reef; and ratio of coral to macroalgae across reefs (PDF)

List of metabolite standards targeted in this study and their corresponding calibration curve outputs (R^2, number of points, LOD, and LOQ); sample metadata and metabolite quantification results; coral exometabolome metabolite comparison between current study using BC derivatization and four nonderivatized SPE datasets; statistical results (PERMANOVA, Kruskal−Wallis, and Wilcoxon rank sum test) for benthic composition differences between reefs and bay; statistical results (PERMANOVA, Kruskal−Wallis, and Wilcoxon rank sum test) for flow cytometry and nutrient differences between reefs and bay; statistical results (PERMANOVA, Kruskal−Wallis, and Wilcoxon rank sum test) for metabolite differences between reefs and bay; metabolite summary showcasing median, average, and standard deviation of metabolite concentrations across reef replicates, the number of replicates detected in, and their corresponding LOD/LOQs; and Spearman correlation results between benthic composition proportions and metabolite concentrations (XLSX)

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https://doi.org/10.1021/acs.jproteome.4c00049
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C.C.B. Data analysis-B.M.G. and C.C.B. Writing-B.M.G. and C.C.B. Editing-all authors.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Nadège Aoki and Justin Ossolinski assisted with field sampling and processing, and Krista Longnecker assisted with sample analysis. We thank Erin McParland for the helpful discussions regarding experimental design. Samples were collected using the National Park Service permit VIIS-2021-SCI-0002. This work was supported by an OCE National Science Foundation award to A.A. (#1736288) and a NOAA OAR Cooperative Institutes award to A.A. and E.K. (#NA19OAR4320074).

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