Title: Complementary sampling methods for coral histology, metabolomics, and microbiome

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Running headline (45 char or less): A comprehensive method to assay coral health

Abstract:

1. Underwater visual surveys of coral reefs are the primary method managers use to monitor coral health. However, these surveys are limited to visual signs, such as bleaching and tissue loss lesions, which occur only after significant stress has accumulated. More holistic characterization of coral health can allow for better monitoring of reef changes across natural environmental gradients, in response to anthropogenic stress, and after disturbance events (e.g., disease outbreaks, mass bleaching, dredging, runoff events).

2. Various methods exist to evaluate the health of the coral holobiont that do not depend on visual signs, including histological assessment, microbiome dysbiosis, and metabolic profiles, yet these tools are rarely deployed concurrently. We present a clear, readily deployable protocol for sampling and preserving coral fragments, including (i) extraction of coral metabolites for analysis, (ii) preservation of microbiome DNA for sequencing, and (iii) preservation of coral tissues for histopathology. Combined with visual surveys, these methods provide an unparalleled, holistic characterization of coral health.

3. We provide a field-tested, optimized protocol for conducting coral sampling. This protocol guides the user through concurrent assessments of coral tissue structure and the holobiont microbiome and metabolome, and directs the user to useful resources for downstream data analysis.

4. This protocol facilitates quantitative characterization of coral health beyond visual surveys alone, which is a valuable step forward in reef research and management and will improve our ability to describe, model, and mediate impacts to coral reefs.

Keywords: Coral health, disease, histology, management, metabolomics, microbiome, multidisciplinary, sampling

Introduction:

Corals are ecosystem engineers and foundational species; therefore, coral health is critical to reef function and is a marine management priority. Coral health is most commonly assessed from visual signs, such as disease and bleaching (Raymundo, Couch, Bruckner, & Harvell, 2008; Swanson, Bailey, Schumacher, Ferguson, & Vargas-Ángel, 2018), which only occur after significant stress accumulation within the host (Downs et al., 2002; Heron et al., 2010; Raymundo et al., 2008; Winston et al., 2019). While visual surveys are a rapid and efficient strategy to detect late-stage or large-scale impacts, they provide no detailed characterization of host condition or non-visible indicators of stress. Early detection of declining coral health would provide time to manage reef stressors (Beeden et al., 2012) and potentially mitigate impacts under climate change (Maynard et al., 2015).

A variety of methods exist to characterize components of coral health, but these are rarely combined to more broadly understand the health of the coral holobiont (Work & Meteyer, 2014). Here, we demonstrate simultaneous sampling for histology, microbiome sequencing, and metabolome characterization to leverage their complementary information. Histology of fixed coral tissue is a "gold standard" for assessing coral condition by providing evidence of coral tissue integrity and cell structure and for allowing for the characterization of apoptosis, necrosis, tissue thickness, presence of microorganisms, and algal symbiont characteristics (Gierz et al., 2020; McClanahan et al., 2004; Work & Meteyer, 2014). Technological advances now allow histological assays to be processed quickly and in large quantities (Toledo-Hernández et al., 2014). The bacterial coral microbiome appears highly significant to coral health from larval to adult stages, and dysbiosis of the coral holobiont has been implicated in both disease and bleaching events (Ainsworth et al., 2010; Ainsworth & Gates, 2016; Bourne et al., 2016; Casey et al., 2015; Hernandez-Agreda et al., 2016; Hernandez-Agreda, Leggat, Bongaerts, et al., 2018; Meyer et al., 2019; Pollock et al., 2019; Sweet & Bulling, 2017; van Oppen & Blackall, 2019). Lowered costs of next-generation sequencing now permit detailed characterization of

bacterial 16S ribosomal DNA (rDNA) that comprise the coral microbiome, allowing detection of microbial shifts toward disease states (van Oppen & Blackall, 2019) and close inspection of microbial symbioses supporting the health and function of coral reefs (Bourne et al., 2016; Hernandez-Agreda et al., 2016; Hernandez-Agreda, Leggat, Bongaerts, et al., 2018). Metabolite profiling (metabolomics) has provided insight into coral holobiont functional responses including metabolite variation by genotype (Lohr et al., 2019), temperature-dependence of known coral pathogens (Boroujerdi et al., 2009), and biochemical coral defense (Quinn et al., 2016). Metabolomics uses high throughput approaches such as liquid chromatography coupled with mass spectrometry to examine organic compounds of small molecular weight (e.g., sugars, lipids and secondary metabolites) foundational to cellular processes, for which changes in concentration may indicate cellular disruption. Thousands of these compounds can be rapidly measured from a single sample, making metabolomics a potentially powerful diagnostic tool for assessing environmental impacts to coral health (Bundy et al., 2009; Gordon & Leggat, 2010). As metabolomics are increasingly used to describe coral response to stress, such as extreme temperatures or ocean acidification (Sogin et al., 2016), there is a need to integrate metabolomic responses with physiological assays such as histopathology. While any single assay type (visual, histological, microbial, metabolomic) will produce valuable data on specific components of coral health, combining these methods provides unparalleled capacity to broadly characterize coral condition.

The methodology described here provides a simple protocol for the simultaneous collection of coral fragments for histological assays, microbiome analysis, and metabolomic profiling. These methods are complementary and provide multiple lines of evidence to evaluate coral condition for a better understanding of natural variability and responses to disease outbreaks or other disturbance events. We provide an easy-to-use guide to material preparation, field sample collection and treatment, and supporting information for downstream sample processing and data analysis.

Description and implementation:

Materials:

Sampling and preservation materials (with the exception of methanol) are best stored in sealed containers at cool room-temperature. Methanol is a toxic, flammable liquid and should be stored according to institutional, local, and national guidelines. Here, we describe a sampling "kit" designed for 120 fragments (see Table 1). Material quantities in square brackets (e.g., [60]) in the following sections will vary with the desired number of fragments. The total cost of materials for one kit is approximately $1,400 USD in 2019.

Pre-sampling preparations:

Permitting: Comply with local and international regulations and permitting requirements for collecting and transporting corals. In this protocol, each coral fragment is approximately 4 cm^2 and is broken into two 2 cm^2 samples allowing for two different preservation methods. Larger fragment sizes may be required depending on the species of interest and histological methods used.

Labeling of sample vials: Each of the [120] coral fragments will require one 20 mL glass scintillation vial and one 50 mL conical tube. Therefore, label [120] pairs of one 20 mL glass scintillation vial and one 50 mL conical tube to match the planned sampling protocol (see "Sampling Design"). Additionally, label two 20 mL glass scintillation vials as "Methanol 1 Control" and two 20 mL glass scintillation vials as "Methanol 2 Control".

Preparing fixative (4% paraformaldehyde (PFA) in 3x phosphate-buffered saline (PBS) solution) for microbial DNA and tissue histology (Fig. 1, Steps 1-4): Prepare conical tubes with fixative no more than one week prior to the planned sampling date; use personal protective equipment and sterile techniques to prevent contamination (Hernandez-Agreda, Leggat, Bongaerts, et al., 2018). (Steps 1,2) To

prepare PBS buffer solution, completely dissolve 15 PBS tablets into each of the [22] 500 mL containers of ultra-pure water to reach a final solution of 3x concentration. (Step 3) Break open and empty one 10 mL 16%-PFA ampule into each of the labeled 50 mL conical tubes. (Step 4) Transfer 40 mL of the PBS-buffered ultra-pure water into each conical tube; seal the lid of each conical tube and store at 4°C for up to one week. The PBS can also be prepared in advance (Steps 1,2) and the PFA added immediately prior to sampling (Steps 3,4) to minimize risk of PFA degradation. These steps ("Preparing fixative…", Fig. 1) will use approximately half of the PBS-buffered ultra-pure water; store remaining PBS-buffered ultra-pure water at 4°C for use in later steps of this protocol.

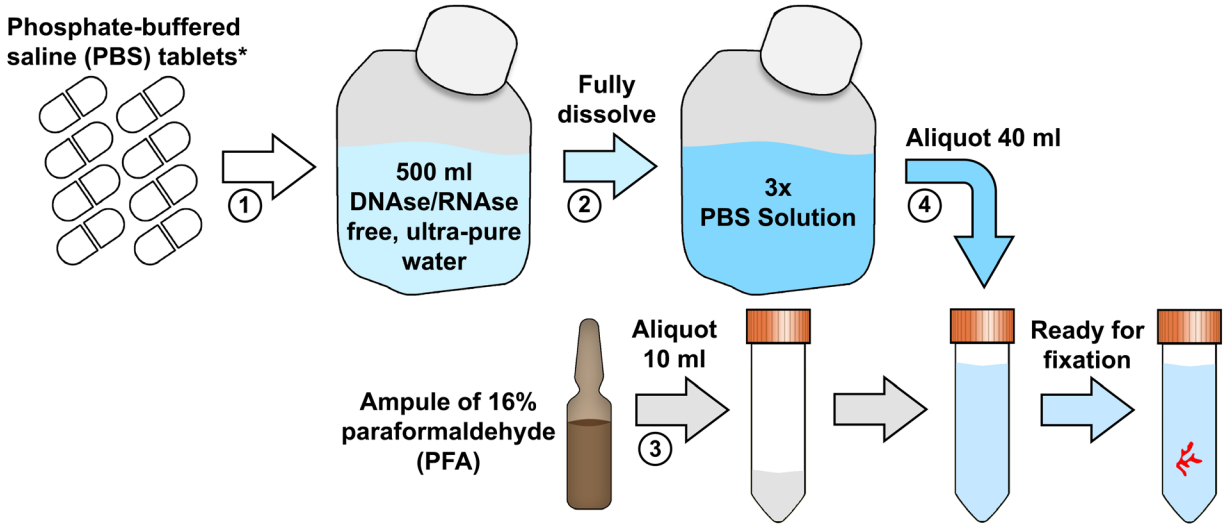

*Figure 1. Fixing samples for microbiome and histology analyses. (1) Place [15] PBS tablets into each 500 mL container of DNAse/RNAse free ultra-pure water, (2) Fully dissolve the PBS tablets to produce a 3X concentration solution, (3) Aliquot 10 mL of 16% paraformaldehyde into each 50 mL conical tube to be used for sampling, (4) then aliquot 40 mL of 3X PBS-buffered ultra-pure water into the conical tube. *NOTE: The number of PBS tablets used to achieve a 3X concentration will vary by manufacturer.*

Preparing spiked methanol for metabolomics (Fig. 2, Steps 1-4): The high-performance liquid chromatography (HPLC)-grade methanol is spiked with 2-aminoanthracene: tracking the spiked methanol used for each sample serves as a control in case of evaporative loss of methanol from the sample vials. Glass vials are recommended to prevent chemical interactions with methanol. (Step 1) Weigh approximately 0.95 mg of 96% 2-aminoanthracene into each of two unlabeled 20 mL glass scintillation vials (these two vials only used for mixing) and record the exact weight of 2-

aminoanthracene placed in each vial. (Step 2) Uncap the first 1 L methanol and extract 1.5 mL using a transfer pipette; (Step 3) dispense this 1.5 mL methanol into one of the glass scintillation vials to suspend the 2-aminoanthracene from Step 1, then (Step 4) pour this suspension back into the 1 L methanol container from which the 1.5 mL methanol was pipetted. The spiked methanol now contains a single, 0.95 mg dose of 2-aminoanthracene. Seal the 1 L methanol container, mix thoroughly, and label this 1 L container as "Methanol 1". Dispose of the used transfer pipette. Repeat steps 2-4 for the second 1 L container of methanol and the second scintillation vial with 2-aminoanthracene, using a new transfer pipette to ensure there is no mixing between the two methanol solutions. Label the second 1 L container as "Methanol 2".

Aliquoting methanol controls (Fig. 2, Steps 5-6): (Step 5) Gather the two 20 mL glass scintillation vials labeled "Methanol 1 Control". Using a clean bottle-top dispenser or serological pipette, dispense 15 mL of Methanol 1 into each of the two "Methanol 1 Control" vials. (Step 6) Next, dispense 15 mL from Methanol 1 into each of [60] 20 mL glass scintillation vials. Switch the bottle-top dispenser to the 1 L container of methanol labeled Methanol-2 and dispense 15 mL into a waste container to clear the bottle-top dispenser. Repeat steps 5-6 for Methanol 2. It is critical that all vials receive the same volume of methanol and only receive methanol from a single source container (Fig. 2). Note on each vial label and on a datasheet which source container was used for each vial (Methanol 1 or Methanol 2). Seal all methanol vials and store at 4°C or cooler for up to one week until sampling.

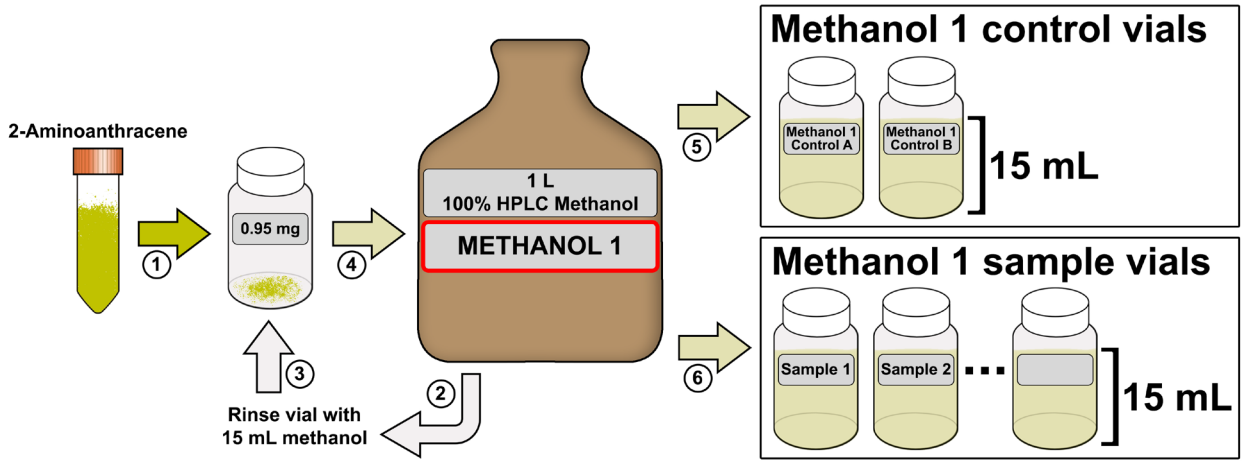

Figure 2. Preserving samples for metabolomic profiling. (1) Weigh 0.95 mg of 2-aminoantracene into each of two glass scintillation vials, (2) aliquot 15 mL from 1 L methanol into vial with the 0.95 g 2-aminoanthracene and (3) mix to suspend, then (4) pour this 15 mL 2-aminoanthracene suspension back into the 1 L methanol. (5) aliquot 15 mL of this spiked methanol into 2 vials labeled Methanol 1, Control A and B and (6) aliquot 15 mL of this spiked methanol into each of 60 labeled sample vials. Repeat this process for each 1 L container of methanol to be used, noting which vials received Methanol 1 vs Methanol 2.

Sampling design:

This sampling protocol has been developed for the collection and preservation of 120 coral fragments. Using sterile forceps each coral fragment (~4 cm^2) is split into two samples (each ~2 cm^2): one is fixed in paraformaldehyde then stored in phosphate-buffered saline for microbial and histological analysis, and the other is preserved in methanol spiked with 2-aminoanthracene for metabolomic analysis. The sampling design determines where and when fragments are collected from source colonies, and if more than one kit is necessary. Coral reef communities are highly variable and tradeoffs in the allocation of sampling effort for ecological experiments and long-term monitoring studies has an extensive literature (Bellehumeur & Legendre, 1998; Edmunds & Bruno, 1996; Legendre et al., 1989; Murdoch & Aronson, 1999; Scott Overton & Stehman, 1996). Most critically, no amount of post-processing can recover a sampling design that is poorly conceived for the primary question of the study. Sampling designs will differ between efforts to establish health baselines within a community and those used to characterize acute impacts such as disease outbreaks or runoff events. When sampling across multiple sites, effort should be made to characterize the environment and benthic community,

maximizing or minimizing site similarity to best meet the research question (Díaz-Pérez et al., 2016; Edmunds & Bruno, 1996). We provide three example sample designs to sample coral disease (Fig. 3) and provide details in Supporting Information 1 – *Sampling Design Considerations.*

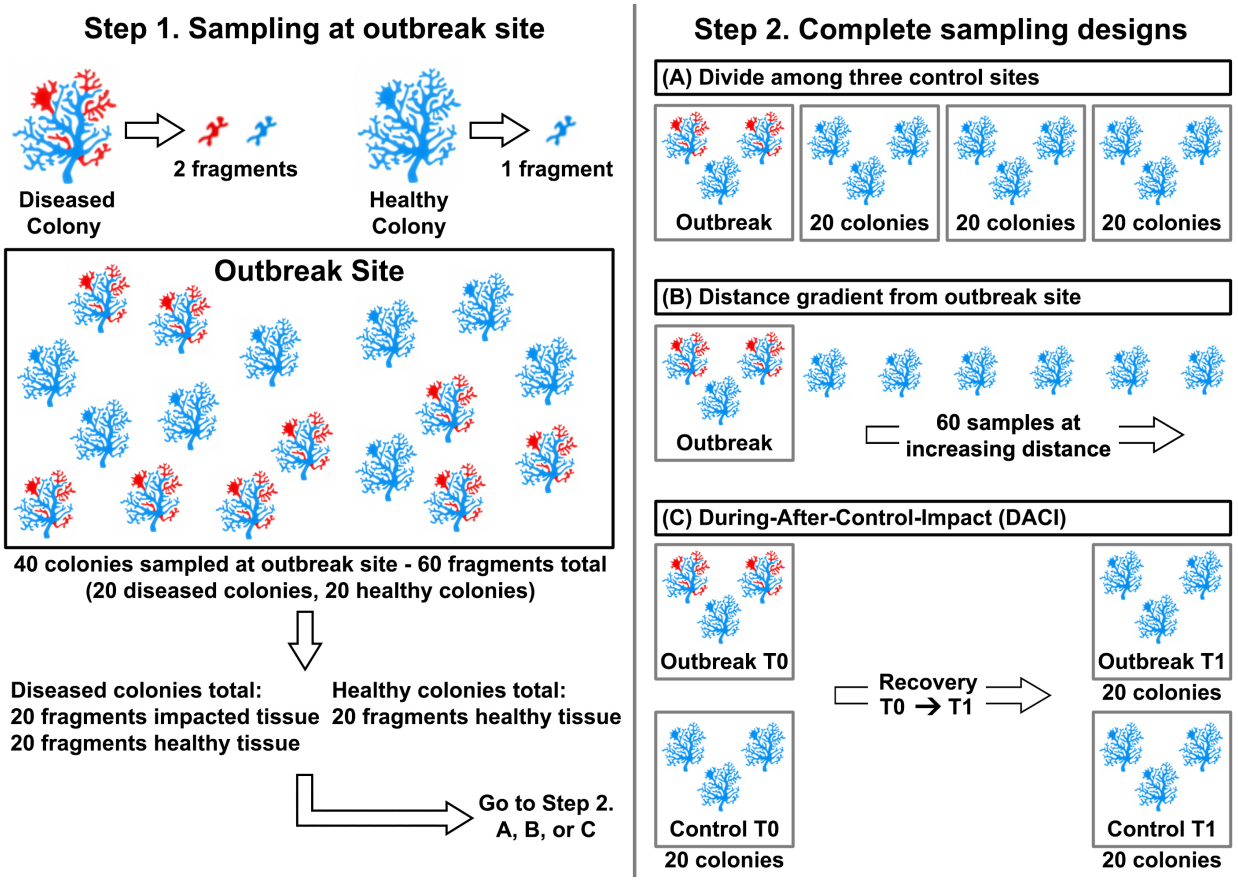

Figure 3. Examples of three coral disease sampling designs. Each design begins with the same initial outbreak sampling (Step 1) to characterize (i) within-colony impacted (red portions of colonies) versus visually healthy tissue (blue portions of colonies) and (ii) diseased (colonies with red and blue) versus visibly healthy colonies (blue colonies) at the outbreak site. Step 2: (A) provides comparison of visually healthy tissue on unaffected colonies at the outbreak site versus three non-outbreak control sites based on ecological site similarity, (B) provides distance-based sampling along a spatial gradient while maintaining disease-vs-healthy contrasts in the affected site, and (C) provides temporal contrast at the disease-occurring sites and at control sites, where T0 indicates the initial survey time point while the outbreak is present and T1 indicates a time point after the outbreak has subsided.

Sample collection:

Ensure all fragment collection plastic bags, PFA canonical tubes, and methanol scintillation vials have been pre-labeled with the site, colony number, and tissue type or other unique identifier. Prepare an accessible shore or vessel-based cooler with ample ice, pre-labeled PFA conical tubes, and pre-

labeled methanol scintillation vials. Once at the sampling site, collect all relevant data for your research question (benthic cover, fish abundance, anthropogenic stressors, water samples, etc.). Data for each sampled colony (such as size, species, health-state, percent of colony affected) should be collected just prior to sampling. Disease lesions should be described according to standard methods (Raymundo et al., 2008). When sampling includes colonies exhibiting disease, we strongly advise that two fragments be taken from each diseased coral colony (Fig. 4); one from visually healthy tissue and one that includes the disease lesion border (Supporting Information 2 – *Sampling Diseased Tissues*).

To sample massive or encrusting coral morphologies, use a small chisel and mallet to remove a ~4 cm^2 region of coral tissue while minimizing skeleton sampled; for branching corals, remove individual branches using bone cutters. Larger fragments may be required for coral species with large polyp sizes and depending on histological methods used, each fragment should encompass multiple polyps. Split the fragment into two samples (~2 cm^2 each; for diseased samples, include the disease lesion border in both samples) and immediately place both into the pre-labeled collection bag (Fig. 4). Limit exposure of samples to air and the amount of time before preservation. If sampling diseased coral colonies, always collect visually-healthy tissues before visually-diseased tissues (i.e., sample from “clean” to “dirty”). Alternatively, sample healthy tissues from all colonies followed by diseased tissues from all colonies or use a different set of sampling tools for healthy and unhealthy tissue types. Clean tools and change gloves before sampling a new colony to limit transmission risk to otherwise healthy corals. The most conservative approach would utilize new tools and gloves between every fragment collected.

After sampling, use sterile forceps to place one sample from each fragment into the corresponding PFA-filled conical tube, and the other into the corresponding methanol-filled scintillation vial. Each fragment collected should result in one PFA-preserved sample (for microbiome and histology) and one methanol-preserved sample (for metabolomics; Fig. 4). Place all sample vials on ice in a cooler

immediately after processing, taking care to avoid breakage of glass vials. Store PFA-preserved samples at 4°C and methanol-preserved samples at 0°C as soon as possible.

For PFA-fixed samples (microbiome and histology): Between 14-24 hours after fragment collection, pour off the PFA fixative from each sample, taking care not to lose or touch the coral tissue, and replace with PBS-buffered ultra-pure water (see *Preparing fixative* and Fig. 1) up to the 50 mL mark on the conical tube so as to adequately cover the sample. Follow all local regulations when disposing of waste 4% PFA solution. Store these samples at 4°C until further processing – do not freeze.

For methanol-extracted samples (metabolomics): Allow coral samples to extract in methanol for at least two weeks. There is no need to remove the coral from the methanol.

Sample processing: For detailed notes on processing and analysis of samples for histology, microbiome, and metabolomics see Supporting Information 3 – 5. Additional literature is available in Supporting Information 6 – *Grey Literature on Coral Health Methods.* Samples fixed in 4% PFA following this protocol are sufficient for both histology and microbiome analysis. Samples extracted in methanol are for metabolomic analyses.

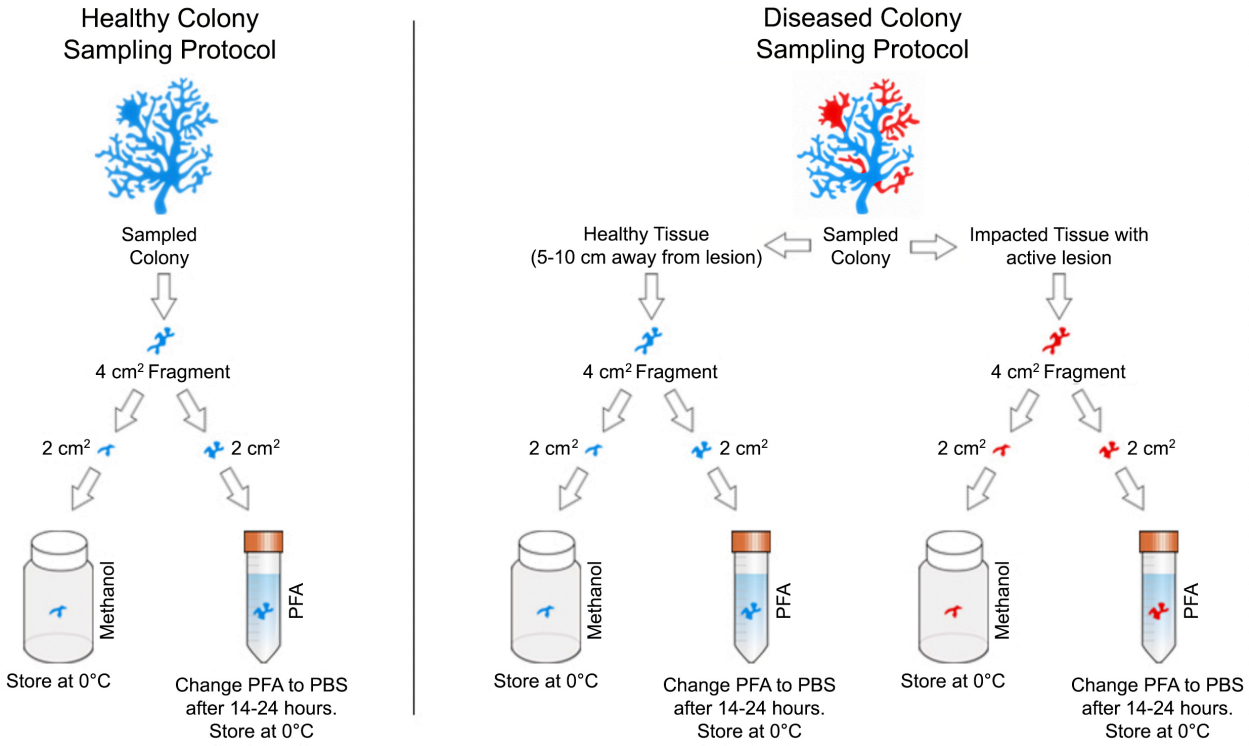

Figure 4. Depiction of fragment splitting for healthy (left) and diseased (right) coral colonies. Each fragment collected from a colony is split in half to produce a sample fixed in paraformaldehyde (PFA, for histology and microbiome) later stored in phosphate-buffered saline (PBS) and a methanol-extracted sample (for metabolomics). While a 4 cm^2 fragment is utilized here, larger fragment sizes may be required for corals with large polyp sizes. Both samples from diseased fragment should include a portion of the disease lesion border.

Discussion:

As coral health declines worldwide, improved and timely characterization of impacts to the coral holobiont will become increasingly important for research and management (Ainsworth et al., 2010; Bourne et al., 2016; Brainard et al., 2013; Cinner et al., 2016; Costanza et al., 2014; Miller et al., 2009; Sokolow, 2009). Inability to quantitatively link responses of the coral holobiont to changing environmental conditions prevents a deeper understanding of reef health and coral disease. While valuable, visual assessments are largely limited to the detection of disease lesions or bleaching, which represent late-stage responses to stress accumulation (Raymundo et al., 2008; Winston et al., 2019). Future coral health research should combine molecular and histological tools, taking a multidisciplinary

approach to detecting non-visible stress and characterizing coral disease.

We have provided one example of a methodology that combines collection and preservation of coral tissue samples suitable for histological analysis, assessment of microbiome dysbiosis, and metabolomic profiling of the coral holobiont. Collection of these samples is minimally invasive and efficiently uses the same fragment for multiple assay types. The described materials can be stored for long periods of time in advance of sampling, enabling rapid response to acute events, such as disease outbreaks. We successfully tested the effectiveness of these methods for multiple coral species (*Porites lobata, Montipora capitata, Pocillopora damicornis*) in Hawaii and Guam. Collected tissues are stable for long periods of time once stored as described, allowing delayed analysis, if necessary. As such, we believe these methods provide a valuable starting point for an improved, standardized survey of coral health that is flexible and viable across coral species.

Analysis of the coral microbiome is a rapidly changing field and requires cautious interpretation. Sampling effort of next-generation sequencing used to classify 16S ribosomal DNA has known limitations and requires careful application of standardization or rarefaction procedures to meet the needs of research questions without imparting unintended bias (Bolyen et al., 2019; Caporaso et al., 2012). For example, while preservation of coral in PFA allows fragments to be used for both histology and microbial analyses this technique may also cause underestimation of mucus-borne taxa (van Oppen & Blackall, 2019). Previous microbial research has demonstrated that PFA-preserved and flash-frozen samples are comparable (Hernandez-Agreda, Leggat, & Ainsworth, 2018), and we believe the refrigerated storage and stability of PFA-preserved samples is an advantage over flash-frozen samples. While we advocate for the use of end-to-end sequencing facilities to limit user-error by researchers or managers unfamiliar with these methods, we do not suggest that this reduces the requirement for researchers to thoroughly understand all steps and implications of data decisions made.

Metabolomic profiling presents exciting opportunities for studying coral health, but also

requires careful interpretation. First, observed metabolites represent the coral host animal, its microbiome, and organisms present in the coral skeleton. Without microdissection, metabolomic patterns observed should be considered in light of this untargeted sampling. Many metabolites identified through Liquid Chromatography Mass Spectrometry may not be characterized, cannot be linked to specific metabolic processes, or may be representative of correlated molecular pathways. Furthermore, metabolomic signatures can be sensitive to both coral genotype (Lohr et al., 2019) and small-scale environmental gradients in some species (Leggat, W. unpublished data). As such, use of metabolomic data requires targeted research questions and sampling designs.

At present, histology can be time-consuming and requires specialized equipment and expertise, though these constraints have been reduced recently (Toledo-Hernández et al., 2014). A valuable standalone tool to describe coral health (Work & Meteyer, 2014), histology also provides critical context to clarify microbial and metabolomic findings (Sogin et al., 2016; Work & Meteyer, 2014). Both metabolomic and microbial data can be highly sensitive to local abiotic or biotic factors and may conflict. Histological analysis provides a third axis of description, allowing users to determine host tissue health, zooxanthellae density, and degree of necrosis or apoptosis. Histology details coral host responses and can improve interpretation of metabolomic and microbiome datasets, which represent a mixed sample of host and microbiome, or the microbiome alone, respectively.

Using histology, microbiome analysis, and metabolomics in combination provides a modern approach to more thoroughly describe coral health beyond visible signs alone. These methods allow for the efficient collection of samples immediately following acute events (e.g., a disease outbreak) or along environmental gradients and have been field-tested at management scales. The methods we describe here are intended to serve as a starting point to many downstream analyses or monitoring strategies, opening the door to a more quantitative understanding of coral holobiont health.

Acknowledgements:

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Author Contributions:

Greene, Leggat, Ainsworth, Caldwell, Donahue, and Heron designed the sampling methodology. Greene developed the presented sampling "kit" and led the writing of the manuscript. Donahue, Caldwell, Greene, and Raymundo led field testing of the presented collection methods. Moriarty, Ainsworth, Leggat, and Greene led processing and analysis of samples collected as part of field testing. All authors contributed critically to the design of the presented methods, manuscript drafts, and gave final approval for publication.

Data archiving statement:

Data collected while developing these protocols is part of multiple ongoing research efforts. When published, these data (histology, metabolomics, and microbiome) will be archived in a publicly-accessible figshare repository (https://figshare.com/). Sequence data will be additionally archived in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). We expect to publish the first of these reports in 2021, cite this manuscript, and archive data as described above at that time.

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Table 1. Bill of materials for one sampling kit including per-unit and per-kit cost in USD in 2019. Kit described is for 120 fragments (see Sampling Design).

ITEM	SUPPLIER	PART #	UNIT COST	UNITS / KIT	PER-KIT COST
Vial labels (1,700 count, cryolabels)	VWR	89097-572	$82	0.15 (244 labels)	$12
50 mL sterile conical tubes (500 count)	VWR	21008-951	$134	0.25 (120 tubes)	$34
Phosphate-buffered saline (PBS) tablets (200 count)	VWR	97062-732	$79	1.65 (330 tablets)	$130
Bottle-top dispenser (for methanol)	VWR	470134-948	$241	1	$241
2-Aminoanthracene (powder, 1 G)	Sigma-Aldrich	A38800-1G	$31	0.002 (~2 mg)	< $1
Methanol (100%, HPLC)	Sigma-Aldrich	646377-1L	$68	2 (2 L)	$136
Quart-size ziplock bags (126 count)	Amazon / Generic	N/A	$11	1 (126 bags)	$11
20 mL glass scintillation vials (500 count, foiled polyethylene cap liner)	Amazon / Wheaton	N/A	$184	0.26 (126 vials)	$48
10 mL ampule of 16% paraformaldehyde (PFA) (10 count, liquid)	Fisher Scientific	50880487	$32	12 (120 ampules)	$384
500 mL ultra-pure distilled Water (10 count, DNAse/RNAse free or equivalent)	Fisher Scientific	10-977-023	$164	2.2 (22 containers)	$361
Nitrile gloves (100 count)	Fisher	19-130-1597	$30	1	$30
Face masks (300 count)	Fisher	19-088-129	$92	0.02 (6 masks)	$2
Safety glasses	Fisher	19-181-514	$4	2	$8
3.2 mL transfer pipettes (500 count)	Fisher	13-711-20	$106	0.02 (≥ 2 pipettes)	$2
				PER-KIT TOTAL:	Approx. $1,400