M.S. THESIS PROPOSAL

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This is to certify that this student's M.S. Supervisory Committee had read and approved the M.S. Thesis Proposal titled: **Investigating the effect of thermal bleaching stress on heavy metal concentrations in the reef-building coral *Montipora capitata***

Approval of Supervisory Committee:

POSITION	PRINTED NAME	SIGNATURE	DATE
CHAIR	Jacqueline Padilla-Gamiño	Jacqueline	April 16, 2023
MEMBER	Lisa Rodrigues	Lisa Rodrigues	April 21, 2023
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Investigating how thermal bleaching stress affects heavy metal concentrations in the reef-building coral *Montipora capitata*

Introduction:

Coral reefs build the most biologically diverse marine ecosystems on the planet that support tens of millions of people worldwide with food, storm protection, and other services (Moberg and Folke, 1999). While corals can consume food (e.g., zooplankton, detritus) through their polyps, most reef-forming corals rely primarily on photosynthetic algal symbionts in their tissues for their energy needs (Muscatine et al., 1981). However, ocean warming in the last few decades has triggered unprecedented rates of coral bleaching, in which thermally stressed corals expel their algal symbionts (Hughes et al., 2017). Bleaching stress can stunt growth and reproduction and may cause coral starvation and death (Loya et al., 2001; Ward et al., 2000). Although bleaching can be lethal for many corals, some species, like the hermaphroditic, reef-building *Montipora capitata*, often tolerate and recover from bleaching events and continue sexual reproduction despite thermal stress (Cox, 2007).

Regardless of their capacity to recover from bleaching, reef corals must also contend with stressors such as heavy metal pollutants. Heavy metals such as copper, lead, and arsenic from industrial emissions, antifouling paints, oil spills, and fuel discharge can affect the health of coral reefs (Berry et al., 2013; Guzmán and Jiménez, 1992). Heavy metals can produce acute or chronic toxicity, affecting critical biological processes in corals such as respiration (Howard et al., 1986), gamete fertilization (Hédouin and Gates, 2013), and larval settlement (Reichelt-Brushett and Harrison, 2000), sometimes causing physiological stress and bleaching (Harland and Brown, 1989; Howard and Brown, 1984). Paleoclimate studies have correlated bleaching with trace metals in corals' skeletal interface, hypothesizing that mucus secretion and coral tissue retraction along the skeletal interface could raise skeletal metal concentrations during bleaching events (Li et al., 2017). However, nothing is known about how bleaching stress could affect heavy metal concentrations in recovering corals' tissue or their subsequent offspring (i.e., gametes/larvae) (Guzmán and Jiménez, 1992; Reichelt-Brushett and McOrist, 2003).

Heavy metals are integrated into corals' tissues and skeleton (Guzmán and Jiménez, 1992; Reichelt-Brushett and McOrist, 2003; Trumbauer et al., 2022) primarily by ingesting particulate and food sources (i.e., by heterotrophy) (Bryan, 1980; Howard and Brown, 1984). Therefore, higher concentrations of copper, cadmium, and zinc have been found in corals that rely more on heterotrophy than photosynthesis (Berry et al., 2013). When corals such as *M. capitata* bleach and lose their algal symbionts, they gain energy by relying more on heterotrophy until they can recover their symbionts as a photosynthetic food source (Fig. 1) (Grottoli et al., 2006; Palardy et al., 2008). Thus, corals recovering from bleaching stress might bioaccumulate more heavy metals from zooplanktonic/detrital food sources than on a photosynthesis-supported diet. Heavy metals have also been shown to transfer to coral gametes (i.e., eggs and sperm) (Reichelt-Brushett and McOrist, 2003). Therefore, increased metal consumption via heterotrophy during bleaching stress might lead to higher metal concentrations within bleached corals' tissue, skeleton, and gametes.

Tracking the effects of thermal stress on heavy metals within corals could also clarify the alternative pathways of metal accumulation within the mutualism between the coral host and its algal symbionts. Previous work has shown that the algal symbionts within coral host tissue (Family: Symbiodiniaceae; LaJeunesse et al., 2018) accumulate most heavy metals including iron, arsenic, manganese, copper, zinc, cadmium, and lead at higher concentrations than the coral host (Reichelt-Brushett and McOrist, 2003). Similarly, metal exposure studies have found that

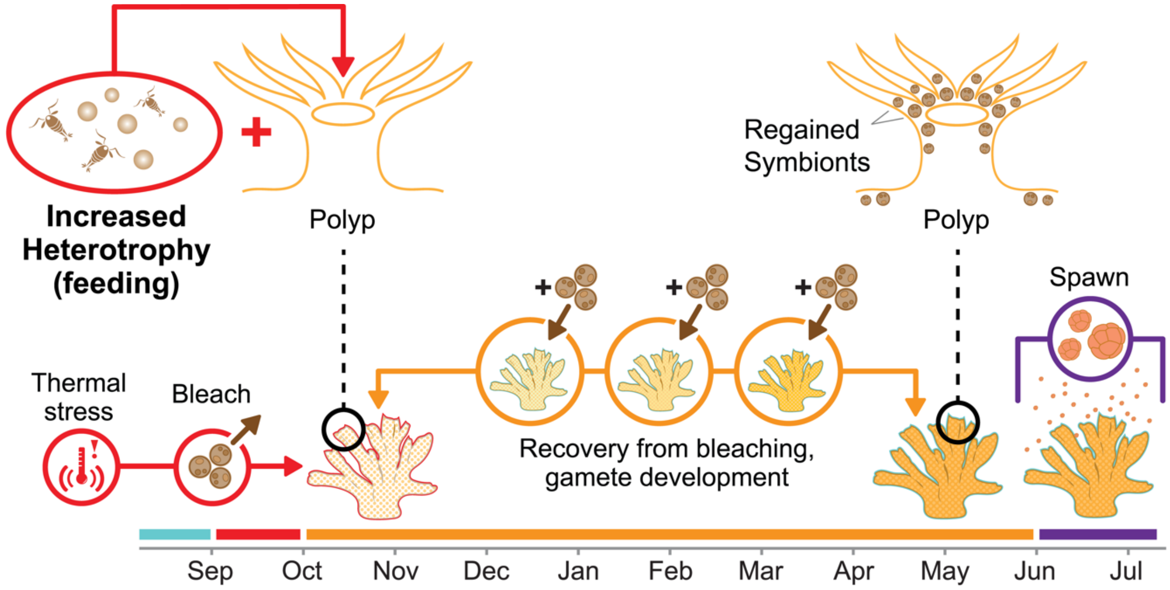

Figure 1. Diagram illustrating the progression of thermal stress, bleaching, heterotrophic plasticity (i.e., increased feeding), and symbiont recovery in *Montipora capitata*. Increased feeding may cause *M. capitata* colonies to ingest and incorporate more heavy metal contaminants in their tissues and gametes during recovery of their symbionts relative to healthy colonies.

anemones containing symbionts accumulate higher concentrations of some metals, including zinc and cadmium, relative to symbiont-free conspecifics (Mitchelmore et al., 2003). Within symbionts, heavy metals such as iron, manganese, zinc, copper, and cobalt can function as trace nutrients necessary for processes such as photosynthesis and antioxidant production (Ferrier-Pagès et al., 2018; Reich et al., 2020). Bleaching might initially eject many of these algal-concentrated metals from coral tissues. However, host heterotrophy during bleaching can pass essential metal micronutrients to the remaining symbionts (Ferrier-Pagès et al., 2018), so subsequent increases in coral heterotrophy may drive higher metal transfer to both the host tissue and the new symbionts as they are recovered. Analyzing metal concentrations in both the host and symbiont fractions of coral colonies during bleaching stress and recovery could clarify how metals are accumulated within the host or, alternatively, transferred to the algal symbionts.

Furthermore, many coral species like *M. capitata* transfer symbionts into their eggs prior to spawning (Padilla-Gamiño et al., 2011). While high concentrations of heavy metals such as zinc have been found in coral eggs (Reichelt-Brushett and McOrist, 2003), it is not known if parental symbiont transmission can expose eggs to excess heavy metal contaminants. Tracking differences in heavy metals stored within both host and symbiont components of coral tissue and eggs could reveal how metals gained during bleaching events affect host-driven processes, such as coral reproduction, relative to symbiont-driven processes, such as photosynthesis.

Objectives:

My research will investigate an unexplored intersection of the effects of climate change and anthropogenic pollutants on vulnerable reef ecosystems. I hypothesize that (1) after thermal stress (bleaching), corals increase the incorporation of heavy metals in their tissues and skeleton due to increased heterotrophy and (2) colonies that bleach will incorporate/translocate higher concentration of heavy metals into their eggs. Tracking the tissue concentration of symbionts after bleaching, and the metal concentrations within the symbiont fraction of each colony, I will clarify how metals are accumulated and stored in symbionts relative to the host during and after bleaching. For all analyses, I will focus on ten metals of environmental concern and/or known bioaccumulation (Fig. 2A), including zinc, copper, arsenic, and cadmium because they have the highest environmentally toxic impact (Berry et al., 2013; Haynes and Johnson, 2000). My proposed research has the following three objectives:

1) To determine the extent to which coral colonies bioaccumulate metals in their tissues/symbionts and translocate metals to their gametes, I will measure metal concentrations in field *M. capitata* colony and egg-sperm bundle fractions as well as in surrounding sediments as a proxy for environmental metal exposure (Fig. 2A).

2) I will thermally stress subsets of *M. capitata* colonies (Fig. 2B) to determine if bleached colonies accumulate more metals in their tissue and skeleton during recovery relative to unbleached colonies (Fig. 2C).

3) To determine if bleached corals transfer higher metal concentrations to eggs than unbleached corals, I will collect the egg-sperm bundles from the bleached/unbleached coral pairs during the spawning season after bleaching (i.e., the June/July spawn in the subsequent year after thermal stress) and compare their heavy metal concentrations.

Methods:

Collection of coral colonies, gametes, and sediment for relative metal concentrations

To determine relative heavy metal concentrations in field *M. capitata* host tissue and symbiont populations, I will collect fragments (~6-8 cm) from 10 colonies growing within 20 m of the eastern shoreline of the Hawaiian Institute of Marine Biology (Kāne'ohe Bay, HI). Each colony will be collected about 2-5 m apart to maximize the chance of sampling genetically independent colonies. Branching fragments will be chosen from branch-dominated colonies of relatively the same symbiont coloration (i.e., to avoid bleaching or disease as a confounding factor). All fragments will be cut using metal gardening shears and stored at -80 °C.

To determine ambient heavy metal concentrations in field *M. capitata* spawn samples, field egg-sperm bundles collected from Kāne'ohe Bay within proximity of the Hawaiian Institute of Marine Biology during the summer spawn (June/July new moon; Padilla-Gamiño and Gates, 2012) will be stored in 50 mL centrifuge tubes in ambient seawater and allowed to break and hydrate. Hydrated eggs will be pipetted into 2 mL tubes, where they will be transferred and rinsed with 1 um filtered sea water to separate sperm. To measure the ambient metal concentrations of Kāne'ohe Bay sediments from the same collection period, I will also collect 100 cm^3 of bay sediment from the same reef.

Controlled bleaching experiment

In August 2023, I will collect 25 small (25 cm diameter), healthy *M. capitata* colonies near the Hawaiian Institute of Marine Biology (Kāne'ohe Bay, HI). I will divide each colony in half (Fig. 2B) and allow each half to acclimate in separate tanks. In Sep. 2023, coinciding with the warmest period of bleaching events in Hawaiian waters (Axworthy et al., 2022), I will expose one half of each colony to elevated water temperatures (30 °C) for one month to induce bleaching while holding each colony's other half at ambient temperatures (~27 °C). After bleaching, I will return each colony to its original reef location and collect small fragments from each colony every month until bleached colonies have re-acquired their symbionts (by January the following year;

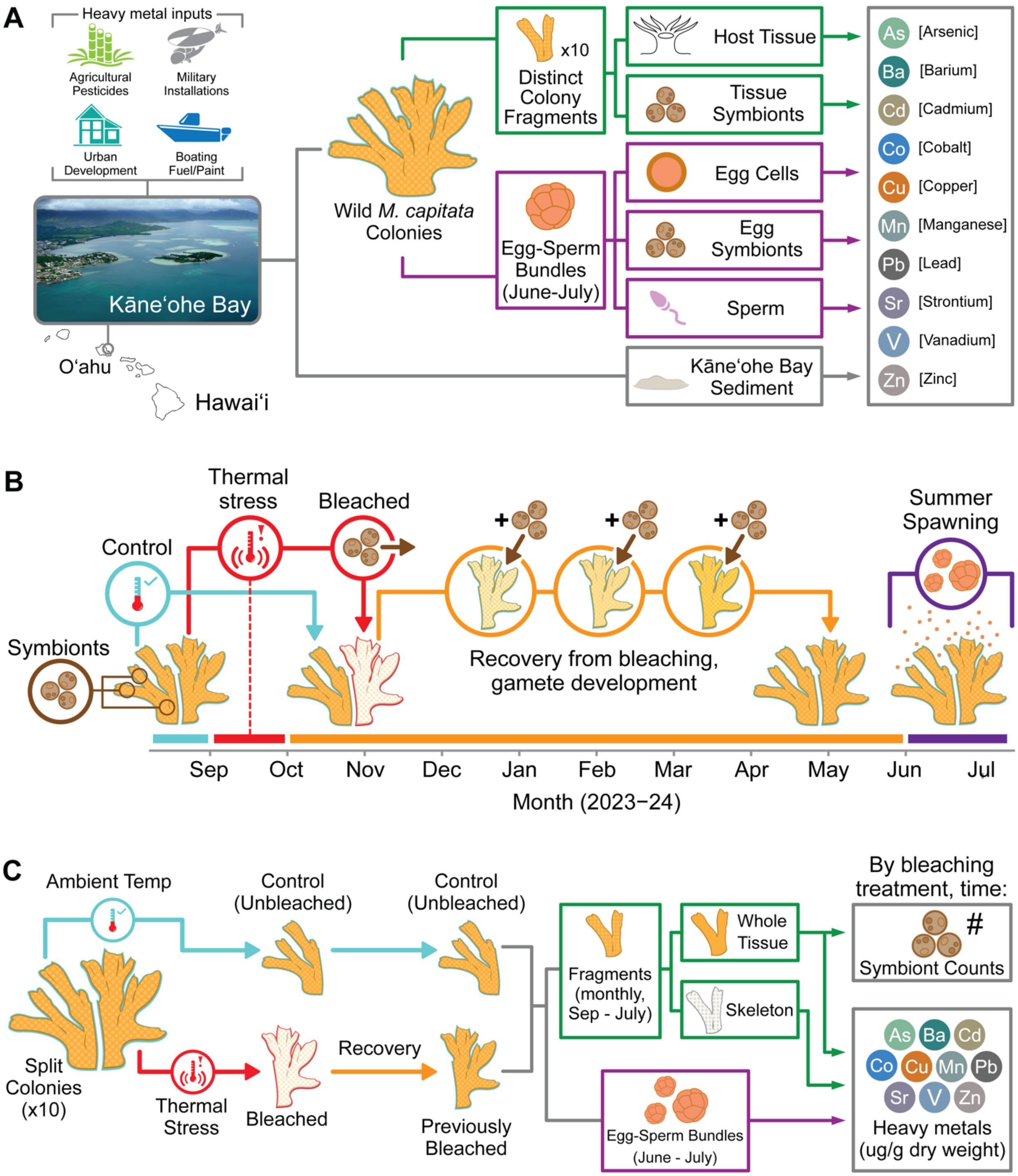

Figure 2. Conceptual summaries of data collection for objectives 1-3. (**A**) Summary of potential heavy metal inputs to reef ecosystems of Kāne‘ohe Bay, Hawai‘i, outlining collections for baseline measurements of metal concentrations in *M. capitata* colony specimens and bay sediments (*Objective 1*). Image credit: James Round (NASA). (**B**) Chronological summary of the thermal stress experimental design. Thermal stress and bleaching (Sep-Oct) coincides with the historically observed period of bleaching events in Hawaiian waters. (**C**) Summary of data collection and analyses from the thermal stress experiment (*Objective 2-3*). All heavy metal measurements to be collected via ICP-MS.

(Rodrigues and Grottoli, 2006). One week prior to spawning (June/July new moon; Padilla-Gamiño and Gates, 2012), I will re-collect all colonies, transfer them to isolated tanks, and collect their spawn (egg-sperm bundles). During collections, I will be advised by Dr. Jacqueline Padilla-Gamiño (University of Washington) and assisted by Dr. Lisa Rodrigues (Villanova University), two experts in coral physiology who have collected fragment and spawn samples from the proposed study system using the same bleaching experimental methods for trophic and reproductive analyses (Axworthy et al., 2022; Rodrigues and Grottoli, 2006).

Sample Processing

In the lab, I will remove the coral tissue from each fragment and then separate host tissue from algal symbionts (see Rodrigues and Grottoli, 2006). Since calcification rates in bleached and healthy *M. capitata* differ in the months after recovery but have only been quantified by weight (Rodrigues and Grottoli, 2006), I will use an Alizarin Red dye to stain the skeletons of a subset of test corals undergoing the same bleaching experiment and monitor their monthly growth rate (Eyal et al., 2019). Monthly re-application of stain will allow me to account for variations in growth between bleached and healthy colonies and across time. After removing the overlying tissue from each fragment, I will then use a Dremel to shave off the outermost layer of experimental coral skeletons corresponding to each month's mean growth estimate to compare skeletal material for metal concentrations.

I will dissolve all coral host tissue, symbiont, and skeleton samples, as well as each spawn sample, in HNO_3 solution using a MARS 6 microwave digestion system. Digested samples will all be run on an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) instrument at Villanova University to measure ten heavy metals of environmental concern and/or known bioaccumulation: arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), copper (Cu), manganese (Mn), lead (Pb), strontium (Sr), vanadium (V), and zinc (Zn) (Fig. 2C) (Berry et al., 2013; Ferrier-Pagès et al., 2002; Haynes and Johnson, 2000; Reich et al., 2020; Reichelt-Brushett and McOrist, 2003). I will place added focus on measured concentrations of zinc, copper, arsenic, and cadmium because they have the highest toxic impact for corals (Berry et al., 2013; Haynes and Johnson, 2000).

Statistical Analysis

As a contextual baseline, I will first use matched-pairs analyses to compare host tissue and symbiont metal measurements to determine how metals are distributed between host and symbiont components of both coral adult tissues and gametes (*Objective 1*). Correcting for metal concentrations measured for each initial colony before splitting and bleaching, I will then use matched-pairs analyses to determine whether bleached corals exhibited an increase in metal concentrations in a) tissue, b) symbionts, and c) skeleton over the recovery period relative to unbleached controls (*Objective 2*). I will conduct an additional set of matched-pairs analyses to determine whether bleached corals incorporated higher metal concentrations in their egg-sperm bundles than unbleached controls (*Objective 3*).

Results:

By measuring the symbiont concentrations within coral host tissue and concentrations of heavy metals across components of the coral colony, I will determine how the accumulation and storage of heavy metals within corals changes in response to bleaching stress. Since many heavy metals are primarily stored in corals' algal symbionts (Reichelt-Brushett and McOrist, 2003), I expect thermal stress and subsequent bleaching to initially reduce the concentration of many heavy metals throughout the coral colony. However, Ferrier-Pagés et al. (2018) have found that corals fed during bleaching stress transferred essential metal micronutrients such as Fe, Mg, and Mn from their food to their remaining symbiont populations. Therefore, I anticipate that subsequent increases in heterotrophy (as recorded by Grottoli et al., 2006; Palardy et al., 2008) will increase concentrations of heavy metals in both coral host tissue and recovered symbionts relative to control colonies. While coral skeletons typically hold lower concentrations of heavy metals than overlying tissue (Li et al., 2017; Reichelt-Brushett and McOrist, 2003), I expect some metals detected in skeletons such as barium (Reichelt-Brushett and McOrist, 2003) and copper, manganese, and lead (Li et al., 2017) to increase in the outer growth of bleached coral skeletons during bleaching recovery. Because bioaccumulation of heavy metals has been shown to vary seasonally in corals (Trumbauer et al., 2022), my control colony halves will account for seasonal trends in ambient heavy metal bioaccumulation relative to bleached colony halves. Finally, I expect that increased concentrations of heavy metals in colonies recovering from bleaching will be transferred to the egg-sperm bundles released during the subsequent year. My analyses will clarify whether egg-sperm bundles are more likely to accumulate some heavy metals relative to others. For example, potentially toxic metals with high lipid affinity, such as arsenic (Azizur Rahman et al., 2012), could accumulate at higher concentrations within the lipid-rich eggs of *M. capitata* (Padilla-Gamiño et al., 2011), with further implications for offspring viability and survival.

Interpretation:

This study will characterize the interaction of both bleaching stress and heavy metal bioaccumulation in an important reef-building coral species from Hawaii. Previous studies have hypothesized that corals that increase rates of heterotrophy (Grottoli et al., 2006) and increase nutrient sharing among regions of the colony (Palardy et al., 2008) to recover from bleaching events may have greater survivorship in the future. However, if bleaching-induced heterotrophy increases bioaccumulation of potentially toxic heavy metals within corals, both stressors must be considered to improve predictions for coral survivorship and fitness. My findings could lead to further studies of potential links between climate change and pollutants in corals, such as relationships between ocean acidification and heavy metal bioavailability or between thermal bleaching stress and microplastic bioaccumulation (Axworthy and Padilla-Gamiño, 2019).

My analyses of heavy metals across coral colony fractions could clarify whether metals are either contaminants or trace nutrients. For example, while cadmium is generally considered a non-essential and potentially toxic metal (Berry et al., 2013; Haynes and Johnson, 2000; Mitchelmore et al., 2003), at least one cadmium metalloenzyme used for photosynthesis has been characterized in marine diatoms (Xu et al., 2008). If cadmium is found primarily in coral symbionts, and if concentrations of cadmium in symbionts return to control colony concentrations (and no higher) after recovery from bleaching, then cadmium may be a trace nutrient necessary for normal symbiont function. Such discoveries would improve our understanding of the coral host-symbiont mutualism and essential nutrient cycling in response to environmental stressors like bleaching.

Significance:

This research will help Hawaiian and other communities identify and address anthropogenic threats to reef corals crucial for fisheries, ecotourism, and other services. The watersheds surrounding Kāne'ohe Bay have been used for various purposes including rice and sugarcane agriculture, urban development, and military installations, all of which can introduce toxic heavy metal contaminants to the Bay (Devaney et al., 1982; Hunter et al., 1995). Previous studies have detected high concentrations of lead, copper, and zinc in oysters at the mouth of Kāne'ohe Bay (Hunter et al., 1995). Furthermore, historical use of sodium arsenite pesticides on sugar plantations during the first half of the 20th century has elevated soil concentrations of arsenic throughout the Hawaiian Islands (Hue, 2013). My study will not only determine ambient concentrations of heavy metals in *M. capitata* corals but also measure whether bleaching stress could amplify heavy metal stressors experienced by these corals. Previous work correlating bleaching with increased heavy metals in coral skeletal growth was conducted on only one pair of colonies collected during a bleaching event (Li et al., 2017). By bleaching colonies and tracking their recovery in a controlled experimental setting, I can further determine whether increased metal concentrations in monthly coral skeletal growth bands detected during and/or after bleaching events are linked to metal concentrations in the overlying tissue and attributable to the bleaching event. If so, detecting elevated metal concentrations in past growth bands of coral skeletons could reveal previous bleaching events. Heavy metal measurements of distinct coral skeletal growth bands could then be used by other coastal communities throughout the globe as monitoring tools to assess reef health and the long-term effects of coral bleaching.

I hope to extend my research to community programs and outreach activities for Hawaiian citizens and government organizations. I will participate in Hawaii's regular Environmental Advisory Council meetings hosted by the state's Department of Health in Honolulu ("Environment Advisory Council," 2022) to apply my findings to coastal management and reef protection. I plan to work with the committee members of the council to share my findings at public meetings and contribute to local efforts to mitigate sources of heavy metal contaminants such as boat fuel discharge and antifouling paints (Berry et al., 2013; Guzmán and Jiménez, 1992). If I find high arsenic concentrations in coral tissue and gametes, my results can inform efforts to remediate agricultural soils and reduce legacy inputs of arsenic into coastal reef ecosystems, such as by planting Chinese brake fern (*Pteris vittata* L.) shown to efficiently capture soil arsenic in Hawai'i (Hue, 2013). With the He‘eia National Estuarine Research Reserve of Kāne‘ohe Bay ("He‘eia National Estuarine Research Reserve," 2019), an organization partnered with the Hawaiian Institute of Marine Biology (HIMB), I can integrate my findings with brake fern planting and harvesting programs that empower communities to restore coastal habitats and improve Hawaiian water quality. With HIMB faculty, I will use my digital illustration and animation skills and fieldwork footage to build modules for teachers across Hawai'i educating students on the values of reef conservation. I will also develop lessons on ocean pollutants for local elementary school teachers and build class activities using the NSF-LTER Bilingual Children's Book *Kupe and the Corals*, written by Dr. Padilla-Gamiño and translated into Hawaiian, Spanish, Tahitian, French and Paumotu (Padilla-Gamiño, 2014). Through these efforts, my research can provide benefits for local communities and environmental health that will enable us to better protect reef systems in the future.

Timeline / Workplan

Quarter	Progress / Goals
Winter 2021	Gathered frozen samples from NSF bleaching experiment (2017-18), tested sample processing and analysis on the ICP-MS of Villanova University's Department of Geography and the Environment.

Spring 2021	Processed and analyzed all egg-sperm bundle samples of NSF bleaching experiment (*Objective 3*), ran statistical analyses comparing bleached and nonbleached gamete metal concentrations
Summer 2021	Processed and analyzed all adult coral tissue and skeleton samples of NSF bleaching experiment (*Objective 2*)
Fall 2021	Ran statistical analyses comparing bleached and nonbleached gamete metal concentrations
Winter 2022	Submitted abstract detailing results from Objectives 2-3 to present at the 15th International Coral Reef Symposium (ICRS), Bremen, Germany, July 2022. Generated figures representing these results.
Spring 2022	Offered an oral presentation position at ICRS 2022 for this study's results. Generated more figures and presentation materials.
Summer 2022	Presented all current findings at ICRS 2022. Collected coral colony fragments and spawn material from June – July to complete more general background goals of *Objective 1*.
Winter-Spring 2023	Process and analyze materials from summer 2022 on an ICP-MS at Villanova University to complete *Objective 1*.
Summer 2023	Collect final round of spawn material and wild colony fragments for metal comparisons of metals in eggs/egg-symbionts vs. adult tissue/adult-symbionts. Process samples via ICP-MS at Villanova University.
Fall 2023	Finish writing manuscript. Submit manuscript to *Scientific Reports*.

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