THE IMPACTS OF PERFLUOROOCTANOIC ACID (PFOA) EXPOSURE ON HAWAIIAN CORALS

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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For my family, friends, professors, and mentors. Thank you for the knowledge and support that got me through this process

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a persistent organic pollutant (POP) that is characterized by fluorinated alkyl chains and resistant to all forms of environmental degradation. One of the most abundant compounds of per-and poly-fluoroalkyl substances (PFASs) in the natural environment, PFOA has been detected in water and sediment samples across the globe and acquired significant attention for its potential health implications and effects. Previous studies have suggested significant negative impacts on metabolic functions in humans, animals, and plants, but no study has yet quantified the impact on coral reef populations. This study investigates the effects of PFOA exposure on both early life phases and in molecular responses in Hawaiian reef-building corals, *Montipora capitata* and *Porites lobata,* respectively. Both physiological conditions, and molecular indicators of stress were measured with Western blot methods to analyze the expression of select coral proteins with xenobiotic metabolizing enzymes. No significant differences in fertilization success or larval survival were observed between treatments of environmentally relevant concentrations of PFOA in *M. capitata* gametes and larvae. Six molecular biomarkers were examined in Western blots for indications of sublethal stress in *P. lobata* adult samples. The study did not find significant indications of stress in the coral animal itself, but it did suggest impacts to heat shock protein, glutathione reductase, and cytochrome p450 expression. Future experiments should implement longer-term exposures and use more specific molecular techniques to get a clearer understanding of the threats involved with PFOA exposure, and to observe if exposure impacts the ability of adult corals to form gametes and perform spawning events. This information will aid in the

management and regulation of PFOA and other PFASs environmental and human health protection.

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LIST OF ABBREVIATIONS AND SYMBOLS

CYP1A1: Cytochrome p450 1A1

DI: Deionized water

EDC: Endocrine disrupting compound

FSW: Filtered sea water

GPx-1: Glutathione Peroxidase 1

GSR: Glutathione Reductase

HSP60: Heat shock protein 60

IC-gel: non-toxic Insta-Cure Cyanoacrylate gel

MHI: Main Hawaiian Islands

NWHI: Northwestern Hawaiian Islands

PBST: Phosphate buffered saline with Tween® 20

PFASs: Per-and poly-fluoroalkyl substances

PFOA: Perfluorooctanoic acid

pg/L: picogram per liter

ng/L: nanogram per liter

mL: milliliter

mg: milligram

µg: microgram

PFOA: Perfluorooctanoic acid

PFOS: Perflurooctonate sulfonate

POP: Persistent organic pollution

Ppb: Parts per billion

Ppt: Parts per trillion

PTFE: Polytetrafluoroethylene, Teflon®

PVDF: Polyvinylidene fluoride (membrane)

Rpm: Revolutions per minute

SELENBP1: Selenium binding protein

$SOD1^{-}$: Superoxide dismutase 1

°C: Degrees centigrade

s: seconds

h: hours

Z-fix: Zinc formalin solution

1.0 INTRODUCTION

1.1 INTRODUCTION TO CORAL REEFS

Coral reefs built by scleractinian corals have dominated shallow tropical marine systems for 200 million years, and are vital ecologically and to global economies, cultures, and for their ecosystem services. Only making up 0.1-0.5% of the ocean floor, they harbor nearly a third of the world's marine life and are the most biodiverse ecosystem on the planet (McAllister, 1991). Coral reefs support nearshore fisheries that provide food for millions of individuals and natural product of biomedical interest (Sorokin, 1993). In Hawai'i alone, they are estimated to have a total value of US$10.3-16.4 million and provide more than 7 million meals annually (Grafield, et al., 2017). Reefs are also the primary line of defense in coastal protection, preventing damage to coastlines and coastal communities. The annual risk-reduction benefits of coral reefs in the United States are estimated at US$1.8 billion (Reguero, et al., 2021). A large amount of the United States reef area is concentrated in the Hawaiian Archipelago, including both the Main Hawaiian Islands (MHI) and Northwestern Hawaiian Islands (NWHI) (Figure 1), making Hawai'i extremely vulnerable to the consequences of declining reef systems (Cesar, Beukering, 2004).

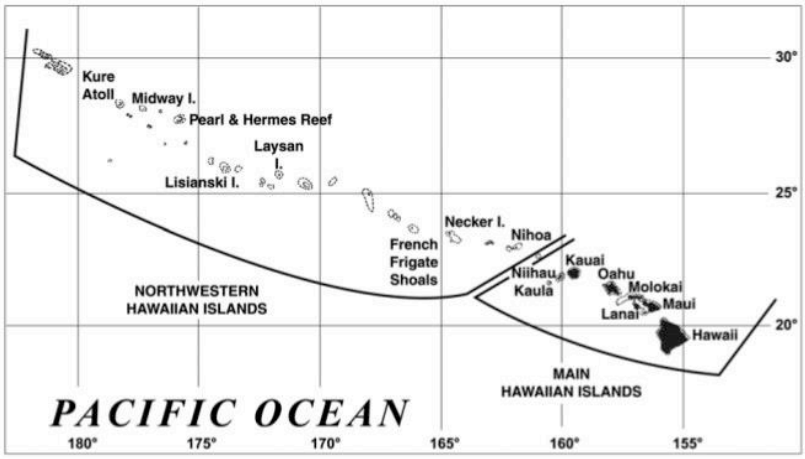


Figure 1: The Hawaiian Archipelago (Kahea Environmental Alliance, 2021)

The Hawaiian creation chant—the *Kumolipo*—depicts corals as the first living organism from which all other life evolved. Additionally, corals were used in traditional Hawaiian medicine and in the construction of sacred temples *(heiau)* and fishing shrines *(ko'a)* (Gregg, et al., 2015). The connection between humans and nature in Hawaiian culture gave them a deep understanding of their surrounding environment; they even recognized the methods of coral reproduction centuries before Western scientists made their first observations. Hawaiians also recognized how their activities directly impacted local ecosystems—including the sea—and utilized conservation techniques to preserve them, a skill that has lost weight in modern societies (Gregg, et al., 2015).

1.2 GLOBAL CORAL REEF STATUS

While reef-building corals can be resilient and demonstrate from acute natural disturbances, anthropogenic disturbances, often chronic in nature, have made them increasingly at risk (Good, Bahr, 2021). It is estimated that only 6% of coral reefs globally will remain unaffected by anthropogenic impacts such as climate change, ocean acidification and pollution (Good, Bahr, 2021). With climate change, thermal anomalies have become more intense and frequent, causing bleaching in corals as they expel their endosymbiotic algae that provides them with 95% of their nutrients (Hoegh-Guldberg, et al., 2007). Increased ocean acidity due to anthropogenic carbon deposition also inhibits the formation of aragonite coral skeletons and can lead to brittle corals that are increasingly vulnerable to erosion, grazing, and storm damages (Hoegh-Guldberg, et al., 2007).

The success of coral reefs is dependent on both the health of adult colonies, and their ability to reproduce and recruit new individuals in the population (Richmond, et al.,

2018). Scleractinian corals reproduce predominantly through mass spawning events where eggs are fertilized and embryos develop in the open water column. Reduced water and bottom quality interferes with their ability to utilize the chemical signaling that synchronizes spawning events, and facilitates fertilization success and subsequent larval development and recruitment (Richmond, et al., 2018). While conservation efforts do exist, they generally fail to protect reefs from land-based sources of pollution, and traditional techniques to assess reef health are limited to bleaching/mortality observations that do not provide sufficient information for policy reform (Richmond, et al., 2007; Downs, et. al, 2005). As intervention and remediation strategies are most effective when they can be implemented before outright coral mortality, data on stress at sublethal levels are valuable for proper remediation.

Cellular diagnostics can now be used to create a more complete picture of reef health by determining specific cause-effect relationships of coral reef decline with clinical biomarkers. These biomarker proteins are known markers of metabolic stressors, such as oxidative stress and detoxification in model organisms of rats and rabbits, but can also be extended to corals. This is especially true when looking at the implications of exposure by chemical pollutants, which can often interfere with the viability of coral reefs without killing them entirely (Downs, et. al, 2005).

1.3 PERFLUOROOCTANOIC ACID (PFOA)

Perfluorooctanoic acid (PFOA) is a fluoropolymer within the chemical subgroup, per-and poly-fluoroalkyl substances (PFASs), a group of chemicals first manufactured by DuPont in the 1940s. PFOA is widely used and released industrial and commercial

applications, such as during the production of protective coatings in textiles, paper, aqueous fire-fighting foams, electronics, insecticides, surfactants, consumer houseware, and in the production of other chemicals like tetrafluoroethylene (commonly known as Teflon®) (Emmett, et. al, 2006). Characterized by fluorinated alkyl chains, the strong electronegativity and small atomic size of fluorine provides PFASs with enhanced production capacity, surface activity, and water and oil resistant properties compared to traditional hydrocarbons (Wang, et al., 2017). The strength of the carbon-fluorine covalent bond also makes PFASs resistant to environmental, biological, and photochemical degradation (Emmett, et al. 2006). Evidence suggests that the chemicals' persistence in the natural environment aids in the prevalence and spread of the compound, and it has now been detected in even some of the most remote locations on the globe. (Emmett, et. al, 2006).

Of the PFAS compounds, PFOA has gained significant attention as it is one of the most commonly detected PFAS species and is both directly manufactured for commercial use, and is a byproduct in the manufacturing and partial degradation of other PFAS compounds (Wang, et al., 2017) Pathways of PFOA exposure in the natural environment include discharge from manufacturing facilities, use of consumer and household products, wastewater treatment plant (WWTP) discharge, and contaminated landfill leachates (Mueller, et al. 2020). Yamashita et al., analyzed open ocean water samples taken across the Pacific and Atlantic Oceans, and detected PFOA and perfluorooctanoate sulfonate (PFOS)—the two most prevalent and researched subspecies of PFASs—in 80% of the samples analyzed (2005). A limited number of studies in humans, rats, and fish have observed that PFOA and other PFASs are endocrine disruptors—with effects of exposure

ranging from hepatomegaly, necrosis in pregnancy, increased liver toxicity, high blood pressure and cholesterol, decreased sperm count, and tumors of the liver, testes and pancreas (Emmet, et. al, 2006; Ye, et al., 2009).

1.4 PFOA AND CORAL REEF ECOSYSTEMS

It is well understood that coral reefs are highly susceptible to the effects of local pollution. Due to the widespread prevalence of PFOA and the numerous studies that have demonstrated concerns for its negative effects, it is vital to gain an understanding of the potential implications for coral reef ecosystems. As an endocrine disrupting compound, it can be assumed that the negative implications of PFOA exposure would extend to coral animals, but up to this point few studies has quantified the relationship between exposure to PFOA and the biological effects on coral species. The goals of this study were to quantify the impact of PFOA exposure on fertilization success, and larval survival of *Montipora capitata* and the physiological and sublethal indicators of stress on adult *Porites lobata* individuals. One 2006 study sucesfully applied clinical biomarkers of cytochrome P450 1A1 (CYP1A1), heat shock protein 60 (HSP60), selenium binding protein-1 (SELENBP1), superoxide dismutase 1 (SOD1), glutathione peroxidase-1 (GPx-1), and glutathione reductase (GSR) to analyze the metabolic impact of marine fuel oil to *Pocillopora damicornis* (Rougée, et al., 2006). Therefore, I hypothesized that the same biomarkers could be used to detect sublethal stress in *P.lobata* samples exposed to PFOA.

2.0 METHODS

2.1 STUDY POPULATION

M. capitata, also known as the rice coral, was the target species for observing the effect of PFOA exposure on both fertilization success and survival rates of planula larvae. *M. capitata* is one of the most abundant reef-building corals in the MHI and has high phenotypic plasticity. They are also a hermaphroditic broadcast spawner that releases egg-sperm bundles in mass quantities during spawning events in the summer months (Padilla-Gamiño, et al., 2012). *M. capitata* was thus selected for this study for its' accessibility and relevance to determining the overall threats to coral reef reproduction on O'ahu.

For the exposure of PFOA on adult corals, *P. lobata,* or lobe corals, were the study population. As a massive coral, *P. lobata* has been seen to have higher survival rates in mass bleaching events compared to branching corals like *M. capitata* (Levas, et al., 2013). By analyzing a more resilient coral, signs of bleaching and/or stress may be considered more relvant to coral populations as a whole and give a clearer idea of the potential implications of the respective impacts of PFOA exposure. If *P. lobata* shows signs of stress in response to PFOA exposure, more sensitive coral species such as *M. capitata* may also be affected.

2.2 SAMPLE PREPARATION

PFOA samples were prepared by making a 100 ppm solution of PFOA in 0.2 µ filtered sea water (FSW). The PFOA stock solution was then diluted with FSW to the desired concentrations of 0.01 ppt, 4 ppt, 70 ppt, and 142 ppt, respectively. The exposure concentrations were selected based on the USEPA established water health advisory level of 70 ppt (0.07 µg/L) for drinking water, and 2005 ocean sampling data from across the

Pacific Ocean. Throughout the Pacific, concentrations ranged between highly industrial areas like Tokyo Bay, which has concentrations of PFOA as high as 192,000 pg/L (0.192 µg/L), the lowest concentrations were in Eastern Pacific surface water (15 pg/L or .000015 µg/L) (Yamashita, et. al, 2005). Based on this information, the exposure gradient applied ranged from some of the lowest Pacific concentrations, the approximate concentration in the western pacific region, and the highest detected PFOA level of the region, in addition to a control group.

An unpublished study from Kewalo Marine laboratory in 2018 observed fertilization success of *M. capitata* at 4 ppt, 70 ppt, and 142 ppt, therefore the concentrations were repeated, and one lower concentration (0.01 ppt) was added to determine the extent by which any PFOA exposure could have negative implications on coral viability (Messengei, 2019). As there is a lack of degradation mechanisms for PFOA, and the concentrations created were extremely low, there is varying confidence in the consistency of the desired concentration amounts, one additional fertilization experiment was done with concentrations 1000 times as strong as the other exposure groups (0.01 ppb, 4 ppb, 70 ppb, 142 ppb). One larval experiment also added a 70 ppb treatment to account for effects at 1000 times the EPA safe water level.

2.3 *MONTIPORA CAPITATA* FERTILIZATION ASSAYS

Gametes were collected from *M. capitata* colonies in Kāneoʻohe Bay, Oʻahu, Hawaiʻi on June 20, 2020, June 21, 2020, July 20, 2020, June 9, 2021, and July 8, 2021. Five gamete traps were placed over large colonies with engorged polyps in the bay (Figure

2) and collected after spawning, which began around 9:00pm. The egg-sperm bundles were mixed from two to three separate colonies to fertilize the gametes.

Figure 2: Gamete trap over coral colony

PFOA concentrations of 0.01, 4, 70, and 142 ppt were prepared in 49 mL solutions. In addition to the exposure concentrations, FSW was used as a control treatment group. Assembled in clear, glass jars with Teflon® lined lids, there were 6 repetitions per treatment. Upon collection of the gametes, approximately 10-12 egg-sperm bundles from 2-3 separate *M. capitata* colonies were placed in each treatment jar. After 5 hours, ~10 embryos from each replicate were placed in 1 mL of 10% zinc formalin fixative (Z-fix) for the summer 2020 assays. For summer 2021, that number was increased to ~20 embryos in the 1mL Z-fix solution, freezing the embryos in the 16-cell developmental stage. Successful fertilization of an egg-sperm bundle is visibly apparent under a light microscope, where cells at the 16-cell division stage would have a clover-like structure, and unfertilized gametes would remain in a smooth, spherical shape (Figure 3). Shapiro-

Wilks test for Normality, Levene's test for variance and Wilcoxon Kruskal-Wallis by ranks test were calculated for each collection day using R.

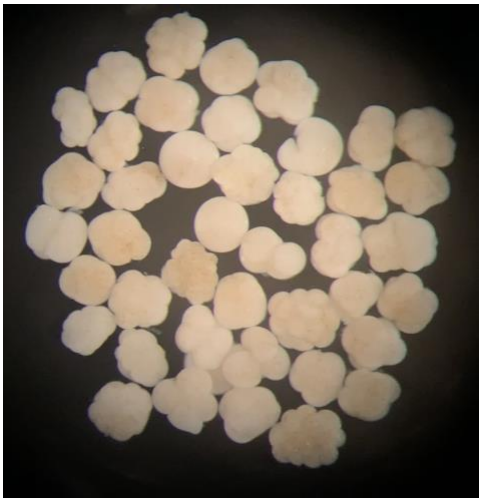


Figure 3: Sample of *M. capitata* gametes of varying fertilization status at 5 h developmental stage

2.4 *MONTIPORA CAPITATA* LARVAL EXPOSURE TO PFOA

Upon gamete collection for fertilization assays at Kāne'ohe Bay, excess gametes from several colonies were placed into a cooler filled with FSW and left to develop into planula larvae. Using the same treatment exposure gradient of 0.01, 4, 70, and 142 ppt with an FSW control group, nine-day old larvae from the most recent gamete collection were placed in 10 mL glass jars with Teflon® lined lids. For summer 2020 collections there were ~10 larvae per treatment with 6 replicates; survival was quantified after 24, and 48 hours. For the July 2021 collections, one additional treatment of 70 ppb—1000 time the EPA safe water level—was added for additional assessment. ~10 larvae were placed in each exposure group with 5 replicates per treatment. After 48 hours the larvae were observed for survival rates. Larval survival was determined by quantifying the number of larvae intact and swimming in each replicate.

2.5 *PORITES LOBATA* ADULT EXPOSURE TO PFOA

P. lobata colonies, which were grown at Kewalo Marine Laboratory, were fragmented into nubbins, and placed on small ceramic tiles using non-toxic Insta-Cure Cyanoacrylate Gel (IC-gel) coral frag glue. The fragmented nubbins were left in a continuous flow seawater table for a month to recover and on day 0 of the exposure period, the colors of each coral nubbin were noted using the Hawaiian Ko'a card (Figure 4).

Figure 4: Hawaiian Koʻa Card (coral reef ecology lab)

Between December 4-11, 2020, the *P. lobata* nubbins were exposed to FSW, 0.01, 4, 70, 142 ppt for a period of 3, and 7 days, respectively. The test chambers were 2 L glass beakers with 2 *P. lobata* nubbins per beaker and 5 replicates each for the 3-day exposure (Figure 5). After the 3-day exposure period, one nubbin was removed from each beaker for further analysis and the other left for the 7-day exposure. Upon removal of the first nubbin after the 3-day exposure, a complete water change was performed for each beaker. Throughout the exposure period, daily observations were made on physical signs of stress (bleaching, polyp presence and retraction, and mucus production) for each coral. At the

end of each exposure period the nubbins were flash frozen using liquid nitrogen, and stored at -80°C.

Figure 5: Adult Exposure to PFOA experimental setup

2.6 SDS-PAGE AND WESTERN BLOTS WITH *P.LOBATA* ADULT EXPOSURE

2.6.1 PROTEIN EXTRACTION

Frozen coral tissue was crushed with a mortar and pestle into a fine powder for protein extraction and placed in 1.5 mL Eppendorf tubes stored at -80°C. To begin the protein extraction, 200 µl of 6 M urea in 50 mM Ammonium bicarbonate (NH_4HCO_3) was added to each sample. Each sample was homogenized for a minimum of 30 s per sample using a handheld homogenizer until the solution was properly blended, with careful attention paid to ensure the temperature of each sample did not increase significantly. The ground samples were then centrifuged at 10,000 rpm for 20 minutes at 4°C, and the supernatant was transferred to separate Eppendorf tubes, with 10 µl per tube and frozen at -80°C.

2.6.2 PROTEIN QUANTIFICATION

To quantify the amount of protein in each 10 µl sample, a dye working solution was assembled using 200 µl of a homogenization buffer and 1 µl dye for each sample, mixed by vortexing. 190 µl of the solution was aliquoted to 3 Qubit® assay tubes for standards and 10 µl of each standard to an assay tube, mixed again by vortexing. 180-199 µl of this working solution was added to assay tubes for the extracted protein samples, 1-20 µl of each sample (according to working solution volume) was added to each tube, giving each sample tube a total volume of 200 µl. Each sample was then incubated for 15 minutes and the resulting protein quantification was read in a Qubit® 2.0 Fluorometer.

2.6.3 SDS-PAGE AND WESTERN BLOTTING

Using the supernatant samples from the urea protein extraction (not the 10 µl dye sample), samples were thawed and centrifuged at 10,000 rpm for 10 s to ensure each protein was fully mixed and at the bottom of each tube for easy extraction. The amount of protein samples and loading dye was calculated using a 1:4 ratio, respectively. Once calculated, and the proper amounts of protein sample and loading dye were added to Eppendorf tubes, the samples were centrifuged at 10,000 rpm for 10 s and placed onto a heat block set at 95°C for 5 minutes to denature the proteins. To separate the protein for observation, the entire sample and loading dye mixture was loaded into a fifteen-lane Mini-PROTEAN® TGX™ precast gel (Bio-Rad, Hercules, CA). Once ten samples (5 selected at random from the 3-day exposure, and 5 from the 7-day exposure) and a positive control of HeLa whole cell lysate (Santa Cruz Biotechnology, Dallas, TX) were added to the appropriate wells in the gel, the gel was left to run at 80 V for approximately 25 minutes, then voltage was

increased to 110 V for 60 minutes. While not always necessary, my experimental run included an additional ten minutes at 120 V.

After the run was completed, the proteins were transferred from the gel to PVDF membranes, and left for 2 h at 4°C under a current of 100 V. The membranes were rinsed with deionized (DI) water and washed with a phosphate buffered saline with Tween® 20 (PBST) solution for 5 minutes. Membranes were blocked in a 5% nonfat dry milk solution and placed onto a rotary table with a cover for one hour with 12 µl of the following primary antibodies from ThermoFisher Scientific: anti-GSR (IgG rabbit clone, PA5-70004, 1:1000 dilution), GPx-1 (IgG rabbit clone, PA5-30593, 1:1000 dilution), Anti-HSP-60 (IgG rabbit clone, PA5-34760 1:200 dilution), anti-CYP1A1 (IgG rabbit clone, PA5-14213, 1:1000 dilution), anti-SELENBP1 (IgG rabbit clone, PA5-37332, 1:1000 dilution), and anti-SOD1 (IgG rabbit clone, PA1-30195, 1:2000 dilution), dilutions were performed with PBST. After the membranes were blocked, a rinse was performed with DI, and followed with four PBST washes for a duration of 15, 10, 5, and 5 minutes, respectively, changing the PBST solution with each wash.

Once the primary antibodies had been prepared, the membranes were incubated with goat anti-rabbit secondary antibodies for two hours at room temperature on a rotary table. Again, the membranes were rinsed with DI, and followed with four PBST washes for a duration of 15, 10, 5, and 5 minutes, respectively, changing the PBST solution with each wash. For imaging of the blots, Pierce® Enhanced Chemiluminescence (ECL) Western Blotting Substrate, and a C-DiGit® blot scanner were used. Image Studio® software was used to quantify the net intensity of the band signal. The respective

band intensities were normalized to the intensity of the HeLa whole cell lysate (control ladder

3.0 RESULTS

3.1 STATISTICAL ANALYSES

Data was analyzed using Rstudio 1.4.1564 IDE software (Rstudio, PBC). Nornality of the distribution of the data was assessed by performing Shapiro-Wilk W test, and homogeneity of variance tested by performing Wilcoxon Kruskal-Wallis by ranks test. An alpha level of 0.05 was used for all hypothesis test statistics.

3.1.1 FERTILIZATION ASSAYS WITH *M. CAPITATA*

For each collection day, embryos were observed at the 5 h mark at the 16-cell stage of development. Since the data sets did not meet the requirements of normality, ANOVA could not be performed, and Wilcoxon Kruskal-Wallis by ranks tests were used to compare the median percentages of successful fertilization among treatments. No significant statistical difference in fertilization success was found among treatments, nor was there consistency in trends between collection days (6/20/20: χ^2: 7.6089, p-value: 0.107; 6/22/20: χ^2: 13.319, p-value: 0.009817, 6/10/21: χ^2: 0.4582, p-value: 0.9774, 7/7/21: χ^2: 6.2701, p-value: 0.1799). Across collection days, fertilization rates among the 142 ppt ranged from 22% to 100% while FSW ranged from 14.2% to 100%. One collection day had a weak, but visible decrease in fertilization rates with increasing concentrations (Figure 6). Although there was non-significance in the results, abnormal development was observed in some gametes; although they were fertilized, they did not develop into the traditional clover-like structure.

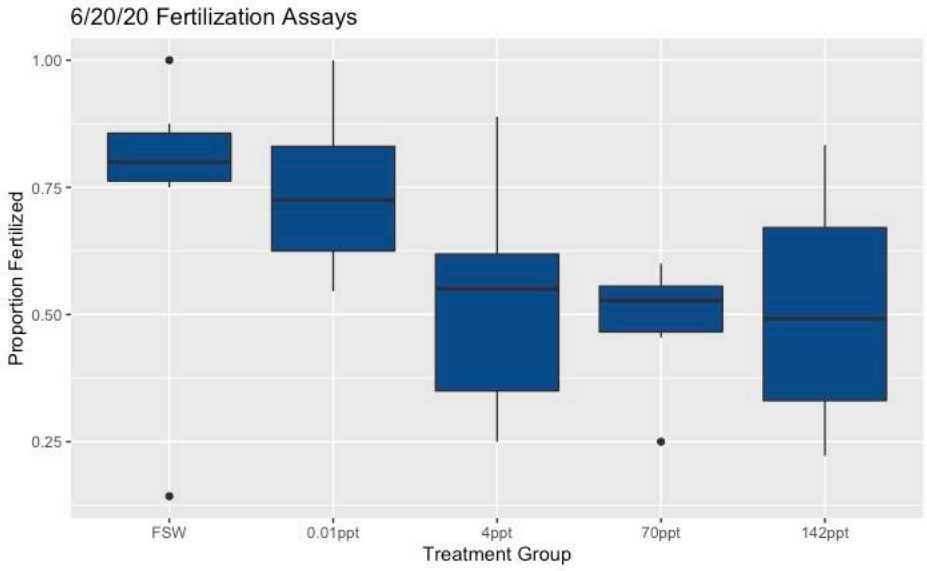


Figure 6: Boxplot of *M. capitata* Fertilization Success with exposure to PFOA (June 20, 2020)

3.1.2 *M. CAPITATA* LARVAL EXPOSURE TO PFOA

Larval survival was quantified after both 24, and 48 h with percent survival ranging from 80-100% within the control group, 70-100% in the 0.01 ppt group, 80-100% in the 4 ppt group, 30-100% for 70 ppt, and 50-100% for 142 ppt after 24 hours. After 48 h the range was 40-100% in the controls, 50-100% in 0.01 ppt, 76.9-100% in 4ppt, 60-100% for 70 ppt, and 30-100% for 142 ppt. There was no significant difference in percent survival among the 5 treatments tested (χ^2: 3.3753 p-value: 0.4971).

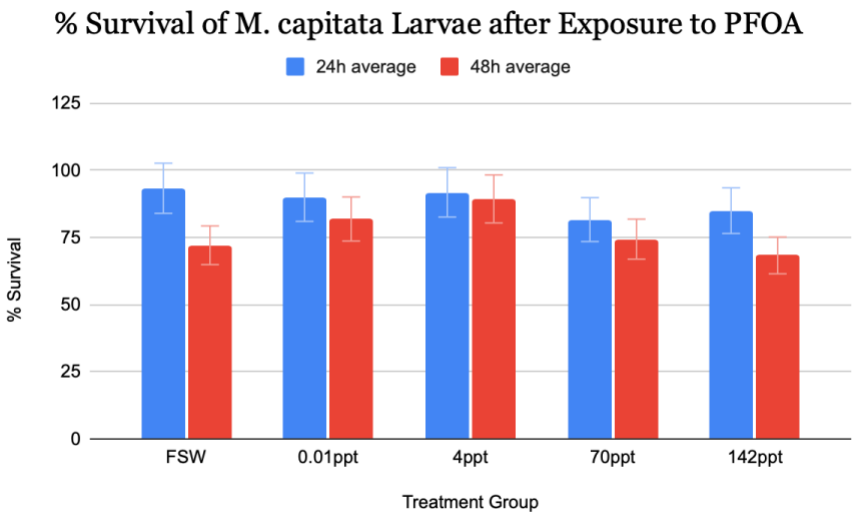


Figure 7: % Survival of *M. capitata* larvae after 24, and 48 hours of PFOA exposure

In July 2021, the exposure concentrations were repeated and one additional treatment of 70 ppb was added to analyze effects of PFOA at 1000 times the EPA safe water level, and quantified after 48 h. There was no significant difference in percent survival among the treatments tested (χ^2: 6.7036 p-value: 0.2436).

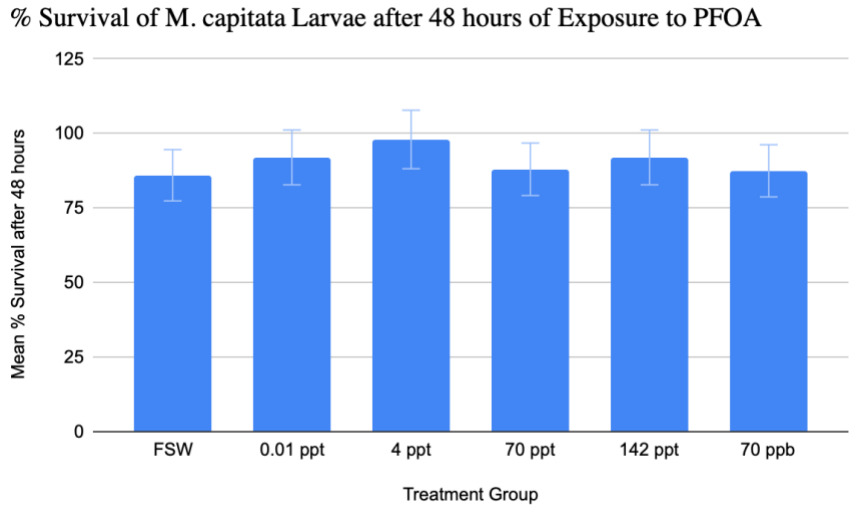


Figure 8: Percent Survival of *M. capitata* larvae after 48 hours of PFOA exposure. July, 2021

3.1.3 PHYSICAL OBSERVATIONS OF *P. LOBATA* EXPOSURE TO PFOA

At day 0 of PFOA exposure, the color of *P. lobata* nubbins were scored with the Hawaiian Ko‘a card with a 16-17 color range. By the end of the 3-day exposure, the largest color difference was noticed in one nubbin in the 4 ppt treatment group, with parts of the nubbin having a color score of 14. The control samples experienced no significant change in color, while the 0.01 ppt group had slight change, with color ranging between 15-17, 4 ppt group between 14-16, 70 ppt 15-17, and 15-16 for the 142 ppt exposure. After 7 days, FSW ranged from 14-17, 0.01 ppt from 14-17, 4 ppt from 13-17, 70 ppt from 13-17, and 142 ppt from 13-16.

Additionally noted was the presence of coral polyps and their reactivity—beakers were tapped gently to observe whether or not coral nubbins would retract their polyps. At day 0 all coral polyps were present and retracted, but after three days of exposure to PFOA there was observable change in all treatment groups, including the control. One replicate for the control group had no physical color change, but the polyps were not out on either sample. Of the 6 nubbins in each of the FSW and 0.01 ppt groups, half had polyps exposed between the different beakers, all nubbins were able to retract their polyps. In the 0.01 ppt treatment two replicates had all polyps and one replicate only had 1 polyp present, all polyps retracted. The samples were also observed for mucus production, however this was only seen in select samples of the exposure treatments. 3 of the 6 4 ppt replicates produced mucus, 4 of 6 under 70 ppt, and 2 out of 6 had heavy mucus production with exposure to 142 ppt.

Table 1: Physical observations of *P. lobata* at day 3 of the exposure period

Day 3 Exposure Observations

Treatment	Polyp Presence	Retract?	Mucus Presence	Koa Card Value
FSW	0	-	none visible	16/17
FSW	2	Y	none visible	16/17
FSW	1	Y	none visible	16/17
0.01 ppt	1	Y	none visible	15/16
0.01 ppt	2	Y	Mucus Presence (both)	15/16
0.01 ppt	2	Y	none visible	15/16/17
4 ppt	0	N	none visible	14/15/16
4 ppt	2	Y	Mucus Presence	~16
4 ppt	2	N	none visible	15/16
70 ppt	1	N	Mucus Presence	15/16/17
70 ppt	1	Y	none visible	15/16/17
70 ppt	2	Y	Mucus Presence	15/16/17
142 ppt	2	Y	heavy mucus presence	15/16
142 ppt	2	Y	none visible	15/16
142 ppt	1	Y	none visible	15/16

After the 7-day exposure, all samples in the control group had polyps present that were able to retract, only one sample from the 0.01 ppt group had polyps out that did retract. The 4 ppt group had some polyp presence on each sample, however the polyps were only present on the edges of each sample nubbin, one sample was unresponsive and did not retract. 2 of the 3 samples in the 70 ppt had polyps present, with one also only being on the sides of the nubbin, the polyps on both samples did retract. The 142 ppt group had 2 samples with polyps on only part of the nubbin, and only one had polyps that retracted. There was no mucus present on any individual coral sample across treatment groups at the end of the exposure period, which may be attributed to the water change after the 3 day exposure.

Table 2: Physical observations of *P. lobata* at Day 7 of the exposure period

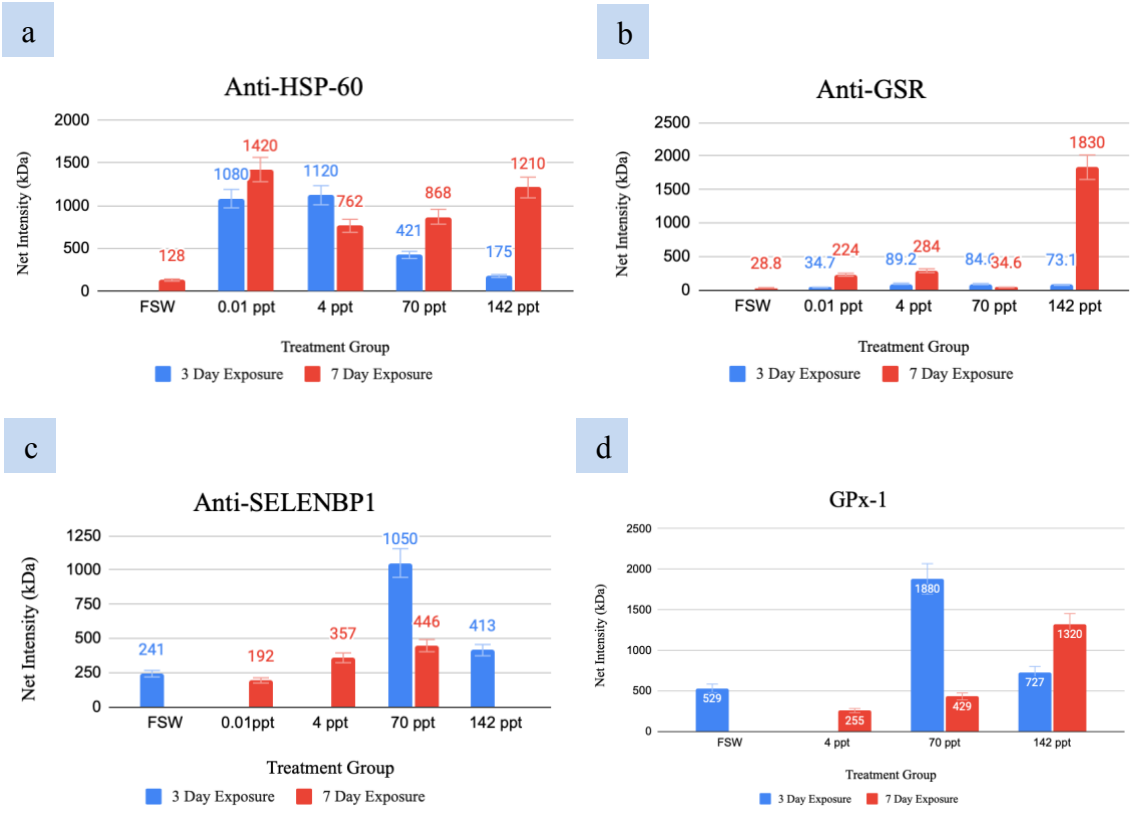
Day 7 Exposure Observations

Treatment	Polyp Presence	Retract?	Mucus Presence	Koa Card Value
FSW	1	Y	none visible	15/16
FSW	1*	Y	none visible	14/15/16
FSW	1	Y	none visible	16/17
0.01 ppt	0	.	none visible	14/15
0.01 ppt	0	.	none visible	16/17
0.01 ppt	1	Y	none visible	14/15/16
4 ppt	1*	Y	none visible	13-17
4 ppt	1*	Y*	none visible	14/15/16
4 ppt	1*	N	none visible	~16
70 ppt	1*	Y	none visible	13/14/15
70 ppt	0	.	none visible	16/17
70 ppt	1	Y	none visible	13/14/15
142 ppt	1*	Y	none visible	13-16
142 ppt	0	.	none visible	13-16
142 ppt	1*	N	none visible	14/15/16

3.1.4 MOLECULAR RESPONSE

During the 3-and 7-day exposure period some visual signs of stress were observed, but results varied among treatment and their replicates. In order to quantify more specific impacts of PFOA exposure to coral health, one replicate from each treatment group and exposure period was selected for protein analysis to compare molecular indications of stress in adult coral samples. Of the antibodies tested, the following were able to succesfully recognize antigens on *M. Capitata* proteins; anti-HSP60, anti-SELENBP1, anti-CYP1A1, anti-GSR, and anti-GPX. Anti-SOD1 did not experience succesful binding upon visualization of the Western blot image. Some antibodies did have higher success in specific bonding, and not all treatments in each anaylsis had visible expression of the selected antigens. The antibodies with the most success in binding were anti-GSR and anti-

HSP60, but even in those samples the 3-day control sample was not visible. All recognized proteins had no apparent differentiation in band length among treatment groups relative to the HeLa Lysate control ladder. All distributions were analyzed in R using Shapiro-Wilks test for normality, Levene's test for homogeneity of variance; as all groups failed to meet normality requirements, Kruskal-Wallis Rank test was performed to compare Western blot net intensity between samples. No test was performed to observe differences in treatment net intensity between the 3-and 7-day samples, as the protein samples were not taken from the same exposure replicates and their expression therefore may vary. Each antibody treatment did not have significant differences in expression



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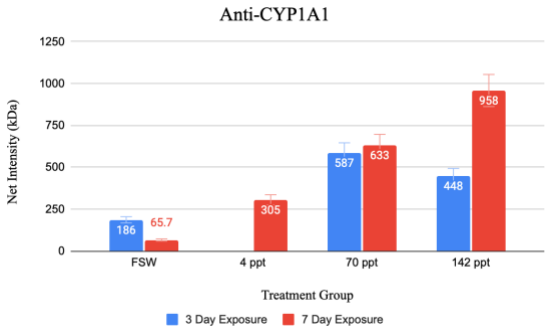

Figure 9: Biomarker protein expressions in *P. lobata* with exposure to PFOA after 3, and 7 days, (a) Anti-HSP-60, (b) Anti-GSR, (c) Anti-SELENBP1 53kDa, (d) GPx-1 (e), Anti-CYP1A1

Table 3: Kruskal-Wallis Rank by Sums Test result per each antibody treatment with both the 3-and 7-day Exposure Periods

Antibody Tested	3 Day		7 Day	
	χ^2	p-value	χ^2	p-value
HSP-60	3	0.3916	4	0.406
Anti-GSR	3	0.3916	4	0.406
Anti-SELENBP1	2	0.3679	2	0.3679
GPx-1	2	0.3679	2	0.3679
Anti-CYP1A1	2	0.3679	3	0.3916

4.0 DISCUSSION

4.1 BIOLOGICAL IMPLICATIONS OF PFOA EXPOSURE

Globally, PFOA is a recalcitrant compound that poses significant biological threats. Due to the wide application and lack of degradation mechanisms, concentrations of PFOA in the natural environment will only accumulate over time and exacerbate the stressors we already know to exist. Reef environments are some of the most susceptible when it comes

to chemical nonpoint source pollution, and they are already extremely threatened by larger global changes. It has been suggested that even if certain pollutants do not physically affect adult colonies, they could still be responsible for negatively impacting reproductive success and development of gametes and planula larvae (Richmond, et al., 2018). Therefore, this study aimed to quantify the potential implications of the ubiquitous contaminant on various phases of the coral life cycle to gain a better understanding of what regulations and remediation efforts should be implemented to maximize the survival of a vulnerable and vital marine species.

Although no studies have yet quantified the impact of PFOA exposure on reef systems, health impacts in other organisms have been studied since the late 1970s. In humans, exposure has been linked to liver enlargement, increased cholesterol, elevated blood pressure, hypertension, thyroid disorders, disorders in pregnancy, decreased birth weight, significant differences in testosterone and estradiol production in men, decreased sperm count and tumors of the testes, liver, pancreas and mammary tissue. In addition, it has been found that PFOA occupational workers had a 2-fold increased risk of developing kidney cancer (Steenland, et al., 2010). While toxicity effects may differ between species, PFOA immunotoxicity has been observed in both humans and animals (Li, et al., 2016). Aside from biological stress on humans, and animal species such as rats, humans, zebrafish and other mammals, one study found negative impacts on germination rate, and root and shoot growth with uptake of PFOA in lettuce roots (Li, Li, 2021).

4.2 IMPACT OF PFOA ON CORAL FERTILIZATION

In coral spawning events, expelled gametes are very likely to come into contact with a wide range of contaminants. Being the most susceptible to changes in environmental parameters, this has been proven to interfere with the overall success of fertilization among colonies (Richmond, et al., 2018). In prior studies, other land-based contaminants, including pesticides, heavy metals, and petroleum products have significantly reduced fertilization and settlement rates in corals at low concentrations and cause damage to coral gametes (Diu, 2016). During the few *M. capitata* spawning events in both the summer of 2020 and 2021, there was high variability in fertilization success, however ~2,300 embryos were scored over the duration of this experiment, giving a statistically substantial sample size that is comparable, but slightly larger than similar coral fertilization studies (eg., Damiani, 2020; Victor & Richmond, 2005; Hedouin and Gates, 2013).

In this study, *M. capitata* egg-sperm bundles were exposed to 0.01 ppt, 4 ppt, 70 ppt, 142 ppt, 0.01 ppb, 4 ppb, 70 ppb, and 142 ppb of PFOA. Results in this experiment suggest that there is not a significant impact on reducing fertilization success; however, an unpublished study performed at Kewalo Marine Laboratory in 2019 did find a significant deleterious effect of PFOA exposure on coral fertilization with exposure to the same concentrations (Messengei, 2019). Variations in fertilization success may be due to a multitude of factors; including low sperm motility from pollution, ocean warming, acidification, and varying surface salinity—although salinity was measured prior to the beginning of each spawning event (Albright, Mason, 2013). While reproductive failures may be prevalent among spawning experiments, there has been 100% fertilization observed

in *M. capitata* controls using the same protocols as this experiment, demonstrating that the protocol used is still viable as a measure of fertilization success (Diu, et al., 2015).

4.3 IMPACT OF PFOA ON CORAL LARVAE

Because previous studies on the effects of endocrine disrupting compounds (EDCs) on coral reefs observed decreased rates of larval survival, I hypothesized that exposure to PFOA would result in the same effect (Rougee, et al., 2006). Other EDCs, such as select pesticides or organophosphates, are lipophilic and planula larvae are composed primarily of lipids and have relatively low protein concentrations (Arai, et al., 1992). In contrast to other toxicants studied for effects on larval mortality, PFOA is not a lipophilic compound and in other mammalian species has been seen to accumulate primarily in the liver, blood and kidneys whereas lipophilic compounds typically accumulate in the fatty tissue of an organism (Steenland, et al., 2010). Since larvae are composed mainly of lipids it is sensible that the 1-2 days of exposure to PFOA performed in this study had no significant effect on larval survival. PFOA and other PFAAs has been observed to have protein-binding characterisitcs, therefore molecular analyses would be a stronger assessment tool to analyze the sublethal effects on coral viability (Wang, et al., 2017). No molecular analyses were performed on larvae samples as prior experiments have been unable to extract the amount of protein that would be sufficient for molecular diagnostic assays (Damiani, 2020).

4.4 CELLULAR DIAGNOSTICS

In order to assess for signs of sublethal stress in coral samples after exposure to PFOA, a series of clinical biomarkers were applied and analyzed based off expression. This study found no statistical significance in differences among treatments, however there is clear upregulation of anti-GSR and Anti-CYP1A1 in the 142 ppt treatment group after 7 days of PFOA exposure compared to the FSW treatment. Glutathione reductase (GSR) aids in the removal of xenobiotics and organic peroxides in cellular metabolism and can be used to measure signs of oxidative stress in corals. Cytochrome p450 (CYP1A) is a xenobiotic response protein and has been observed to exhibit upregulation under stress, and therefore increases in xenobiotic metabolism (Titshammer, 2017). A study on the response of *P. damicornis* to fuel oil exposure also found an increase in CYP1A expression after the exposure period was completed (Rougee, et al., 2006). GSR and glutathione peroxidase-1 (GPx-1) also protect cells from oxidative damage by reducing hydrogen peroxide radicals to water enzymatically (Lubos, et al., 1997). A study analyzing thermal stress in *Symbiodinium* species, the algal symbiont of coral individuals, observed increased expression of GSR with exposure to sublethal levels of increased heat (Krueger, et al., 2014).

While there was not statistical significant differences between treatments, there is indications of upregulation of HSP60 in some treatments of PFOA in both the 3-and 7-day exposures. HSP60 is a molecular chaperone protein that acts as a protective mechanism against thermal stress and other stressors, exposure to stress causes an increase in expression (Sørensen et al., 2003). HSP60 is commonly used as a biomarker to detect stress in corals. SELENBP1 had the weakest correlation between net band intensity and treatment

groups. SELENBP1 plays an important role in detoxification pathways and participates in intra-golgi transport (Porat, et al. 2000). $SOD1^-$ is the primary enzyme involved in cellular antioxidant activity, converting oxygen O_2^- to H_2O_2, and is often upregulated with exposure to oxidative stress (Krueger, et al., 2014), there was not successful binding in the $SOD1^-$ antibody application in this experiment, future studies should be repeat the methods of this study to quantify if there is a significant upregulation of $SOD1^-$ with exposure to PFOA.

Past studies have observed a variety of biological impacts with PFOA exposure, including heptoxicity, genotoxicity, immunotoxicity and neurotoxicity. PFOA was found to increased intracellular reactive oxygen species (ROS) by 150% and increased oxidative DNA damage (Yao and Zhong, 2005). One study on lettuce (*L. sativa)* found no significant increase found in SOD- or GPX production with exposure to PFOA, which is consistent with the results of this study. However, there were other noticeable implications on root metabolomes, with 53 metabolites observed with significant change—it was ultimately concluded that PFOA is linked to metabolic disorders and oxidative stress in *L. sativa* (Li, Li, 2021). This poses additional concern for coral reef systems, as a holobiont with algal components, any impact on the animal or algal symbiont will have an overall impact on the coral individual.

The application of the clinical biomarkers in this experiment, while not producing statistically significant variation, does indicate some degree of metabolic stress in *P. lobata* samples. The findings in this study do indicate that corals do respond to PFOA at a metabolic level by shifting protein expression for various functions. Future experiments should consider more specific factors of the corals themselves, and should be expanded to other coral species, as different genotypes may exhibit varying responses.

Coral reproduction is highly dependent on the interplay of pro-oxidants and antioxidants and is a source of ROS generation. ROS production and detoxification plays a critical role in reproduction, specifically in gamete production, fertilization, and embryo development. Oxidative stress can reduce embryo growth and decrease fertilization rates. The same compounds utilized as clinical biomarkers in this experiment are all vital in regulating ROS impacts on egg and sperm viability and healthy embryo growth (Murphy, et al, 2019).

4.5 LIMITATIONS

Molecular diagnostic techniques are extremely valuable in detecting sublethal indicators of stress in various organisms and Western blot methods have been used in past experiments to detect proteins in corals. However, this application is relatively new and therefore has various limitations. No cnidarian-specific antibodies are commercially available, and those that may be created for that purpose are expensive, and take time to design and obtain. Downs (2006) did design cnidarian-specific antibodies which reduced non-specific binding and was ultimately more successful than applying non-specific antibodies as was done in this experiment. In the absence of cnidarian-specific antibodies, this experiment used non-specific polyclonal antibodies in hopes that there would be more success in protein-binding than monoclonal varieties. Even with this effort, there are clearly constraints to this method and inconsistencies in results, which can be observed with the failure of some applied antibodies to successfully bind across extracted protein samples. Chemiluminescence was used in this experiment for detection of net band

intensity, which is a method sensitive enough to detect very small concentrations of protein (Alegria-Schaffer et al., 2009).

While Western blot techniques are relatively inexpensive and easy to perform, there are clearly limitations to the protocol when used for coral proteins. Western blotting, and SDS-PAGE are also relatively older forms of genetic analysis and are not as sensitive as newly developed methods. Future studies would benefit from using more specific molecular techniques, such as Liquid chromatography with tandem Mass Spectrometry (LC-MS/MS), which allows for the quantification of the entire proteome of an organism, and would provide a much more thorough picture of the metabolic pathways within the coral sample and give a more detailed response of changes with PFOA exposure (Kitada, et al., 2008).

Additionally, corals are incredibly complex organisms. As a holobiont, there are multiple factors that may influence stress mechanisms aside from PFOA exposure alone. While adult exposures were controlled to the same closed environment, there still may have been minor discrepancies that would interfere with adult coral health. This is especially the case with the fertilization success aspect of this project, as gametes were collected directly from Kāne`ohe bay, and general ocean conditions and seasonal variation could have significant impacts on gamete development and spawning outside of PFOA exposure.

The final, but arguably most important limitation of this study was that the concentrations worked with were extremely small and required multiple dilutions. Testing for PFOA concentrations is expensive and requires specialized equipment that was unavailable within the lab that was worked with (Rodriguez, et al., 2020). It is very likely that there were variations in actual PFOA concentrations in the exposures, which may have

drastically impacted the results of this experiment. Future studies should implement detection technology to ensure that the desired exposure concentrations are what the sample populations are being exposed to.

4.6 FUTURE STEPS

In this study, PFOA was not observed to have significant impacts on fertilization succces. However, the protein-binding characteristics of the compound and its reproductive impacts on other organisms suggest that there are still substantial threats to reproduction and recruitment success. Future studies should focus on whether PFOA influences the ability of adult corals to produce gametes and coordinate spawning events between colonies. To test this would require a longer-term exposure of PFOA to *M. capitata* colonies before and during their gametogenesis cycle.

Another important aspect of coral health that needs to be researched more deeply is the impact of PFOA exposure on the zooxanthallae that lives within the coral animal. Since research has suggested that PFOA has metabolic impacts on both animals and plants, it would be beneficial to quantify how PFOA exposure impacts photosynthetic efficiency in the algal symbiont; which can be performed with pulse-amplitude fluorometry (PAM).

5.0 CONCLUSION

Coral reef populations around the globe have been faced with extreme challenges and already suffer from mass bleaching events and decreased reproductive capacity. By limiting the effects of locally sourced pollution, we are encouraging healthier reef building corals that may be more resilient to changing global conditions. Although there was not a

significant impact of PFOA exposure on coral health in this study, the compound still poses a great threat to marine systems and other organisms. This study identifies potential methods for future studies of toxicant exposure on marine health, but more specific and longer-term studies with PFOA exposure will need to be performed to properly identify the threats of the poorly understood ubiquitous contaminant at various phases of the coral life cycle. The study also highlights the need for specific biomarker development that will allow for more accurate depictions of sublethal levels of stress in reef-building corals. It is important to identify the effects of known toxicants on coral reefs so that we can properly implement management strategies that will preserve reef health, the ecosystem they provide, and protect the communities that are reliant on their success.

APPENDIX: SUPPORTING INFORMATION

LIST OF TABLES

LIST OF FIGURES

Table S1: Mean and Standard Deviation of Fertilization Success in *M. capitata* gametes exposed to PFOA collected on 6/20/20

Treatment Group	Average % Fertilization Success
FSW	83.75 ± 29.96
0.01 ppt	71.31 ± 16.76
4 ppt	50.63 ± 23.45
70 ppt	52.76 ± 12.61

142 ppt	62.917 ± 23.74

Table S2: Mean and Standard Deviation of Fertilization Success in *M. capitata* gametes exposed to PFOA collected on 6/22/20

Treatment Group	Average % Fertilization Success
FSW	52.98 ± 25.12
0.01 ppt	71.61 ± 10.74
4 ppt	88.92 ± 10.59
70 ppt	91.51 ± 7.91
142 ppt	76.12 ± 30.54

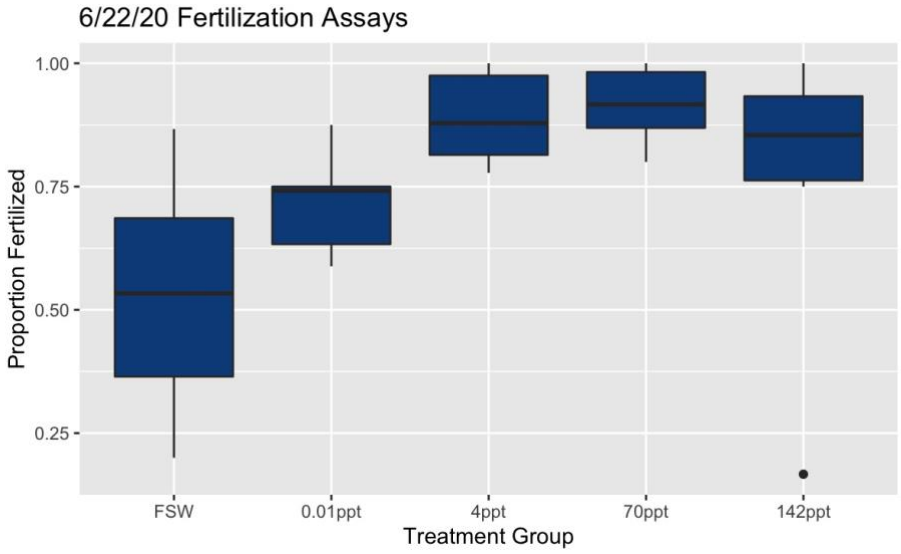

Figure S1: Boxplot of proportion of fertilization success in *M. capitata* gametes exposed to PFOA on 6/22/20

Table S3: Mean and Standard Deviation of Fertilization Success in *M. capitata* gametes exposed to PFOA collected on 6/10/21

Treatment Group	Average % Fertilization Success
FSW	81.79 ± 14.27
0.01 ppt	77.34 ± 20.41
4 ppt	84.52 ± 12.40
70 ppt	88.56 ± 37.02
142 ppt	74.64 ± 28.07

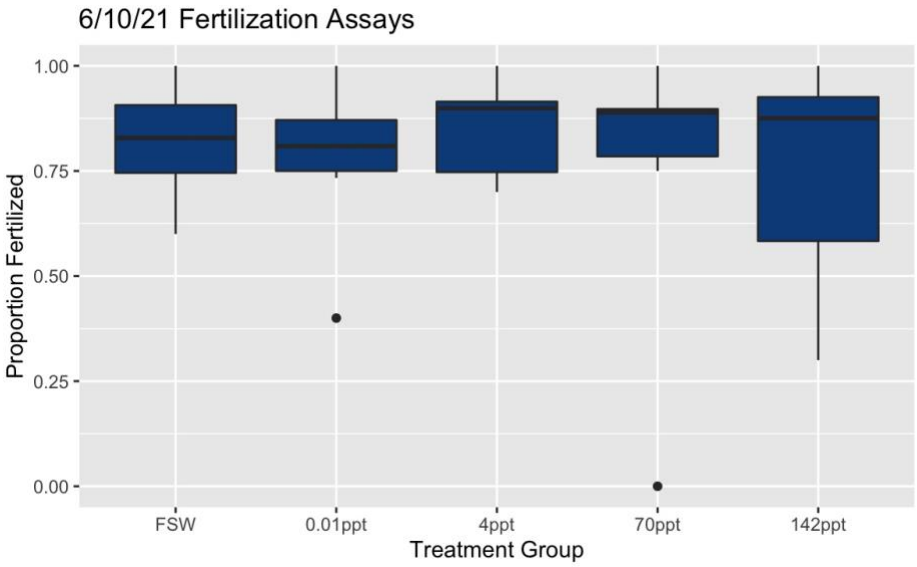

Figure S2: Boxplot of proportion of fertilization success in *M. capitata* gametes exposed to PFOA on 6/10/21

Table S4: Mean and Standard Deviation of Fertilization Success in *M. capitata* gametes exposed to PFOA collected on 7/7/21

Treatment Group	Average % Fertilization Success
FSW	61.78 ± 41.98
0.01 ppb	3.33 ± 5.77
4 ppb	18.33 ± 40.21
70 ppb	29.76 ± 39.23
142 ppb	15.78 ± 37.42

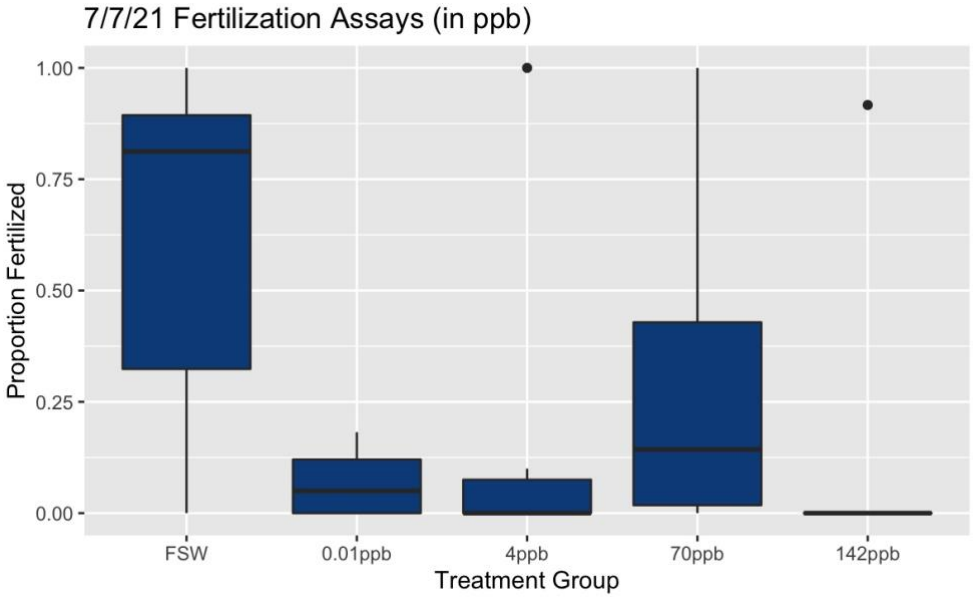

Figure S3: Boxplot of proportion of fertilization success in *M. capitata* gametes exposed to PFOA on 6/10/21

Table S5: Mean % Survival of *M. capitata* larvae. June, 2020

Treatment Group	% Survival after 24 h	% Survival after 48 h
FSW	93.33 ± 8.16	72.12 ± 24.82
0.01 ppt	90 ± 15.49	81.9 ± 21.22
4 ppt	91.82 ± 7.50	89.38 ± 10.84
70 ppt	81.67 ± 27.87	74.4 ± 14.68
142 ppt	85 ± 17.61	68.33 ± 26.39

Table S6: Mean % Survival of *M. capitata* larvae after 48 h. July, 2021

Treatment Group	% Survival after 48 h
FSW	86 ± 8.94
0.01 ppt	92 ± 8.37
4 ppt	98 ± 4.47
70 ppt	88 ± 10.95
142 ppt	92 ± 8.37
70 ppb	87.5 ± 13.42

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