ABSTRACT

Title of Thesis:	DETERMINING THE TOXICITY OF THE UV FILTER OXYBENZONE IN THE HARD CORAL, *GALAXEA FASCICULARIS*
	Annaleise Jane Conway, Master of Science, 2021
Thesis Directed By:	Professor Carys L. Mitchelmore Marine Estuarine Environmental Sciences

Despite limited toxicological studies of UV filters in corals, legislative activity in Hawaii and other locations has led to bans on the sale and/or use of sunscreens containing the active ingredient oxybenzone (BP-3). The few published coral toxicity studies on BP-3 are difficult to compare due to varied methodology and inconstant acute and chronic toxicological responses. Therefore, I conducted repeated acute (96-hour) toxicity tests with copper and a common hard coral, *Galaxea fascicularis,* based on standard invertebrate toxicity testing guidelines to determine the species' utility as a standard testing organism as well as the utility of copper as a positive control. This was followed by acute and chronic (28-day) toxicity tests with BP-3 using the same methodology to determine this compound's toxicity. Multiple endpoints pertinent to risk assessments (mortality and growth) and additional biological endpoints were examined. Using these results, preliminary risk quotients of BP-3 and *Galaxea fascicularis* were calculated.

DETERMINING THE TOXICITY OF THE UV FILTER OXYBENZONE IN THE HARD CORAL, *GALAXEA FASCICULARIS*

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2021

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Dedication

This thesis is dedicated to my mother, Marianne Conway. Thank you for showing me how to be a strong, smart, confident woman.

Acknowledgements

Thank you to my advisor, Dr. Carys Mitchelmore, as well as the rest of my committee, Dr. Michael Gonsior and Dr. Andrew Heyes, and my mismatched lab group Cheryl Clark, and Cynthia Ross for help with forming this thesis, conducting research and analyzing data. Being Carys' only student, I quite literally could not have done this without you all.

I would like to thank my funding sources (CBL Solomon's House fellowship and Johnson & Johnson) for supporting this project as well as SETAC, SETAC CPRC and the GEC travel awards for helping me to present my findings. Thank you to Dr. Emily Brownlee, Elaine Szymkowiak, Thomas Brewer, John Spinicchia, Nhu Chau, Ava Chrisler, Rustin Pare and Tanner Lowe from Saint Mary's College of Maryland for help with coral culture and my daily water pick-ups. Thank you to Dr. Kurt Reynertson (Johnson & Johnson), Dr. Emily Burns and Dr. Iain Davies (PCPC) for their support, advice, and companionship during SETAC 2019.

Thank you to Dr. Tom Miller and the faculty and staff of CBL for just being a wonderful community and helping to make sure I had everything I need for all of my experiments. A special thank you to Jerelle Jesse, Skyler Golt and the rest of the students and FRAs from CBL for being my support group. Moving to a new place where I know no one is difficult but you all made it so much easier. I have somehow made it out of this process at least as sane as I entered and I mostly owe that to all the trivia, happy hours, and Students4Students meetings.

Thank you so much mom and dad for always supporting me and pretending you're okay with me moving around the country to pursue my education and to my

siblings and their families for giving me so many excuses to go back home. Thank you to the Ritters, my new family, for welcoming me so quickly and giving me a home away from home.

And, finally, thank you to my fiancé and favorite dog dad Kevin Ritter for always giving me a reason to smile. I came down to Maryland for a master's degree and found my future.

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List of Abbreviations

ASW	Artificial Seawater
BP-3	Benzophenone-3 (oxybenzone)
CBL	Chesapeake Biological Laboratory
ECHA	European Chemicals Agency
ECx	The effect concentration at which x% effect is observed compared to the control group
EPA	United States Environmental Protection Agency
LCx	The concentration of a compound that will lead to mortality of x% of the test organisms
LOEC	Lowest-Observed Effect Concentration
MEC	Measured Environmental Concentration
NASL	Nutrient Analytical Services Laboratory
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Co-operation and Development
PAM	Pulse-Amplitude Modulated
PAR	Photosynthetically Active Radiation
PNEC	Predicted No-Effect Concentration
QA/QC	Quality Assurance/Quality Control
RQ	Risk Quotient
SMCM	St. Mary's College of Maryland
UV filter	Ultraviolet light filter

Chapter 1: Introduction to UV filters and their impacts to corals

1.1 Coral Reefs and UV Filters

Coral reefs are one of the most biologically and economically productive ecosystems in the world partially due to their role as habitat to commercially important fish species (Brown et al., 2006). However, reefs also draw visitors from around the globe. One estimation by Spalding et al. (2017) suggested that there are approximately 70 million trips taken annually to reef destinations generating an industry that is worth approximately US$ 36 billion. Due to these and other human activities, coral reefs are being negatively impacted by a combination of stressors at the global and local levels, including increases in sea surface temperatures and other climate change ramifications, disease, nutrient inputs, and chemical contamination (Hughes and Connell, 1999; Ban et al., 2014). The most recent global reef monitoring report from 2008 stated that 20% of coral reef area has been lost with an additional 35% to be lost by 2048 assuming no changes to current practices (Wilkinson, 2008). Other estimations suggest 60% of coral reefs are threatened by both natural and anthropogenic stressors (Hughes et al., 2003; Pandolfi et al., 2003).

Corals are known to live near their thermal limit and show signs of stress when pushed over their thermal limit including expulsion of their symbiont (i.e. bleaching). Although global increases in ocean temperatures are the major driver for declines in coral reef health, local exposure to other stressors may reduce their resilience to climate change driven impacts. For example, exposure to the myriad physical, chemical, and biological pollutants that enter coastal systems via effluents or land runoff results in multiple

stressors for corals to overcome. Numerous chemical contaminants have been detected near coral reefs and shown to impact them at environmentally relevant concentrations (see van Dam et al., 2011). Periodically, a specific class of stressor and its impacts on corals come to the forefront of conversation.

A recent example of this is whether the active ingredients in sunscreens, ultraviolet (UV) filters, cause impacts to coral reef health. This concern was first raised by Danovaro et al. (2008) but only received significant public and political attention after the publication of a laboratory experiment demonstrating impacts of the UV filter oxybenzone (benzophenone-3 or BP-3) to coral planulae (Downs et al., 2016). A handful of studies have investigated this and other UV filters and a recent review has summarized the state of the science regarding the potential risks of organic UV filters to corals (Mitchelmore et al., 2021). Coral reef locations tend to be popular to tourists given their narrow and warm temperature ranges. Therefore, corals are being exposed to potentially dangerous chemical compounds during time periods that may already be stressful due to being near their thermal limits.

Sunscreen UV filters are classified in two groups, organic chemical UV filters and mineral, or physical UV filters which both prevent the sun's energy from reaching the skin to help decrease the risk for skin cancer and other dermatological concerns. Physical sunscreens (i.e. sunblock) utilize metal oxides such as zinc oxide (ZnO) or titanium dioxide (TiO_2) to reflect UV radiation from the skin. Some questions regarding the environmental impact of these active ingredients to corals have been raised over the past decade (Corinaldesi et al., 2018; Fel et al., 2019); however, various groups have directed consumers toward physical sunscreens deemed "reef-safe" (Reef Safe Sunscreen Guide,

2020) when research supporting these recommendations thus far has been inconclusive (Fel et al., 2019).

Organic chemical UV filters are a wide variety of compounds which protect skin or various products from UV light damage through reactions that convert UV light energy to heat energy. Although UV filters are most often tied to sunscreen use, these compounds, and specifically BP-3, are also commonly used in other products like cosmetics, paints, and plastics (Briasco et al., 2017; Lyon, 2013). BP-3 is a broad-spectrum UV filter meaning it protects from both UVA and UVB radiation and, in the US, is one of the only FDA-approved chemical UV filters that does so. Therefore, omitting this ingredient from sunscreens means scrambling to approve another compound to fill this role. BP-3 has been given the majority of attention by the general public because of its potential for coral toxicity as well as studies linking endocrine disruption in multiple species to this compound (Krause et al., 2012). Because of its infamy in the media, its importance in the sunscreen industry (and resultantly, to human health), and its potential for negative environmental impacts, BP-3 is the compound chosen as the focus for this thesis and the evaluation of its toxicity to corals.

1.2 Environmental Concentrations

In general, UV filters can enter the marine environment through point or non-point (diffuse) sources. Point sources include sewage outfalls or a person wading into the water while nonpoint sources include land runoff that washes land-based chemicals into the ocean. Downs et al. (2016) has suggested between 6,000 and 14,000 tons of sunscreen lotion are released in coral reef areas annually. However, the calculation of these

estimates was not clear. It seems that this estimation was based off of a calculation by Danovaro et al. (2008) proposing that 16,000 to 25,000 tons of sunscreen are used annually in tropical countries and approximately 25% of total sunscreen application is washed off during swimming and bathing and therefore between 4,000 and 6,000 tons of sunscreen per year is released in reef areas. However, this is not the whole story. Some sunscreen will also be rinsed off of the body and a percentage will be broken down in sewage systems resulting in either more or less toxic products, leaving the rest to be released into waterways (Margot et al., 2015).

Because of the direct release of sunscreen into the environment from coastal visitors, UV filters present a unique problem. The input of these chemicals is highly variable and depends on hydrology of the water body, time of day and time of year, and the number of people engaging in coastal recreation which may be impacted by water and air temperature. Furthermore, transport and fate of these compounds is not well understood. There are also many UV filter compounds that could be present in a number of combinations and concentrations in a given sunscreen product. This makes prediction of concentrations nearly impossible and so relies on monitoring to establish the presence of these UV filters in a particular location to inform policymakers of what the true risk to the marine ecosystem is.

Of all the UV filters studied to date, BP-3 is the most studied and often the one detected most frequently and at the highest concentrations. Currently there are twelve studies detailing concentrations of BP-3 in coral reef environments (Bargar et al., 2015; Downs et al., 2016; Tashiro and Kameda, 2013; Tsui et al., 2014; 2017; 2019; Mitchelmore et al., 2019; Kung et al., 2018; Goksøyr et al., 2009; Horricks et al., 2019;

He et al., 2019a; Schaap and Slijkerman, 2018) and many more describing its concentrations in other environmental matrices (e.g. freshwater, biota, wastewater, etc.). These concentrations vary widely from below the limit of detection to 1.395 mg/L (BP-3; Downs et al., 2016). Concentrations are generally on the low end of this range, often in the ng/L or below range, with a few exceptions in the µg/L range. However, the high end of these (mg/L range) exist only in the study by Downs et al. (2016) for a single sample (i.e. the site did not have replicate sampling for confirmation). Due to the concentrations of total dissolved organic carbon (DOC) in reef waters (< 1.0 mg/L; de Goeij et al., 2008; Tanaka et al., 2011), these concentrations, while alarming, are unlikely and represent distinct outliers in the dataset available for BP-3 concentrations in seawater near coral reefs (summarized in Mitchelmore et al., 2021). There are also a number of caveats in the bulk of the existing UV filter monitoring data which make the dataset less reliable in determining the risk of UV filters to corals. For example, many studies do not take replicate samples (Bargar et al., 2015; He et al., 2019a) or simply do not report this aspect (Goksøyr et al., 2009; Horricks et al., 2019; Tsui et al., 2014; 2019; Downs et al., 2016; Schaap and Slijkerman, 2018) or fail to report important information like the material of the bottle used to collect samples (Tashiro and Kameda, 2013; Tsui et al., 2017). Unfortunately, even with these caveats, the highest reported concentration has caused alarm in conjunction with results from coral toxicity testing and its use as the measured environmental concentration (MEC) used in environmental risk assessments of BP-3 in corals.

1.3 Current Literature on the Toxicity of BP-3 to Corals

These concerns over the impacts of UV filters to corals were first raised by Danovaro et al. (2008) but gained more attention following the Downs et al. (2016) study which showed that BP-3 caused bleaching and death in *Stylophora pistillata* coral larvae and in an *in vitro* test system using isolated non-symbiotic coral gastrodermal cells from a number of coral species over short time periods (<24 hour) using nominal concentrations of 2.28 µg/L to 228 mg/L BP-3 (Downs et al., 2016).

There are currently nine published studies on the impacts of UV filters to corals. Of these, six studies focus on the impacts of BP-3 in corals. As coral is a non-standard toxicity test organism, no U.S. Environmental Protection Agency (EPA) or Organization for Economic Co-operation and Development (OECD) toxicity test guidelines exist for coral species. Because of this, there is a large variety of methods that have been employed to determine the impact of UV filters to corals. Since there is limited literature on this topic, it is important to consider the results and methods of each of these studies. Only one of these studies attempted to follow a standard testing design for acute toxicity testing in the larvae of a hard coral species (Downs et al., 2016). Out of those tests that used adult corals, none of them achieved mortality from BP-3. This means that a lethal concentration causing 50% mortality (LC50), the most common acute endpoint used in risk assessment, cannot be calculated for adult hard corals. However, many sublethal endpoints have been studied both in acute and chronic testing. These include polyp retraction (He et al., 2019a; Stien et al., 2020), bleaching (e.g. visible bleaching or algal density; Downs et al., 2016; Danovaro et al., 2008; He et al., 2019a; Wijgerde et al., 2020), growth (McCoshum et al., 2016; Wijgerde et al., 2020), and many others. The

only study showing coral growth impacts due to BP-3 was on a soft coral species but the authors did not appropriately convey dosing concentrations (McCoshum et al., 2016). Another study attempted to examine BP-3's impact on growth of the hard coral *S. pistillata,* but the authors failed to see an effect at a nominal concentration of 1 µg/L BP-3 over 6 weeks (0.06 µg/L measured; Wijgerde et al., 2020).

Unfortunately, this set of data omits many standard facets of toxicity testing. For example, most of these tests were not repeated. Those that were contained just a single preliminary test with a single definitive test (He et al., 2019a). As these are unique tests, it is impossible to determine if these results were unique as well. Furthermore, many studies failed to include analytical confirmation of exposure solutions (Danovaro et al., 2008; Downs et al., 2016; McCoshum et al., 2016; Stien et al., 2020) or when this was completed, were not able to maintain concentrations close to nominal throughout the exposure (He et al., 2019a; Wijgerde et al., 2020). Finally, none of these tests include a positive control. Although positive controls are not routinely needed for standard toxicity testing, when testing a species that is not routinely used (e.g. coral), it is important to be able to compare the sensitivity of the species to intra- or interspecific counterparts as well as determine the reproducibility of tests (OECD, 2019).

Conducting toxicity tests with standard methods is critical to ensure reproducible and representative results are obtained and that there are quality assurance and control (QA/QC) measures in place leading to robust, reproducible, high-quality data on the impacts of BP-3 to corals. Standard protocols are also critical for result comparison between compounds to determine if certain UV filters are more detrimental to the health of an organism as well as to determine the differences between species sensitivity of a

particular UV filter. Without standard methods, these comparisons are difficult, if not impossible, to make (for a review see Mitchelmore et al., 2021).

1.4 Environmental Risk of BP-3 to Corals

Environmental risk determination is an important decision-making tool. Despite only a handful of studies investigating the impact of UV filters to corals and limit data on exposure, no formal risk assessment having been conducted, and no standard risk assessment framework for these organisms, concern remains. This concern for corals exposed to UV filters has gained support from policy makers and the public leading to bans on the sale of certain UV filters including BP-3 and octinoxate based on the precautionary principle in a variety of US locations including Hawaii (SB 2571; State of Hawaii Senate, 2018), US Virgin Islands (Bill # 33-0043; US Virgin Islands, 2019), and a locally proposed ban in Key West, Florida (Recently overturned; Ordinance File #18-3253; Key West City Commission, 2019) as well as international bans in Palau (SB 10-135; Republic of Palau, 2018) and Bonaire (Ministries of the Netherlands, 2020). However, more recently, doubt has been raised about the validity of this concern from legislative bodies and scientists (see Czajka, 2019 for a summary of opposing views) and following results from other researchers that have shown much lower toxicity for UV filters than previously reported (e.g. He et al., 2019a; 2019b; Wijgerde et al., 2020).

Risk assessments allow us to use measured values combined with assessment factors to predict the concentration at which compounds are not detrimental to species or communities in order to both prioritize mitigation strategies and inform stakeholders of potential risks. Risk quotients (RQs) comparing measured environmental concentrations

(MECs) to predicted no-effect concentrations (PNECs) derived from toxicity endpoints combined with an assessment factor have been calculated for a handful of UV filters in four studies to date (Tsui et al., 2014; 2017; He et al., 2019a; 2019b) with the majority showing no immediate risk (i.e. RQ <1; Mitchelmore et al., 2021). However, these results are based on a combination of environmental concentrations and toxicity test data with poor QA/QC and a lack of standard procedures.

1.5 Goals and Hypotheses

The overall goal of this thesis is to investigate the toxicity of BP-3 to a hard coral species. To achieve this goal, the first step needed is to create a standard acute toxicity test using the hard coral *Galaxea fascicularis* and the framework provided by the EPA and OECD aquatic testing guidelines. Then, we will use this standard acute test to calculate an LC50 for BP-3 in this species. Using these results, we will determine the appropriate dosing for a chronic exposure study to quantify the sublethal growth impacts of BP-3 on a hard coral for the first time. This collection of data will allow us to calculate a risk quotient of BP-3 to *G. fascicularis* to help determine the environmental risk of this compound. This will help to make more informed decisions surrounding UV filters and corals. It is expected from the literature that BP-3 will not lead to significant mortalities in *G. fascicularis* at environmentally relevant concentrations but may result in some sublethal effects. It is not expected that BP-3 poses significant environmental risk to this species.

Hypotheses:

1) *Galaxea fascicularis* will provide replicable results for toxicity testing.

2) An LC50 for BP-3 in this species will be achieved but will occur above the majority of measured environmental concentrations as well as the limit of solubility for this compound.
3) Chronic testing will demonstrate significant growth impacts due to BP-3 above the majority of environmental concentrations but below its limit of solubility.
4) The risk of BP-3 to *Galaxea fascicularis* will be minimal as calculated by the risk quotients.

Chapter 2: Exploring the utility of *Galaxea fascicularis* as a standard toxicity species for coral through acute copper exposures

2.1 Introduction

Frameworks for testing the toxicity of chemical contaminants have been established in regulatory bodies such as the U.S. Environmental Protection Agency (EPA) or Organization for Economic Co-operation and Development (OECD). These frameworks consist of standardized testing methodology on a handful of species acting as representatives for larger groups which means a limited number of test organisms are available to cover the diversity of organisms that exist. These guidelines are not chemical-specific, but provide general guidelines to test a variety of compounds on standard testing organisms. There are very few standard marine toxicity species versus freshwater and, furthermore, corals are unique symbionts which do not fit neatly into these representative categories. Even if suitable algae and invertebrate studies are carried out, they likely will not account for the interdependency of the intricate host-symbiont-microbial holobiont relationship.

Arguably the closest standard test organism to coral currently used is the marine invertebrate, mysid shrimp (*Americamysis bahia*) which is a tropical crustacean. However, cnidarians, especially corals and other symbiotic species, are fundamentally different from these organisms in many ways including reproduction, growth (especially in reef-building species), their sessile adult phase, as well as impacts to the algal symbiont that do not affect the coral host. Other studies, most notably by Howe et al.

(e.g. Howe et al., 2012; 2014a; 2014b), have explored using the anemone *Aiptasia pulchella* as a potential symbiotic cnidarian standard test organism. Although this may be a viable choice, it is worthwhile to consider a coral species, especially a reef-building species, due to their importance as keystone species as well as their fragility in the current environment due to a variety of biological, chemical and physical stressors. Although an anemone or a soft coral species may be more amenable to laboratory testing and capture nuances with the symbiotic partnership, they are not calcifying organisms and may not accurately represent reef building corals.

Therefore, the hard coral, *Galaxea fascicularis* was chosen as a potential standard test organism. This species is a shallow-water coral native to the Red Sea and Indo-Pacific region and is characterized by its large, green-tipped polyps (Hoeksema and Cairns, 2020). This species is sold to the general public for saltwater aquaria due to its relative ease of cultivation and growth in artificial seawater. Colonies will continually bud new polyps over time resulting in a seemingly never-ending supply of new test organisms without having to spawn or retrieve new samples from the wild. This can be especially important as permitting is required for collection in many locations and some species cannot be collected at all. This allows for testing even at the most remote laboratories and decreases the environmental impact of testing. Also, because of its comparatively large polyps which are usually up to 10 mm in diameter (Veron, 1986), as opposed to a coral like *Acropora spp.* with much smaller polyps that are approximately 2-3 mm in diameter (Veron, 1986), *G. fascicularis* provides easy observations of individual polyps making estimations of mortality and other visible responses (e.g. polyp retraction) of the coral host straightforward without needing additional specialized equipment.

Reference toxicants which have been used historically include a variety of compounds, most notably, metals (e.g. silver, cadmium, zinc, and copper; US EPA, 2002). To begin to explore the feasibility of using *G. fascicularis* as a standard test organism for corals, the reference toxicant copper was chosen as the chemical contaminant for a number of reasons. Firstly, copper of sufficient purity is easily obtained by a number of sources in multiple forms. Next, the analysis of copper in seawater is a well-established and relatively straightforward process. Copper is also a common contaminant in coastal areas including in areas with coral reefs (van Dam et al., 2011). Finally, copper was chosen because it has been used in a number of studies on coral to date (e.g. Sabdono, 2009; Reichelt-Brushett and Harrison, 1999; 2000; 2004; 2005; Negri and Heyward, 2001; Bielmyer et al., 2010; Mitchelmore et al., 2007; Reichelt-Brushett and Michalek-Wagner, 2005; Esquivel, 1986). The literature on the toxicity of copper (or any compound) to *G. fascicularis* is extremely limited; however, there is one study characterizing the acute (96 h) mortality of *G. fascicularis* to copper which gives some point of reference (Sabdono, 2009).

After consulting all standard acute toxicity testing guideline parameters for aquatic invertebrates and vertebrates from both OECD and EPA (Table 1), it was decided that a 96-hour acute toxicity test to achieve an LC50 (lethal concentration causing 50% mortality) should provide a good initial baseline for the sensitivity of this species and would be able to show the reproducibility of this test as it is the standard length for marine invertebrate (i.e. mysid) testing. This also is the longest of the acute durations and, making daily mortality observations, calculations for tests of a shorter duration could be made to compare to other studies. The test methods employed were primarily based on

the EPA Mysid Acute Toxicity Test (US EPA, 2016b) as this is the closest current standard species but some changes in lighting scheme, salinity, and endpoints explored were made to accommodate the needs of this particular species (Table 1). Guidance from EPA and OECD general guidelines for toxicity testing (US EPA, 2016a; OECD, 2019) were also taken into account. The biological endpoints recorded included mortality, polyp retraction, as well as a number of bleaching-related endpoints to help better describe the impact of copper to the algal symbiont.

The goal of this study is to begin investigating the utility of the hard coral species *Galaxea fascicularis* as a standard test species for toxicity testing by using copper as a reference toxicant in three full-scale repeated acute (96 h) static renewal exposures. This will demonstrate the reproducibility of response in this species as well as provide initial information about its sensitivity in comparison to other hard coral species.

Table 1. Summary of acute toxicity testing guidelines from EPA and OECD aquatic studies. S = static, SS = semi-static (static renewal), FT = flow-through, SD = species-dependent, NR = not reported, NA = not applicable, Imm. = Immobilization, DT = during test.

Guideline	OECD No. 203	OECD No. 202	OECD No. 201	OECD No. 235	OECD No. 236	OCSPP 850.1025	OCSPP 850.1035	OCSPP 850.1045	OCSPP 850.1055	OCSPP 850.1075	OCSPP 850.4500	This Study
Test Organism	Fish (various)	Daphnia sp.	Alga and Cyanophyta	*Chironomus sp.*	Fish (various, embryo)	Oyster	Mysid	Penaeid Shrimp	Bivalve (Embryo-Larval)	Fish (various)	Alga	*G. fascicularis* (coral)
Test type	S, SS or FT	S	S	S	S or SS	FT	SS or FT	SS or FT	S	S, SS or FT	S	SS
Duration (h)	96	48	72	48	96	96	96	96	48	96	96	96
Temp C (± DT)	SD (± 2)	18 to 22 (± 1)	21 to 24 (± 2)	SD (± 1)	26 (± 1)	20 (± 2)	25 (± 1)	23 (± 1)	SD (± 1)	SD (± 2)	SD (± 2)	25 (± 1)
Light intensity (lux)	540-1000	*****	SD	500 to 1000	540 - 1080	540-1080	540-1080	540-1080	540-1080	540-1080	60 µmol/m²/s	89 – 210 µmol/m²/s
Photoperiod (h light)	12 to 16	16^	SD	16^	12 to 16	12 to 16	12 to 16[&]	12 to 16[&]	12 to 16	12 to 16	14 to 24	12
Salinity (ppt; ± DT)	SD	NA	NA	NA	NA	20 (± 2)	20 (± 2)	20 (± 2)	20 (± 2)	SD (± 2)	30 (± 5)	33 (± 2)
pH (± DT)	6.0 to 8.5	6 to 9 (± 1.5)	SD (± 1.5)	6 to 9 (± 1.5)	6.5 to 8.5 (± 1.5)	7.5 to 8.5	7.5 to 8.5 (± 1)	7.5 to 8.5 (± 1)	7.5 to 8.5 (± 1)	7.5 to 8.5 (± 1)	NA	8.0 (± 0.2 SD)
Organism/conc.	7	20	NA	20	20	20	20	20	15-30 mL	7[@]	10000 cells/mL	12 polyps
Min. Replicates	1	4	3	4	1/well	2	2	2	2	1[@]	4	4
Exposure Concentrations	≥ 5 geometric series ≤ 2.2	≥ 5 geometric series ≤ 2.2	≥ 5 geometric series ≤ 3.2	≥ 5 geometric series ≤ 2.2	≥ 5 geometric series ≤ 2.2	≥ 5 in geometric series	≥ 5 in geometric series	≥ 5 in geometric series	≥ 5 in geometric series	≥ 5 in geometric series	≥ 5 in geometric series	5 in geometric series of 2
Vehicle concentration	OECD No. 23	OECD No. 23	OECD No. 23	100 uL/L or 100 mg/L (lowest)	100 uL/L	<0.1 mL/L	<0.1 mL/L	<0.1 mL/L	<0.1 mL/L	<0.1 mL/L	ND	NA
Endpoint(s)	96 h LC50	48 h Imm.	EC50 growth	EC50 Imm.	96 h LC50	96h IC50 shell growth reduction	96 h LC50	96 h LC50	48h EC50 mortality or abnormality	96 h LC50	96h IC50 & NOEC/IC05 yield, growth rate, algal density	96 h LC50 (+ additional endpoints)

*None if water is unfiltered, feeding supplement needed if water is filtered
^complete darkness acceptable for compounds prone to photodegradation
[&]30-minute transition recommended
[@]10 organisms preferred with 2 replicates

2.2 Materials and Methods

2.2.1 Test Species and Coral Culture Conditions

G. fascicularis were obtained from St. Mary's College of Maryland (SMCM) from a culture system started with at least 8 individual colonies from various sources and maintained at this location since 2002. Artificial seawater (ASW; Instant Ocean® Sea Salt Mix [Blacksburg, VA] in deionized [DI] water) was prepared at 35‰ and allowed to completely dissolve for two days before adding it to the coral culture tanks (see Text S1 for a complete summary of culture conditions and Table S1 for summary of culture water quality parameters[1]). This conditioned ASW was used, unfiltered, as dilution water for all preliminary and definitive exposures as previous tests have shown that coral health was reduced if filtered (0.2 μm) water was used (C.L. Mitchelmore, personal communication). All ASW was kept at exposure temperature and aerated until use.

At least three different parent colonies for each independent experiment were used to provide polyps for toxicological testing. Individual polyps were fragmented from the parent colony and attached on their sides to a plain ceramic poker chip in what was called the "4-star" pattern (Figure S1). Polyps were allowed to recover for 3-4 weeks after fragmentation at the coral culture facility at SMCM before moving them to the Chesapeake Biological Laboratory (CBL). At CBL the polyps were placed into the treatment vessels and acclimated to test conditions for 24 hours. Corals were not fed during the acclimation or exposure periods.

[1] Appendix A contains all supplementary text, Appendix B contains supplementary tables and Appendix C contains supplementary figures.

2.2.2 Chemicals

Copper (II) chloride ($CuCl_2$, CAS# 7447-39-4, 97%) for exposure solutions and analytical standards was obtained from Sigma-Aldrich, St. Louis, MO. Dissolved fractions were preserved with nitric acid (Baker Instra-Analyzed, 69-70%). Filters were digested with the same acid along with hydrogen peroxide (Baker Analyzed, 30%). For copper exposure solutions, a stock solution of $CuCl_2$ dissolved in control water at the high concentration (1.0 mg/L copper) was prepared daily and diluted to make all other exposure concentrations. To prepare definitive test solutions, culture water was dosed as pooled replicates then split into individual exposure vessels.

2.2.3 Test Setup

Toxicity testing was based on the EPA guidelines for Mysid Acute Toxicity Testing (US EPA, 2016a) and EPA and OECD general guidelines for toxicity testing (OECD, 2019; US EPA, 2016b) with modifications in parameters such as lighting scheme and salinity to reflect appropriate conditions for coral health.

Exposures were carried out in 2.0 L glass beakers with aeration to drive circulation in the vessel as adequate water flow is essential for the health of *G. fascicularis* (Schutter et al., 2010). Vessels were loosely covered with plastic wrap to prevent evaporation, provided full-spectrum illumination on a 12:12 light:dark cycle, and kept in a water bath set to maintain a coral exposure temperature of 26 ± 1 °C which was monitored continually using a HOBO data logger. Photosynthetically active radiation (PAR, μmol m^{-2} s^{-1}) was measured daily to confirm spectral quantity. Daily water quality (temperature, dissolved oxygen, salinity, pH) was performed on pooled replicates of new

(i.e. immediately after solution preparation) and aged (i.e. after 24 h of coral exposure, before renewal) solutions for each concentration using a YSI instrument. Daily water quality and PAR are summarized in Table S2.

Before water changes each day, images of each chip and readings with a Junior PAM (Pulse-Amplitude-Modulation) fluorometer (Heinz-Walz, Effeltrich, Germany) were taken for analyses described in section 2.6. Seawater samples of pooled replicates from definitive exposure solutions were taken daily. Unfiltered samples for confirmation of new (n = 2 per concentration) and aged (n = 1) exposure concentrations were refrigerated until the processing and analysis described in section 2.5. Additional seawater samples were filtered through a 0.7 µm Whatman GF/F glass fiber filter (47 mm). The filter and filtrate were independently frozen at -80 °C for chlorophyll-α/phaeophytin analysis and additional water quality analyses (nitrates, nitrites, ammonia, phosphates, and alkalinity; Table S3), respectively.

On day 0 of definitive exposures, additional coral polyps that were not part of the exposure were photographed, measured with the Junior PAM, and then immediately frozen at -80 °C for tissue analyses. After observation on Day 4 of definitive exposures, all test corals were immediately frozen at -80 °C for the same purpose. Additional test setup detail can be found in Text S2.

2.2.4 Toxicity Tests

2.2.4.1 Range-Finding Tests

To determine the concentration range for definitive testing, three preliminary, range-finding acute toxicity tests were carried out without replication of exposure vessels.

Copper toxicity tests were conducted using $CuCl_2$ (10, 1, 0.1, 0.01, 0.001 mg/L Cu; test 1 and 0.01, 0.05, 0.1, 0.5, 1.0 mg/L Cu; test 2) or $CuSO_4$ (10, 1, 0.1, 0.01, 0.001 mg/L Cu; test 3) plus a negative control (dilution seawater only) for each test. The toxicity observed in the two copper compounds was comparable so $CuCl_2$ was employed for definitive testing because it dissolved more quickly in seawater than $CuSO_4$.

2.2.4.2 Copper Definitive Acute Tests

After preliminary testing, copper was used as the primary compound for three definitive acute tests to confirm reproducibility of the results. Definitive tests differed from preliminary tests in that for the definitive tests, the number of organisms was increased, replication was added, concentration range narrowed, and chemical analysis and full endpoint characterization were performed.

Each definitive test was a 96 h, static renewal with a 24 h (daily) renewal interval including a negative control and 5 exposure concentrations (0.063, 0.13, 0.25, 0.50, and 1.0 mg/L Cu) with 4 replicates each. The three tests were carried out in January, February and November 2020.

2.2.5 Chemical Analysis

Analysis of dissolved and particulate copper concentrations were based on the EPA 6020 guideline (US EPA, 2014). Seawater samples were collected in acid-washed polyethylene bottles and filtered through 0.45 μm cellulose acetate filters which were placed at -20 °C until digestion. The filtered samples were preserved with 0.5% nitric acid and refrigerated until analysis of dissolved copper concentrations. For dissolved

analysis, samples were diluted 1 in 100 to lower the salt level and analyzed directly using an Agilent 7500 ICP-MS with helium in the collision cell to reduce interferences. While internal standards were used (Germanium 72) to correct for drift, a subset of samples were spiked with copper to assess ongoing performance as suppression from complex matrices, such as saltwater, can occur. The limit of detection (LOD) and limit of quantification (LOQ) for the dissolved copper fraction were 0.03 µg/L and 0.4 µg/L respectively.

The cellulose acetate filters for analysis of the particulate copper fraction were microwave digested in a Milestone EOTHO-EZ microwave. Filters were placed in a 20 mL quartz reaction vessel with 5 mL concentrated nitric acid for 2 hours, after which 5 mL DI water was added to the vessel. The vessel was covered with a quartz cap and placed inside a Teflon chamber. Ultrapure water (5 mL) and 5 mL 30% H_2O_2 were then added to the outer Teflon chamber. The Teflon chamber was capped and pressure sealed. The vessels were heated to 180 °C over a 20-minute period and allowed to reflux for 20 minutes at 180 °C. Once cooled, the samples were diluted to 15 mL with DI water. A subsample (50-500 µL) of the digest was then diluted to 10 mL and copper was measured using an Agilent 7500 ICP-MS with helium in the collision cell. The LOD and LOQ for particulate copper were 0.03 µg/L and 0.7 µg/L, respectively.

2.2.6 Biological Endpoints

Mortality was assessed daily and LC50s were calculated using a dose response curve discussed in section 2.7. Mortality was qualified visually by the sloughing of tissues as well as the lack of fluorescence under the full-spectrum exposure lights (Figure

S2). Mortality in corals is challenging to assess compared with standard test organisms (i.e. mysid shrimp and larval fish) especially given that tissue is both external to the skeleton and within the oral cavity into which polyps may retract. Placing corals in recovery conditions after testing can ensure if true mortality occurred (i.e. no recovery) but the additional endpoints required for these tests precluded this assessment.

As corals are very different to the current toxicological standard test species, most notably their symbiotic relationship between the invertebrate host and algal symbiont, other assessments were also conducted. These were used to investigate if other endpoints would be more appropriate and/or more sensitive to use for acute coral testing. Daily images were used to determine the degree of polyp retraction by visually scoring them from 0 (no polyps visible, full retraction) to 4 (full extension, polyps appear relaxed and freely moving). This was compared to a quantification of polyp extension using Adobe Photoshop® where one tentacle from each polyp was measured using the "measure" tool from where it became visible above the skeletal cup to its tip and standardized against the diameter of the poker chip (Figure S1). As these two manners of characterizing polyp retraction correlated well (Figure S3), the quantification method was used for statistical analyses to avoid observer bias.

Coral bleaching is a common endpoint often reported both for corals in the field and in the laboratory as an observation of stress. In these experiments, bleaching was assessed in a number of ways. First, it was qualified using the Coral Color Reference Card from Siebeck et al. (2006) then quantified using an image analysis method modified from the same study. Images were standardized using the white poker chip as a white standard and analyzed using Adobe Photoshop® to measure brightness and saturation

composition in an averaged 5x5 pixel selection. One selection was taken per polyp then averaged by replicate (i.e. n = 12 samples). Visual scoring of the polyps correlated well with quantification of saturation (Figure S6) and saturation of the polyp was shown to be a more sensitive value with a larger range of responses over the spectrum of healthy to bleached coral (Figure S1). Therefore, to reduce observer bias, quantified saturation was used in statistical analysis.

Second, the algal pigments chlorophyll-α (chl-α) and phaeophytin in coral tissue were quantified following Yost and Mitchelmore (2010). After defrosting from -80 °C, coral tissue was removed from the skeleton by air-brushing with 0.2 µm filtered ASW (at 35‰ using Crystal Sea® Marinemix [Marine Enterprises, Baltimore, MD]). The resulting tissue slurry was homogenized and either immediately processed for algal pigment analysis or frozen at -40 °C for later assessment of pigment and protein levels. For pigment assessment, 1 mL of the slurry was filtered using a GF/F filter which was then placed into 4-5 mL of 90% acetone and allowed to extract at 4 °C for 12-18 h before processing.

Third, chl-α was measured in the aged exposure water using the Nutrient Analytical Services Laboratory protocol (NASL, 2019). Frozen GF/F filters that had been used to filter exposure water were briefly thawed and placed into a centrifuge tube with 5 mL 90% acetone then placed at 4 °C for 12-24 hours before processing for pigment analyses.

After the refrigeration, all pigment samples (tissue and exposure water) were centrifuged and the acetone was removed, filtered, and placed into a cuvette where absorbance readings were made at the wavelengths 750, 665, 664, 663, 647 and 630 nm

using a spectrophotometer (SpectraMax® PLUS 384 [Molecular Devices, San Jose, CA]). Then, the extract was acidified with 1 N HCl and readings were again made at the aforementioned wavelengths. These results were used in the equations outlined by NASL (2019) to calculate uncorrected chl-α, phaeophytin, and chl-α corrected for phaeophytin. To determine the best manner to standardize tissue chl-α, both surface area and protein content were quantified.

Finally, although not a direct measure of bleaching, a Junior PAM using a saturating pulse was used to measure light-adapted photosynthetic efficiency $\Delta F/F_m$' of each of the polyps using the PAM software (WinControl-3 v 3.29; see Murchie and Lawson, 2013 for a comparison of measures of photosynthetic efficiency). Extended methods for all biological endpoints including surface area and protein calculations can be found in Text S3.

2.2.7 Statistical Analysis

Statistical analysis was performed using R (v. 3.4.2) and R Studio (v 1.2). Statistical significance was determined at the $p < 0.05$ level. Statistics used measured dissolved concentrations from newly made exposure solutions. To determine if there was a dose-dependent effect, the Jonckheere trend test was employed for monotonic data that did not satisfy the assumption of normally distributed data as determined by a Shapiro-Wilks test and confirmed visually. For data that satisfied the assumption of normality, and were non-monotonic, Dunnett's Test was employed.

LC/ECx's, lowest-observed effect concentrations (LOECs), and no-observed effect concentrations (NOECs) are all reported, however it should be noted that endpoints

other than LC50s are generally used for chronic rather than acute risk assessment and are only included to add to the understanding of how copper impacts this species. To determine NOEC and LOEC the package "mixtox" was used (Zhu, 2017) and for LC/EC_x determination and graphing of the dose response curve, the "drc" package was used (Ritz and Strebig, 2011). The dose-response curve was chosen using Akaike information criterion (AIC).

2.3 Results and Discussion

All three acute definitive copper exposures demonstrated similar results across all biological endpoints showing that the effect of copper on *Galaxea fascicularis* is reproducible making this species a good candidate for a standard toxicity test organism (Figure 1). Dissolved copper in new solutions was measured between 79 and 104% of nominal (Table 2). After 24 h, the measured aged stock was between 4 to 38% lower than the new stock (Table S4). This is likely from fractionation to the particulate fraction and uptake by corals as the particulate fraction did increase in the aged solutions (Table S5), however this fraction did not account for all loss over the 24-hour period.

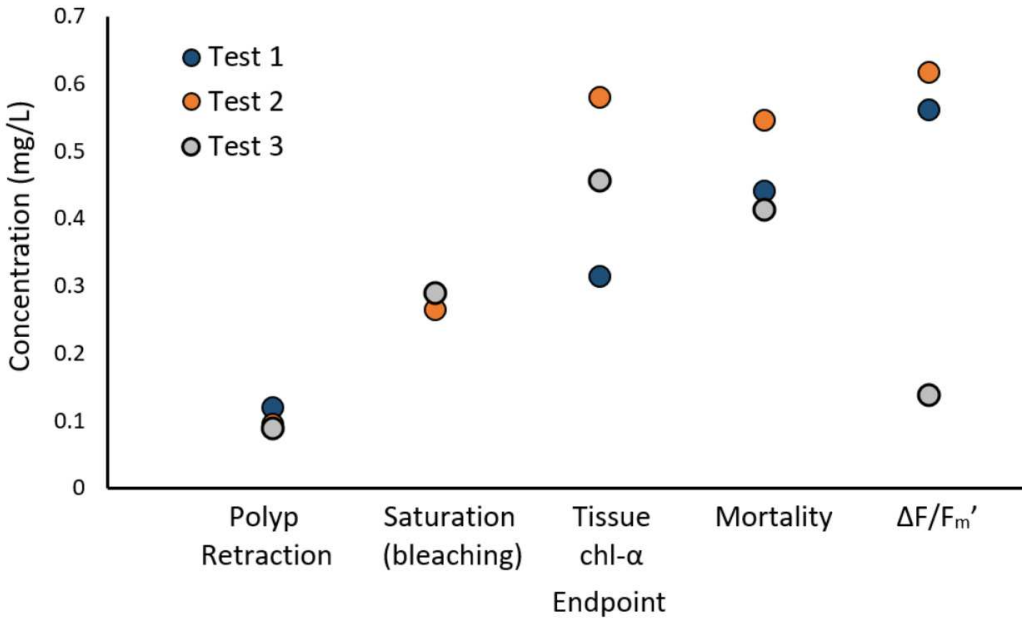

Figure 1. Caclculated EC/LC50s for endpoints of triplicate copper acute tests with *G. fascicularis*. Replication between all three tests was generally good with the exception of $\Delta F/F_m'$ in test 3 and some variation with tissue chl-α. Polyp retraction was seen to be the most sensitive endpoint.

Table 2. Concentrations of new copper solutions (mg/L) for copper test 1, 2, and 3. Concentrations show averages of new solutions over 4 days of testing.

Concentration (mg/L)	Copper Acute #1	Copper Acute #2	Copper Acute #3
Control	0.007 (± 0.001)	0.013 (± 0.003)	0.009 (± 0.003)
0.063	0.053 (± 0.003)	0.065 (± 0.002)	0.054 (± 0.013)
0.125	0.106 (± 0.005)	0.124 (± 0.010)	0.118 (± 0.017)
0.25	0.198 (± 0.021)	0.229 (± 0.025)	0.213 (± 0.044)
0.50	0.387 (± 0.034)	0.474 (± 0.067)	0.419 (± 0.095)
1.0	0.769 (± 0.073)	0.839 (± 0.092)	0.828 (± 0.062)

The LC50 for copper in *Galaxea fascicularis* was calculated as an average of 0.436 ± 0.056 mg/L dissolved Cu which was similar to the 0.511 mg/L calculated in a later test when Cu was used as a positive control discussed in Chapter 3. and is within the acceptability criteria for reference toxicant results (i.e. within 2 standard deviations from the mean; US EPA, 2002). In the case of our testing, the acceptable range would be 0.324 to 0.548 mg/L. Other copper acute tests lasting a variety of durations have been completed (Table S6). However, most studies have looked at mortality in coral larvae

(Kwok et al., 2016; Reichelt-Brushett and Harrison, 2004; Esquivel, 1986) and only one study has examined adult coral mortality (Sabdono, 2009). These papers, especially Esquivel (1986) and Reichelt-Brushett and Harrison (2004) demonstrate similar results of larval LC50s calculated over various time periods with a 96 h larval LC50 ranging from 0.057 (Esquivel, 1986) to 0.107 (Kwok et al., 2016) mg/L Cu (Table S6). However, the calculated LC50 presented here (0.436 mg/L; Table 3) is in conflict with previous adult mortality data from Sabdono (2009) which showed an LC50 at 0.032 mg/L in *G. fascicularis*. Little information about dosing and other methodological specifications are provided by Sabdono (2009) which makes it difficult to ascertain the reason for the discrepancy. When looking at current standard testing organisms (i.e. mysid shrimp and algae), 96 hour LC50 values range from 0.0697 to 0.284 mg/L for *Americamysis bahia* (Science Applications International Corp, 1993; Cripe, 1994; Lussier et al., 1985; US EPA, 1992) and only a single 96-hour algal study could be found for *Chlamydomonas reinhardtii* with an LC50 of 0.808 mg/L (Zhang et al., 2008). The mysid results are similar to the coral results presented elsewhere, but, again, are lower than the LC50s calculated in this study. However, the algae results are actually higher than what is presented here and elsewhere for corals.

Table 3. Summary of endpoints for triplicate copper tests (n = 3). All concentrations are in mg/L and EC/LC50s are given as an average for the three tests ± standard deviation. The most common NOEC and LOEC from the three tests is included, in the case that one of the three tests calculated different a LOEC/NOEC, those values are included in the footnotes. The LC50 in copper positive control was 0.511 mg/L.

Endpoint	NOEC	LOEC	EC/LC50
Mortality	0.25	0.50	0.436 (± 0.056)
Quantitative retraction	NA	0.063	0.054 (± 0.032)
$\Delta F/F_m'$	0.50[A]	1.0[A]	0.453 (± 0.057)
Saturation	0.063[B]	0.13[B]	0.277 (± 0.017)[E]
Tissue Chl-α	0.25[C]	0.50[C]	0.450 (± 133)
Tissue phaeophytin	1.0[D]	NA[D]	NA

[A]Test 3: 0.063 and 0.13
[B]Test 1 was NA and 0.063
[C]Test 3 was 0.13 and 0.25
[D]Test 2 was 0.50 and 1.0
[E]An EC50 for saturation in Test 1 was calculated at 4.01 mg/L and not included in this calculation

It is recommended that further exploration using other lineages of *G. fascicularis* should be completed to determine if this species truly demonstrates a high level of variation in hardiness. Furthermore, even if this species is hardier than others, this variability is common in related species (e.g. coral cell line results in Downs et al., 2016) and may be an asset to a standard organism as it would need to be robust enough to survive in a laboratory culture. An adjustment factor would simply need to be applied to toxicity testing results to account for more sensitive coral species.

Standard acute toxicity tests may also have additional observations recorded outside of death or immobilization. For example, the EPA OCSPP 850.1075: Freshwater and Saltwater Fish Acute Toxicity Test requires that "any abnormal behavior or appearance, and the number of individuals exhibiting these characteristics, should be counted and recorded at the same time as observations of mortality" (US EPA, 2016c). For corals, an obvious behavioral change is polyp retraction. Polyp retraction has often been reported as a behavioral sign of stress with many triggers (e.g. sedimentation stress:

Vargas-Ángel et al., 2006; light stress: Brown et al., 1999). Long-term polyp retraction may reduce feeding for a heterotrophic species like *G. fascicularis* which would in turn decrease nutrient intake (May et al., 2020). Furthermore, this behavior is energetically expensive and may shade symbionts, decreasing their carbon fixation and leading to decreased growth if sustained over longer periods of time. These long-term effects could be exacerbated by negative impacts from the stressor to the symbionts directly. Significant polyp retraction relative to controls was seen in all copper exposure concentrations (Table 3; Table S7) following a dose-dependent response meaning that those corals in high copper conditions retracted their polyps more severely than those at lower concentrations. Due to its sensitivity, dose-dependent response, and potential energetic impacts, polyp retraction may be worth considering as a standard endpoint for sublethal effects to corals in chronic toxicity testing or simply as a supplementary behavioral observation in acute testing.

Bleaching is the most widely reported endpoint in coral toxicity studies and given its field importance, and importance to the maintenance of symbiosis, it is a key endpoint to measure. An increase in visible bleaching and a decline in photosynthetic efficiency of the symbionts (i.e. $\Delta F/F_m$' decline) did not seem to occur in polyps that were not showing signs of severe stress and imminent mortality. Therefore, copper seems to more quickly and severely affect the host rather than the algal symbiont which mirrors the aforementioned toxicity thresholds from standard algal testing. The EC50 of $\Delta F/F_m$' was generally similar to the LC50 and the LOEC/NOEC was identical to those for mortality in the first two tests but gave quite different results in the third possibly due to a slight decrease in laboratory light intensity. For this reason, as well as the specialize equipment

necessary for measurements, this is not suggested to be a high-quality endpoint for standardized testing. Furthermore, Bielmyer et al. (2010) studied the effects of copper to the effective quantum yield (equivalent to light-adapted photosynthetic efficiency) and showed significant impacts *Acropora cervicornis* zooxanthellae at 20 µg/L and to *P. damicornis* at 4 µg/L (Bielmyer et al., 2010) which shows some moderate variation between species.

Only four studies have examined algal endpoints including chl-α (Nyström et al., 2001 [24h NOEC: 11 µg/L] and Alutoin et al., 2001 [14 h LOEC: 30 µg/L]), algal cell density (Yost et al., 2010 [48h LOEC 5 µg/L]), and zooxanthellae density (Jones, 1997 [48 h LOEC: 20 µg/L). Our studies showed tissue chl-α concentrations significantly decreasing at 0.50 mg/L and saturation decreasing at 0.13 mg/L (Table S7). Exposure water chl-α analysis showed a dose-dependent increase in chl-α for the first two days then a subsequent decline to concentrations similar to the control treatment. These results suggest that the corals used in this experiment may be more resistant to copper effects than the variety of species studied in the literature. , Tissue chl-α and image analysis of saturation percentages were both highly replicable endpoints in the three experiments conducted in this study with tissue chl-α appearing more variable and slightly less sensitive than saturation measurements in two of three tests. However, in Test 1, the saturation EC50 was calculated as 4.01 mg/L which is vastly higher than any other endpoint calculation likely because little variation in saturation was seen in this test. Even considering this, saturation analysis can be done relatively quickly and has the capability of being highly repeatable. Because of the ease of analysis, saturation percentage determination may be better suited for routine toxicity testing. However, tissue chl-α

analysis could be added in the event of a compound with a suspected bleaching mode of action or compounds in which preliminary testing showed significant impacts to saturation measures to confirm bleaching.

All of the results in this study lead to important questions of interspecies sensitivity and intraspecies variability when compared to literature values. From these results, *G. fascicularis* seems to be less sensitive than most hard coral species. To investigate this further, a species sensitivity distribution (SSD) was attempted using the information from the EPA ECOTOX database (OPP Pesticide Ecotoxicity Database, 2021; Figure S4). Unfortunately, the only endpoint in this database for *G. fascicularis* is from Sabdono (2009). If we include the data from chapter 3 of this thesis, copper and BP-3 are the only two compounds with results for *G. fascicularis*. Although these compounds have been tested on a variety of other species, there are no other calculated adult LC50s with which to compare these. In the case of the additional endpoints, it is impossible to compare more than a couple of species due to differences in test duration, life stage, and endpoints measured. Recently, data from Turner (2020) demonstrated exposure of five species of corals to three different hydrocarbons in an effort to determine sensitivity. Unfortunately, *G. fascicularis* was not part of this study, but this does provide a framework which is necessary moving forward to determine relative sensitivity of different species and relative toxicity of different compounds without an accepted, standardized coral testing approach.

In order to approximate relative sensitivity, a combined ranking of several studies which compared at least three species was used to build upon "hardiness" rankings from Sprung (1999; Table 4). This includes the aforementioned study on hydrocarbons

(Turner, 2020), a study on the effects of temperature and pCO_2 (Bahr et al., 2016), a study on the combined impacts of UV light and temperature (Ferrier-Pagès et al., 2007), and a final study on temperature sensitivity (McClanahan, 2017). As Sprung (1999) ranked hardiness on a scale of 1-10, the least sensitive genus in each study was given a rank of 10 (i.e. hardiest) and those ranked as more sensitive were given subsequently lower numbers. This assumes the genus which was least sensitive to the stressor is actually the least sensitive of all genera; however, this was necessary in order to provide average rankings and is only used as a tool to help determine where *Galaxea spp.* sensitivity lies. Only genera with at least three rankings (other than Sprung, 1999) were considered, except in the case of *Galaxea spp.* where only one other study ranked the genus of interest. With this ranking system, *Galaxea spp.* achieves an average ranking in the middle of the pack, being the 5^{th} most sensitive genus out of 10 although results for this study would suggest it is far less sensitive than others. However, this ranking is to be taken lightly as there is little data on this genus and all studies used in this ensemble have variable methodology. This again highlights the need for standardized coral testing. It is our belief that following the established and openly available guidelines of OECD and the EPA provides the standardization needed. Using this framework, *G. fascicularis* can then be assessed next to a number of other coral species to determine what assessment factors would need to be put in place in order to take into account this species sensitivity, or lack therefore, in comparison to other reef-building corals.

Table 4. Sensitivity ranking of 10 hard coral genera based on multiple stressors.

	Sprung, 1999	Turner, 2020			Bahr et al., 2016			Ferrier-Pagès et al., 2007	McClanahan, 2017		
Genus	**Overall**	**Toluene**	**Methyl-napthalene**	**Phenan-threne**	**Temp**	**pCO_2**	**Temp+ pCO2**	**UV light+ Temp**	**Temp**	**Sum**	**Avg. Rank**
Siderastrea	6	7.00	9	10						32	8
Acropora	4.7*	8	8	10				8	10	48.7	8.12
Porites	7	6	10	8	8	8	8		10	65	8.13
Pocillopora	7				8	9	9		8	41	8.2
Galaxea	7								10	17	8.5
Solenastrea	7	9	10							26	8.67
Stephanocoenia	9	10	7	9						35	8.75
Montipora	6				9	10	10	10	8	53	8.83
Fungia	8				10	10	10			38	9.5
Leptastrea	9				10	10	10			39	9.75

* Sprung (1999) gave 3 hardiness scores that were species-dependent. This is the average value of those 3 scores (3, 4, 7).

2.4 Conclusions

Three repeated acute toxicity tests with copper were conducted (as well as copper used as a positive control) and produced repeatable results demonstrating that this species would be a good standard testing organism. Furthermore, *G. fascicularis* grows well in laboratory culture conditions and is easily fragmented allowing laboratories without direct access to natural seawater to test on a coral species. However, these results were significantly different than the body of literature, all be it sparse, currently in existence on coral toxicity to copper, suggesting additional testing using an alternate strain of this species will be needed before baseline toxicity values can be determined. Although *G. fascicularis* may not be a highly sensitive species, it is likely one of the more ecologically relevant species to toxicity testing for compounds than may be a part of coastal pollution as they are shallow-water corals and therefore in the zone of highest impact. This standard framework provided robust results and should be used in further testing to make coral toxicity endpoints comparable and sensitivity rankings possible. Furthermore, a standard test using a hard coral like *G. fascicularis* should be considered by EPA and OECD due to the ecological importance of reef-building corals.

Chapter 3: Acute toxicity of the UV filter oxybenzone to the coral *Galaxea fascicularis*

3.1 Introduction

Now that a repeatable standard testing format has been established, the next step is to use these methods to expose *G. fascicularis* to BP-3 in an acute test. Mortality is typically the focal endpoint in acute toxicity tests (i.e. the LC50, the lethal concentration resulting in 50% mortality) as well as the acute endpoint usually used for a formal acute risk assessment (ECHA, 2008). However, no toxicity test performed thus far has achieved an acute LC50 for BP-3 on adult coral fragments. The only LC50s reported in an intact coral are for *S. pistillata* larvae exposed for 24 h (ranging 139 to 779 µg/L nominal concentration BP-3 for light and dark conditions; Downs et al., 2016) in a single unreplicated experiment. In contrast, the study by He et al. (2019a) showed no mortality in *Seriatopora caliendrum* or *Pocillopora damicornis* larvae exposed to up to 1,000 µg/L (nominal) BP-3 for 14 days (a duration implying a chronic, sublethal endpoint test). With only two studies that differ in various aspects of study design, it is challenging to compare these results. Furthermore, mortality of the host coral may not be the only important acute biological endpoint for these complex symbiotic organisms.

For example, bleaching measures (i.e. loss of algal symbiont) have already been used in an informal risk assessment by Tsui et al. (2014). Studies with UV filters to date have enumerated bleaching in a number of ways including the concentration of algal cells within coral tissue (algal density), pigment analysis (e.g. tissue chlorophyll-α content), the efficiency or yield of photosystem II of the algal symbionts using a PAM fluorometer (photosynthetic efficiency), and visible bleaching which can either be a qualitative

assessment or a quantitative measure using image analysis software. For example, Danovaro et al. (2008) showed BP-3-induced bleaching using visual scoring methods on *Acropora spp.* fragments beginning at 24 h in an *in situ* experiment. Furthermore, He et al. (2019a) showed changes in algal density in adult fragments after 7 days at the highest dose (1,000 µg/L nominal BP-3), similar to a recent study by Wijgerde et al. (2020) where BP-3 at their single dose of 1 µg/L (nominal) over 6 weeks did not affect algal density on its own in adult *S. pistillata* or *Acropora tenuis* fragments.

Another endpoint often reported for corals is polyp retraction which has been shown to be one of the more sensitive whole-organism endpoints following UV filter exposures (He et al., 2019a; 2019b; Stien et al., 2019). May et al. (2020) also saw polyp retraction as the most sensitive endpoint in response to polycyclic aromatic hydrocarbon exposure and speculated that because this retraction closes off the gastrodermal cavity, it may lessen or delay toxic effects as the gastrodermal layer is not protected by mucus. This suggests that although this endpoint may not lead directly to negative impacts, it may be a good indication of significant stressor exposure and an attempt for the coral polyp to protect against it.

Many of the existing UV filter studies have failed to include one or more aspects of traditional, standard toxicity test protocols and QA/QC procedures including appropriate water quality, analytical confirmation of exposure concentrations, appropriate dosing schemes to maintain acceptable toxicant concentrations, and a positive control to ensure appropriate, replicable response of the testing organism. Even when aspects like analytical confirmation of exposure solutions are included, they often fall short of recommendations by only measuring at the beginning and end of an exposure even when

significant losses are seen over that time period (He et al., 2019a; 2019b) or failing to analyze new and aged solutions at renewal intervals (Wijgerde et al., 2020) which makes actual exposure concentrations impossible to determine. The different methods used and lack of repeated testing to confirm results has yielded a sparse and conflicting array of toxicity test results of BP-3 on corals. This makes determination of BP-3 risk and, therefore, informed decision making difficult. In order to determine BP-3 toxicity to corals, the triplicate acute toxicity tests in this study used *Galaxea fascicularis*, a shallow-water reef-building coral native to the Red Sea and Indian Ocean (Hoeksema and Cairns, 2020) and followed standard methods and QA/QC procedures modified from the EPA Mysid Acute Toxicity Test (US EPA, 2016b) including analytical verification of exposure solutions and the use of a positive control to determine reproducibility between multiple independent experiments.

The objective of this study was to conduct repeated acute (96 h) toxicity tests with BP-3 using appropriate QA/QC procedures to provide the first LC50 for the UV filter BP-3 in an intact adult hard coral species. The use of three repeated BP-3 tests is a high level of replication that has not yet been demonstrated for UV filter exposure to corals. Results from this work provide a reproducible and reliable estimation of the acute toxicity of BP-3 in a representative hard coral species and are used along with concentrations from a well-controlled monitoring study to calculate a conservative RQ for BP-3 and *G. fascicularis.*

3.2 Materials and Methods

3.2.1 Test Species and Coral Culture Conditions

Test species and culture conditions were identical to that of the copper testing (Section 2.2.1).

3.2.2 Chemicals

Benzophenone-3 (CAS# 131-57-7, 99.96%, Certified Reference Material), diuron (CAS# 330-54-1, >98%), and copper (II) chloride ($CuCl_2$, CAS# 7447-39-4, 97%) for exposure solutions and analytical standards were obtained from Sigma-Aldrich, St. Louis, MO. Deuterated d_5-BP-3 (CAS# 1219798-54-5, >98%) and d_6-diuron (1007536-67-5, >99.0%) used for internal standards for Liquid-chromatography triple-quadrupole mass spectrometric (LC-qqq-MS) quantification were obtained from Sigma-Aldrich, St. Louis, MO.

Unique dosing stocks for BP-3 and diuron were prepared for each exposure concentration at the beginning of each toxicity test using Optima LC/MS grade methanol (MeOH, Fisher chemical, Pittsburg, PA; see Text S4 for justification of solvent choice) and stored at -25 °C. A maximum solubility of approximately 50 mg/mL was achieved for both diuron and BP-3 at room temperature with ultrasonication. Therefore, a maximum of 5 mg/L exposure solution of each compound could be made using the OECD and EPA acute testing guideline of the maximum allowed 100 µL/L solvent carrier (US EPA, 2016a; OECD, 2019). However, for the high concentration of each toxicant, double this guideline (i.e. 200 µL/L) was used to create a 10 mg/L solution and an additional high solvent control was added. For copper, a stock solution of $CuCl_2$ dissolved in control ASW at the high concentration (1 mg/L Cu) was prepared daily and diluted with control water to make all other exposure concentrations.

LC/MS grade methanol, Optima LC/MS 0.1% formic acid (Fisher chemical) and formic acid (>98%, Sigma-Aldrich) were used for all extraction applications except in the first BP-3 study where acetonitrile (LC/MS grade, Fisher chemical) was used instead of MeOH (see section 2.5 for justification).

3.2.3 Test Setup

To determine the appropriate dosing frequency and exposure volume/mass ratios for the static tests, a preliminary investigation was conducted (see Supplemental Text S5 for further details). From these results, a 24-hour renewal was decided with 1 L of exposure water to ensure daily maintenance of BP-3 concentrations and adequate water quality (e.g. dissolved oxygen levels). Following this investigation, preliminary, range-finding acute toxicity tests for the BP-3 definitive tests and positive controls [n = 2 (BP-3), 1 (diuron) and 3 (copper)] without replication of exposure vessels were carried out to determine appropriate concentration ranges before definitive acute toxicity test exposures were conducted. For full details on preliminary investigations, please see Text S5. Subsequently, three repeated independent BP-3 definitive tests were carried out based on the aforementioned standard testing with copper (Chapter 2) with the addition of a positive control (e.g. either copper or diuron).

Exposures were carried out in 2.0 L glass beakers with aeration to drive circulation in the vessel as appropriate water flow conditions are essential for the health of this species (Schutter et al., 2010). Vessels were loosely covered to prevent evaporation and provided full-spectrum illumination on a 12:12 light:dark cycle. Photosynthetically active radiation (PAR, μmol m^{-2} s^{-1}) was measured daily to confirm

spectral quantity. Vessels for all tests were kept in a water bath set to maintain a coral exposure temperature of 26 ± 1 °C which was monitored continually using a HOBO data logger. For all tests, daily water quality was performed on pooled replicates of new (i.e. immediately after solution preparation) and aged (i.e. after 24 h of coral exposure, before renewal) solutions for each concentration using a YSI instrument. Daily water quality (temperature, dissolved oxygen, salinity, pH) and light condition (PAR) are summarized in Table S8.

All observations were conducted on the coral polyps before water changes to avoid any potential impacts due to handling stress. Seawater samples of new and aged pooled replicates were taken daily at each exposure concentration. Samples for analytical confirmation of new (n = 2 per concentration) and aged (n = 1) exposure concentrations were refrigerated in the dark until extraction (within a few hours of sampling) as described below. Additional daily seawater samples from each concentration were filtered through a 0.7 µm GF/F glass fiber filter. The filter and filtrate were independently frozen at -80 °C for chlorophyll-α (chl-α) and phaeophytin analysis and additional water quality analyses (nitrates, nitrites, ammonia, phosphates, and alkalinity; Table S9), respectively.

On day 0 of definitive exposures, observations on additional coral polyps that were not part of the exposure were made and these corals were then immediately frozen at -80 °C for tissue analyses. After observation on Day 4 of definitive exposures, all test corals were immediately frozen at -80 °C for the same purpose. Additional test setup details can be found in Text S2.

3.2.4 Definitive Acute Toxicity Tests

To parallel the design of an EPA/OECD standard acute toxicity test, each definitive toxicity test conducted in this study was a 96 h, static renewal with a 24 h (daily) renewal interval including negative and positive controls and at least 5 exposure concentrations with 4 replicates each (each replicate containing n = 3 coral chips with n = 4 individual polyps on each chip). Positive controls were conducted at the same time as the BP-3 exposure tests and consisted of a 3 concentration by 3 replicate matrix.

The first BP-3 definitive acute toxicity test was carried out in August 2019 and did not include a positive control run in parallel. There were 6 exposure concentrations (0.31, 0.63, 1.3, 2.5, 5.0, and 10 mg/L nominal BP-3) along with a negative control and two solvent controls (100 and 200 µL/L MeOH). The second definitive exposure was performed in September 2019 using the same concentrations and exposure setup as the first definitive exposure but for this test, a positive control, diuron was employed (2.5, 5.0 and 10 mg/L nominal). The third definitive exposure was performed in March 2020. The concentrations and exposure setup were identical to the prior exposures, however the lowest BP-3 concentration was omitted and copper was employed as the positive control (0.25, 0.5 and 1.0 mg/L nominal Cu) because adequate diuron mortality was not achieved in the highest concentration that could be used given the 100 µL/L solvent limit recommended for standard toxicity tests (US EPA, 2016a; OECD, 2019).

3.2.5 Chemical Extraction and Analysis

BP-3 and diuron test exposure water samples and method recovery spikes were extracted using solid-phase extraction with modified protocols outlined in Mitchelmore et

al. (2019) and Carabias-Martínez et al. (2004), respectively. The most notable change from the BP-3 extraction protocol was that MeOH was utilized instead of acetonitrile (ACN) in the latter two exposures because adequate and consistent recoveries for this concentration range were achieved using this simplified method.

Seawater samples (50 mL each) for BP-3 and diuron chemical analysis were filtered through a 0.7 µm Whatman GF/F glass fiber filter which was stored at -20 °C until particulate analysis. Filtered samples were acidified to pH 2 using formic acid (FA), manually agitated, then run through a pre-conditioned HLB cartridge under vacuum at approximately 5 mL/min (Waters Oasis®, 6 cc, 150 mg sorbent; conditioned with 5 mL MeOH and 5 mL 0.1% FA). 0.1% FA (5 mL) was added to the cartridge to remove residual sample solution containing salts followed with 7 mL MeOH to elute BP-3 or diuron from the cartridge into 8 mL borosilicate glass amber vials which were placed at -20 °C until further analysis.

GF/F glass fiber filters were allowed to thaw briefly before the addition of 5 mL ACN and 5 mL DI water. Samples were then shaken at 300 rpm for 24 h then NaCl (0.6 g) and $MgSO_4$ (1.5 g) were added to the sample to remove salt and enhance solvent partitioning. Samples were shaken for 1 h then centrifuged at 3000 rpm for 10 minutes before 2.5 mL of the top layer of supernatant was removed and run through a pre-conditioned HLB cartridge under vacuum at approximately 5 mL/min (conditioned with 3 mL ACN) and retained. An additional 3 mL ACN was run through the cartridge and added to the sample to ensure full recovery. This sample was evaporated under N_2 gas and dissolved in 5 mL MeOH. Before analysis, all samples were diluted with MeOH to

levels that were within the calibration range and spiked with an internal standard of d_3-BP-3 or d_6-diuron.

LC-qqq-MS analyses were performed using an Agilent 6420A LC-qqq-MS (Agilent, Santa Clara, CA). To quantify samples, individual calibration curves for BP-3 and diuron were created in MRM mode using precursor ions (m/z) 229.09 and 233 respectively and product ions 151/105.1 and 160/72 respectively. LOQs for BP-3 and diuron were 0.015 mg/L and 0.074 mg/L respectively with the LODs calculated as LOQ/3 (0.005 mg/L and 0.025 mg/L respectively; see Text S6 for explanation). Due to low levels of BP-3 contamination during the sample extraction method in tests 1 and 2, which appears to be universal and not unique to this method (Saxe et al., 2020), only full analytical results from test 3 will be presented. The uncontaminated samples from the first two days of new samples for test 2 were similar to corresponding samples in test 3 (Table S10). Moreover, observations were similar in all 3 tests so exposure concentrations are expected to be similar in all three BP-3 exposures.

Analysis of dissolved copper is identical to that described in section 2.2.5. Additional details for all chemical analyses and method recovery spikes can be found in Text S6.

3.2.6 Biological Endpoints

Biological endpoint characterization was similar to that of the copper exposures. Mortality was assessed daily and LC50s of all three compounds (BP-3, diuron, and copper) were calculated using a dose response curve discussed in section 2.7. Mortality

was qualified visually by the sloughing of tissues from the skeleton and the lack of fluorescence under the full-spectrum lights (Figure S2).

Although mortality is the regulatory endpoint used from acute toxicity tests, additional biological endpoints were also investigated to determine their utility for acute coral testing and to fully characterize the toxicity of BP-3. Daily photographs were used to determine the degree of polyp retraction by visually scoring them from 0 (full retraction) to 4 (full extension). This was compared to a quantification of polyp extension using Adobe Photoshop®. As these two manners of characterizing polyp retraction correlated well (Figure S3), the quantification method was used for statistical analyses to avoid observer bias.

Coral bleaching was assessed in a number of ways. First, it was qualified using the Coral Color Reference Card from Siebeck et al. (2006) then quantified using the image analysis method modified from the same study. Visual scoring of the polyps correlated well with quantification of saturation (Figure S6). Therefore, to reduce observer bias, quantified saturation was used in statistical analysis. In addition, the algal pigments chl-α and phaeophytin in coral tissue were quantified following methods detailed in Yost and Mitchelmore (2010). Defrosted coral tissue was removed from the skeleton by air-brushing with filtered ASW. The resulting tissue slurry was homogenized and either immediately processed for algal pigment analysis or frozen at -40 °C for protein level assessment. For pigment analysis, 1 mL of the homogenate was filtered then placed into 90% acetone at 4°C for 12-18 h. Sample tubes were then centrifuged and the supernatant was removed, filtered, and placed into a cuvette where absorbance readings were made at 750, 665, 664, 663, 647 and 630 nm using a spectrophotometer. Then, the

extract was acidified with 1 N HCl and readings were again made at the aforementioned wavelengths. These results were used in the equations outlined in NASL (2019) to calculate uncorrected chl-α, phaeophytin, and chl-α corrected for phaeophytin. To standardize coral tissue chl-α, both polyp skeleton surface area and total protein content of the coral tissue were quantified. Finally, although not a direct measure of bleaching, assessment of algal health was conducted using a Junior PAM to measure light-adapted photosynthetic efficiency ($\Delta F/F_m'$) of each of the polyps using the PAM software (WinControl-3 v 3.29). Detailed methods for all biological endpoints are provided in Text S3.

3.2.7 Statistical Analysis

Nominal concentrations were used in BP-3 statistical analyses due to the analytical complications in the first two tests as well as to allow comparisons with the existing literature. Although measured values have been reported in some instances, endpoint determination and calculations have thus far have only been reported in nominal concentrations. This is mostly due to extreme toxicant losses over time and lack of daily exposure confirmation making average exposure concentrations impossible to calculate (He et al., 2019a; Wijgerde et al., 2020). However, the LC50 for test 3 was also reported as the measured concentration of the dissolved fraction in the newly made exposure solutions not corrected for recoveries to provide a more accurate toxicity estimate (Please see Text S6 for explanation). For copper, concentrations used in statistical analysis were averaged from analytical chemistry measurements of new dissolved solutions.

Statistical analysis was performed using R (v. 3.4.2) and R Studio (v 1.2). Statistical significance was determined at $p < 0.05$. Controls were pooled for analysis when there was no statistically significant negative effect of the solvent controls. When there was a difference, statistical significance was determined by comparing to the high solvent control for the high concentration (10 mg/L) and low solvent control for remaining concentrations. To determine if there was a dose-dependent effect, the Jonckheere Test was employed for monotonic data that did not satisfy the assumption of normally distributed data as determined by a Shapiro-Wilks test and confirmed visually. For data that satisfied the assumption of normality and were non-monotonic, Dunnett's Test was employed. LC/ECx's, lowest-observed effect concentrations (LOECs), and no-observed effect concentrations (NOECs) are all reported, as endpoints other than LC50s are generally used for chronic rather than acute risk assessment, these are only included to add to the understanding of how BP-3 impacts this species. To determine NOEC and LOEC the package "mixtox" was used (Zhu, 2017) and for LC/EC_x determination and graphing of the dose response curve, the "drc" package was used (Ritz and Strebig, 2011). The dose-response curve was chosen using Akaike information criterion (AIC).

3.3 Results and Discussion

This study provides the first determination of an LC_{50} for BP-3 in an adult hard coral and did not show any evidence that BP-3 causes bleaching in this species. This study employed the use of replicate experiments, positive controls and analytical verification of the exposure solutions which are facets that have so far been neglected in many UV filter exposure studies on corals. A detailed table of biological endpoints for all

definitive BP-3 tests can be found in Table S11. The positive control, copper, demonstrated reproducible results (LC50 of 0.563 mg/L) during the BP-3 tests, which were similar to triplicate acute toxicity tests performed with copper (LC50 of 0.436 ± 0.056 mg/L dissolved Cu) and is within the acceptability criteria for reference toxicant results (i.e. within 2 standard deviations from the mean; US EPA, 2002).

Because the exposure route of a toxicant is an important consideration and many UV filters are poorly soluble and thus are more likely to bind to particulate matter, dissolved and particulate concentrations of BP-3 in exposure solutions were determined independently. Furthermore, this will help elucidate any supersaturation at higher concentrations where BP-3 did not immediately incorporate into solution without a solvent carrier. Total new BP-3 concentrations (i.e. dissolved and particulate combined) ranged from 5 to 27% of nominal (Table 5) with lower percentages seen in the low (0.063 mg/L) and high (10 mg/L) concentrations. The dissolved fraction of new exposure concentrations varied between 5 and 16% of nominal and clean controls (Table S12). Particulate BP-3 retained on GF/F filters was between 2.7 and 54.4% of average total measured concentrations with an average (new and aged solutions) of 0.155 and 0.434 mg/L for the 5 and 10 mg/L concentrations respectively (Table S14). A low percent contribution of particulate BP-3 is expected as this UV filter has a reasonable water solubility within the lower to mid-level concentration ranges used in this study but due to the lack of method recovery adjustments, this dissolved portion is likely underreported, making the particulate fraction appear more substantial than it truly is.

Table 5. Summary of total concentrations (i.e. dissolved and particulate) of BP-3 in new and aged solutions for BP-3 test 3 with the calculated percent loss over the 24-hour interval. Dissolved and particulate concentrations were determined independently. Extended analytical chemistry results can be found in Tables S7-9. Limit of quantification (LOQ) = 0.015 mg/L.

	New Solutions		Aged Solutions		
Nominal (mg/L)	**Total**	**% Nominal**	**Total**	**% Nominal**	**% Loss**
Control	<LOQ	NA	<LOQ	NA	NA
LSC	<LOQ	NA	<LOQ	NA	NA
HSC	<LOQ	NA	<LOQ	NA	NA
0.63	0.03	5%	0.02	3%	47%
1.3	0.35	27%	0.09	7%	74%
2.5	0.45	18%	0.31	12%	31%
5.0	1.12	22%	0.88	18%	21%
10	1.61	16%	0.54	5%	66%

Other UV filters (e.g. octocrylene) are more lipophilic or hydrophobic and in those cases, determining both particulate and dissolved fractions becomes increasingly important (Mitchelmore et al., 2021). The low recoveries at the lowest concentration and relatively small contribution of particulate BP-3 suggests adsorption to vessel walls and possibly to the corals themselves. The higher concentrations of particulate BP-3 in the high concentrations were likely due to the exposure solutions approaching or exceeding the limit of solubility in the ASW and correlated with a lower percentage of dissolved BP-3 at 10 mg/L. It is also important to note that the dilution ASW is a complex, unfiltered matrix so differences in particulate and dissolved organic matter may lead to differential binding and fractionation (Burkhard, 2000). The increase of particulate BP-3 in conjunction with the lower percent of nominal concentrations in the high concentrations highlights the difficulties of using concentrations above water solubility in acute exposures. Although these tests provide important information about the acute toxicity of BP-3, it is not environmentally relevant as these concentrations are not

expected in natural waters, so sublethal testing at relevant concentrations are required to determine true risk to corals.

New solutions of the high concentration contained only slightly higher BP-3 levels than the 5 mg/L solutions, likely due to supersaturation and consequently coagulation/flocculation in the solution which also likely drove the 50% BP-3 loss from solution over the 24 h renewal periods. Aged solutions declined in concentration by 40-60% in the lowest (0.63, 1.3 mg/L) and highest (10 mg/L) concentrations but remained consistent in the middle concentrations (2.5, 5 mg/L; Table S13) which was not matched by an increase in particulate BP-3. Loss in the lowest concentrations was likely at least partially due to coral uptake which has been demonstrated for BP-3 (Tsui et al., 2017; Mitchelmore et al., 2019) as polyps did not exhibit strong retraction or other signs of stress and so were likely filtering significantly more exposure water than in higher concentrations where significant retraction and tissue loss were observed. However, as UV filters are designed to react with sunlight, this could also be a function of photolytic degradation. This assumption is further supported by the uptake of DOC by corals in their natural environment, even when the DOC is considered to be rather recalcitrant (Nelson et al., 2011).

In other BP-3 exposures to coral, this fractionation was not taken into account (i.e. samples were not filtered before extraction) and chemical analyses were reported as total BP-3 concentration (He et al., 2019a; Wijgerde et al., 2020). The measured concentrations from this study were generally closer to nominal than has been previously reported for other methods (He et al., 2019a; Wijgerde et al., 2020) and, once an appropriate method recovery is established, will likely be much closer to nominal.

Furthermore, the dosing scheme allowed these concentrations to be as closely maintained as possible over the duration of the exposures. Downs et al. (2016) renewed exposure solutions in their larval experiments once at hour 8 of their 24 hour testing period but did not analytically confirm their concentrations while He et al. (2019a) prepared solutions just once and did not renew exposure solutions during their larval (14 day) and adult (7 day) exposures. Exposure concentrations were measured in He et al. (2019a) only at the beginning and end of the exposures. Although the concentrations were similar to nominal at the beginning of the exposure period, they were far lower than nominal (<LOD to 2% of nominal) by Day 7 in nubbin tests and Day 14 of larval experiments demonstrating a failure to maintain consistent exposure. Wijgerde et al. (2020) dosed their header tank for exposure every 48 hours during their 6-week study and measured concentrations twice weekly, however their average measured test concentration (0.06 µg/L) was only 6% of nominal (1 µg/L).

Previous acute toxicity tests with BP-3 in corals have used a variety of species, life stages, experimental designs and duration of exposures but none have been repeated to confirm results leading to LC50s reported from 139 to >1,000 µg/L. He et al. (2019a) recorded adult *S. caliendrum* and *P. damicornis* mortality over 7 days but saw no mortality at the highest concentration (1,000 µg/L) for either species showing similar results to what has been observed in this study. Larval mortality has been explored in *S. pistillata* (Downs et al., 2016), *S. caliendrum,* and *P. damicornis* (He et al., 2019a); however, these two studies report very different results with a 24 hour LC50 in the first study of 139 µg/L and minimal to no mortality in the second study at the highest exposure concentration (1,000 µg/L) over 14 days which did not allow for LC50

calculations. These results likely highlight the variability in species responses and conclusions are difficult to elucidate without standardized testing. The endpoints calculated in Downs et al. (2016) suggest BP-3 is "very highly toxic" according to the US EPA Ecotoxicity Categories for Terrestrial and Aquatic Organisms (US EPA, 2017). However, the He et al. (2019a) results are similar to those presented in this study showing no mortality for adult coral at 1 mg/L BP-3 which would correspond to rating of "moderately toxic" which is a significant difference from a regulatory standpoint.

In our studies, we consistently observed a steep decline in survival at 10 mg/L BP-3 with 100% mortality by day 4 (96 h) resulting in a LOEC of 10 mg/L and NOEC at 5 mg/L (Table 6). The average LC50 for the three tests using nominal values was calculated at 6.53 (± 0.47) mg/L (Table 6, Figure 2) with the LC50 calculated using uncorrected measured dissolved concentrations in test 3 being 0.830 mg/L (95% CI: 0.829 to 0.831). Additionally, by day 4 in all tests, the 5 mg/L treatments had severely retracted polyps, signs of mortality in all polyps (i.e. thinning and sloughing of outer tissue layers), observed mortality in up to 25% of polyps, and cloudy exposure water (likely due to tissue loss and breakdown). Some of these observations were also seen at the 2.5 mg/L concentration but as of 96 h there were no observed mortalities at this concentration in any test. This is the first LC50 calculated for BP-3 in an adult intact hard coral species and these results were highly replicable between the three independent tests.

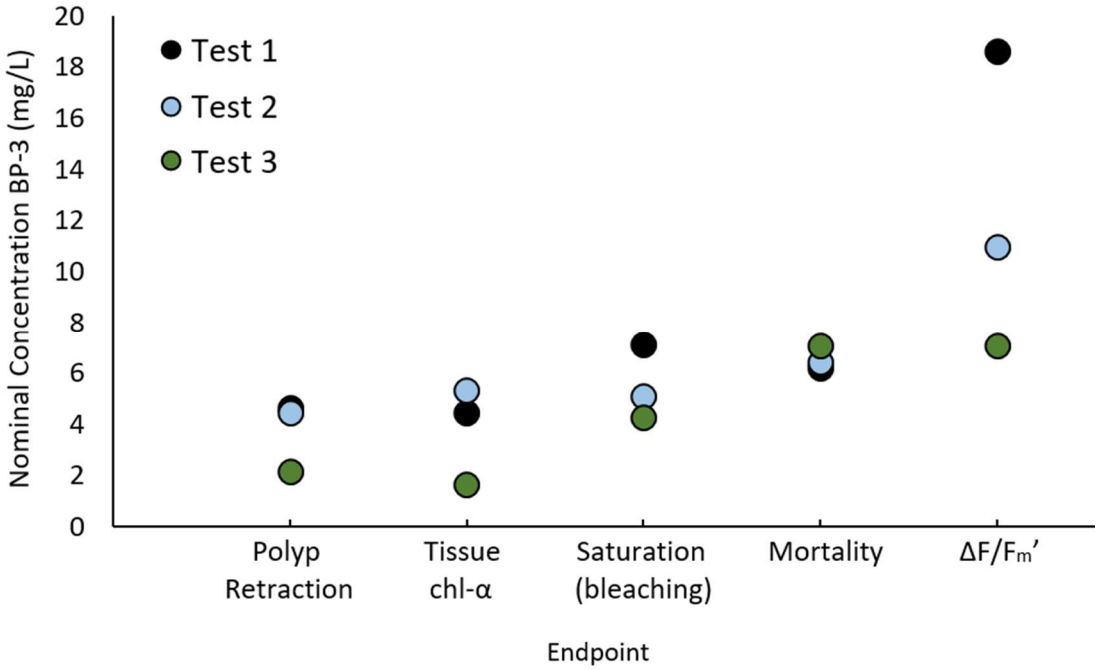

Figure 2. Calculated LC/EC50s for triplicate BP-3 acute tests with *G. fascicularis*. Polyp retraction was seen at low concentrations in all three studies with the lowest average EC50. The LC50 in all three studies showed the lowest variability and was generally seen at higher concentrations. Light-adapted photosynthetic efficiency ($\Delta F/F_m'$) was generally both the least sensitive of the endpoints as well as the most variable between tests which demonstrates the variability of this endpoint with small differences in testing conditions.

Table 6. Summary of endpoints for all BP-3 acute tests (n = 3; mg/L) using nominal concentrations. In the event that NOEC and LOECs vary between tests, the lower concentrations were reported and have an asterisk (*). See table S4 for a summary of endpoints by test.

Endpoint	NOEC (mg/L)	LOEC (mg/L)	EC/LC_{50} (mg/L)
Mortality	5.0	10	6.53 (± 0.47)
$\Delta F/F_m'$	2.5*	5.0*	12.21 (± 5.89)
Polyp Retraction	0.63*	1.25*	3.71 (± 1.35)
Tissue chl-α	2.5	5.0	5.48 (± 1.48)
Saturation (bleaching)	1.25*	2.5*	3.80 (± 1.93)

Mortality of the coral host, however, may not be the only important endpoint in acute assessments. The state of health of corals is uniquely intertwined with the health of their symbionts. Because of their symbiotic nature, the fact that up to 90% (species-dependent) of the fixed carbon required for growth in hard corals comes from algal

symbionts (Muscatine and Porter, 1977), as well as the difficulty in determining host mortality, health and survival of the algal symbiont should also be considered through a variety of bleaching measurements. Commonly, coral health is measured in the field in terms of coral bleaching, which often is due to the loss of the algal symbiont. Some coral species may survive for a time without the symbiont, but generally this loss is detrimental to host coral. However, there are a variety of bleaching measurements available when doing *ex situ* exposures with various strengths and limitations which were explored in this study. The analysis of chl-α in tissue as a proxy for algal content has been used for over 20 years as a manner of determining bleaching in corals (Jones, 1997; Brown et al., 1999). However, this parameter requires some form of standardization. Biological parameters can be normalized by a number of indices including algal cell counts (Jones, 1997; Brown et al., 1999), coral surface area (Siebeck et al., 2006), or tissue protein content (Yost and Mitchelmore, 2010). Only the latter two were explored during this study. Coral surface area (e.g. using foil or wax coating) is difficult to quantify, especially with *G. fascicularis* due to its large individual polyps creating a topographically complex surface. Indeed, Al-Moghrabi et al. (1995) found it problematic to achieve reliable results with any method of surface area quantification with this species. The experiments presented here utilized images of the polyp skeletons to calculate approximate surface area calculations independent of tissue thickness or tissue loss. However, in control individuals, surface area and chl-α concentration were not correlated which suggests that the size of the corals had little impact on the amount of chl-α in their tissues. Protein, on the other hand, was weakly positively correlated with chl-α ($R^2 = 0.1444$) and surface area ($R^2 = 0.1359$) in control corals. However, the tissue

loss seen in these exposures led to significant dose-dependent protein-loss. Therefore, standardizing chl-α with protein removes the variable of tissue loss and shows only chl-α impacts not due to tissue loss (i.e. tissue bleaching) so surface area was additionally utilized to compare the total loss of chl-α including both tissue loss and algal symbiont expulsion. This study demonstrated a dose-dependent decrease in surface-area standardized chl-α that correlated strongly with the protein content (R^2: 0.7 to 0.8; Figure S7). There was some slight decline in protein-standardized chl-α at the higher concentrations (2.5 to 10 mg/L; Figure S8) suggesting additional bleaching. However, this was minimal compared to the total chl-α loss observed.

The photosynthetic efficiency of coral symbionts has been examined for coral exposed to BP-3 (Wijgerde et al., 2020) as well as exposure to multiple other compounds, including UV filters (e.g. Fel et al., 2019) and has been discussed in direct relation to bleaching events due to its impact on chlorophyll fluorescence (Jones, 2005; Cantin et al., 2007). Light- and dark-adapted photosynthetic efficiency ($\Delta F/F_m$' and F_v/F_m, respectively) both have utility for identifying photosynthetic disruptions, the latter being more sensitive. However, for pragmatism, $\Delta F/F_m$' was chosen for this study. Light-adapted photosynthetic efficiency among these three studies was variable, with impacts only at the highest concentrations (5 and 10 mg/L) in the first and second study and in all concentrations in the third. It is unclear what ultimately caused this change in response. However, $\Delta F/F_m$' is impacted by light exposure and the light intensity in test 3 was intentionally lowered slightly to more closely mimic culture conditions which may have made the corals more sensitive to the saturating pulse. This deviation in response among nearly identical studies highlights the importance of using standardized toxicity testing

methods, as this exhibits how slight differences in conditions may have larger impacts to certain endpoints. In Fel et al. (2019), the NOECs for F_v/F_m in *S. pistillata* for a variety of other UV filters were 1 to 5 mg/L (nominal) during a 5-week exposure which is a similar observation to this study on a longer timescale. However, in Wijgerde et al. (2020), although BP-3 alone did not elicit significant responses in most endpoints, there was a significant decrease (4-5%) in F_v/F_m over 6 weeks at just 1 µg/L (nominal) for both *S. pistillata* and *A. tenuis*. The first of these tests, however, did not use other measures to relate these endpoints to visible bleaching and the second did not report any effect from BP-3 on algal density, suggesting BP-3 may have negative impacts on algal health that do not immediately translate to significant bleaching. The variability of this endpoint and its lack of correlation to bleaching demonstrate that this endpoint may not have the highest utility for acute toxicity tests in corals where the mode of action is unrelated to photosynthetic abilities of the algal symbionts like photosystem (II) inhibitors like diuron (Trebst, 1987) where bleaching did occur concurrently with decreased $\Delta F/F_m$' (Figure S9). This endpoint likely has higher overall utility in chronic testing where these subtle negative impacts to algal health would have time to manifest as observable bleaching.

Previous studies have suggested that BP-3 causes coral bleaching using the same quantitative image analysis used in this study (86% at 48 h of 33 µL/L; Danovaro et al., 2008), or using assessment of chlorophyll fluorescence (LOEC of 2.28 µg/L; Downs et al., 2016). However, He et al. (2019a) reported limited bleaching at their highest BP-3 concentration (1,000 µg/L nominal) in *S. caliendrum* larvae and saw no BP-3 related bleaching up to 1,000 µg/L in 14 days in larval *P. damicornis* or in adults of either species. However, our study utilized multiple methods concurrently (image analysis, chl-

α concentration of tissues and dark-adapted photosynthetic efficiency) in an attempt to quantify bleaching response finding some evidence of bleaching at high concentrations of BP-3 (>2.5 mg/L nominal). However, when looking at phaeophytin, an indication of chl-α breakdown, there was no increase concurrently with chl-α loss until the highest concentration (10 mg/L nominal BP-3; Figure 3). This combination of observations demonstrated that this loss of pigment is mostly due to mortality-driven tissue loss in this species and therefore does not support a bleaching mechanism of action. This difference with existing literature is likely due a combination of difference in life stage (i.e. larvae may be more [Downs et al., 2016] or less [He et al., 2019a] sensitive to bleaching), species (i.e. *G. fascicularis* is less susceptible to bleaching) or other methodological differences. For example, both Downs et al. (2016) and Wijgerde et al. (2020) employed the used of dimethyl sulfoxide (DMSO) as their solvent carrier which may increase uptake of BP-3 (Mitchelmore et al., 2021) while Danovaro et al. (2008) did not allow for adequate water flow.

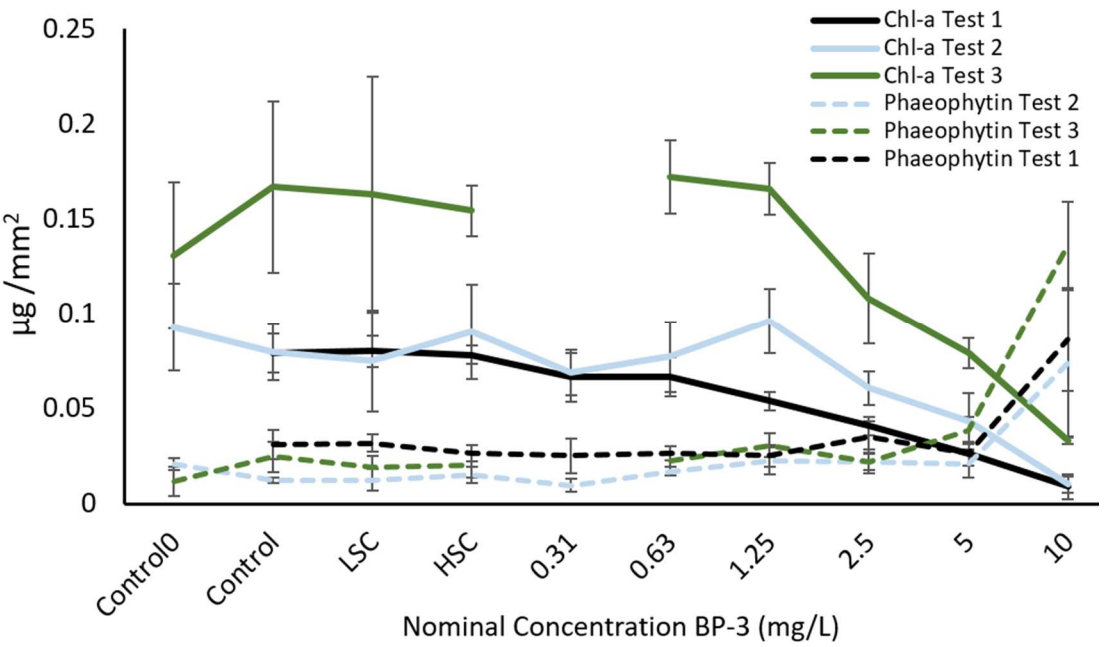

Figure 3. Algal pigments in *G. fascicularis* tissue at 96 h BP-3 exposure. LOECs for chl-α concentration were 2.5 mg/L for tests 2 and 3 and 1.25 mg/L while the LOEC for phaeophytin concentration was 10 mg/L in all three exposures. As 10 mg/L coral polyps exhibited 100% mortality, this data demonstrates the effects of total tissue loss as well as the negative impacts of BP-3 to the algal symbionts at such high concentrations through the increase in phaeophytin suggesting chl-α degradation. This concentration-dependent decrease in chl-α was not seen in exposures where tissue loss was not observed and correlated well with total protein (Figure S7) which demonstrates that this chl-α loss is due to mortality-driven tissue sloughing and is unlikely the mechanism of action for BP-3 in *G. fascicularis*.

Although polyp retraction has not yet been linked directly to adverse individual- or population-level impacts, it is important to note as it has been often reported as a behavioral sign of stress to many triggers (e.g. sedimentation stress: Vargas-Ángel et al., 2006; light stress: Brown et al., 1999). Polyp retraction was demonstrated by He et al. (2019a) in relation to multiple benzophenones (BP-1, -3, -4, and -8) to be the most sensitive endpoint explored, meaning this response was seen earlier and at a lower concentration than any others. However, it should be noted that in some cases, that study reported retraction in just a single fragment at low concentrations (10 µg/L nominal) and it is unclear whether this response was statistically significant (He et al., 2019a). Our

study also suggests it is a highly sensitive and easily observed endpoint which demonstrated dose-dependent retraction in all concentrations in all three tests with an average calculated EC50 of 3.71 (± 1.35) mg/L which is the lowest EC50 calculated in this test (Table 6; Figure 2). Due to its sensitivity, dose-dependent response, and potential energetic impacts, polyp retraction may be worth considering as a standard endpoint for sublethal effects to corals in chronic toxicity testing. Investigation into the links between increased polyp retraction, molecular stress responses, and population-level impacts should be explored to better understand its role in coral health, and to determine whether it would be an appropriate standard endpoint.

Risk assessments require robust data both on relevant environmental concentrations as well as toxicity of the compound in question. However, there is currently no established approach for risk assessments in corals including which endpoints to use, toxicity test guidelines to follow, or the appropriate risk assessment factors to utilize (Mitchelmore et al., 2021). Environmental monitoring data, although still sparse, has expanded in the past decade and includes multiple coral reef environments generally showing low (ng/L) concentrations of BP-3 with a few outliers. Although the data on BP-3 in coral reef environments continues to increase in quantity and quality, there are very few toxicity studies on BP-3 in corals and their results have thus far been incredibly variable due to a general lack of methodological standardization. The LC50 used thus far in risk assessment calculations (Tsui et al., 2017) is 139 µg/L from Downs et al. (2016); however, our results and others (He et al., 2019a; Wijgerde et al., 2020) suggest this may not be an appropriate endpoint to determine acute risk to all life stages and species.

In Mitchelmore et al. (2021), RQs in three tests that calculated BP-3 risk (Tsui et al., 2014, 2017; He et al., 2019a) were standardized to use the same assessment factors (i.e. 1000 for acute data and 100 for chronic data). For BP-3, this resulted in RQ_{worst} that ranged from 0.01 to 31.61. However, the mean RQ of all three of these studies was less than 0.1 which suggest that, except in the case of specific, highly polluted sites (e.g. environmental concentrations presented in Downs et al., 2016), BP-3 does not pose a risk to any of the species tested. To determine the risk of BP-3 to *G. fascicularis*, a RQ was deterministically derived as MEC/PNEC using the most conservative values of this study and an assessment factor of 1000 for consistency with published data (Mitchelmore et al., 2021) combined with a high-quality monitoring study in Hawaii (Mitchelmore et al., 2019). The LC50 value used to calculate the predicted no-effect concentration (PNEC) was 0.830 mg/L which is the LC50 calculated in the third test using uncorrected measured dissolved concentrations and is the most conservative LC50 reported in this study as the uncorrected concentrations are extremely conservative and likely to increase once final method recoveries are determined. Mitchelmore et al. (2019) BP-3 concentrations ranged from <LOD to 142.7 ng/L so this highest measured value of 142.7 ng/L BP-3 was used as the MEC. This resulted in an RQ_{worst} of 0.172 which is within the range of the studies referenced above. This suggests that BP-3 exposure does not pose a risk of acute mortality in *G. fascicularis*. As a next step, chronic, sublethal endpoints should be explored to obtain a clearer view of long-term impacts of BP-3 to corals, since extrapolation from acute studies to chronic effects using assessment factors is more uncertain than directly relying on data from chronic studies. Furthermore, these results do not take into account potential co-stressors (e.g. increased temperature, increased

sedimentation, other chemical pollutants) which may exacerbate negative impacts. Wijgerde et al. (2020) attempted to examine the interaction between BP-3 and temperature, however did not find strong evidence of stressor interactions.

This is the first coral UV filter study to perform repeated definitive acute toxicity tests to provide a robust and reproducible LC50 following a 96 h exposure in an intact adult hard coral species as well as dose-dependent responses in a variety of additional endpoints. Furthermore, this is the first report of a UV filter toxicity test in a coral species to include all QA/QC elements including positive controls, appropriate chemical sampling, and definitive exposure concentrations spanning a range accepted by EPA and OECD standard marine invertebrate toxicity tests guidelines. The copper positive control LC50 matched well with repeated copper definitive acute tests, demonstrating its utility as a positive control for this species which helps assure reproducibility of a testing organism's response and gives an indication of organism health. Additional studies on a variety of coral species and life stages are clearly needed to fully understand BP-3 and other UV filter toxicity taking into account appropriate exposure setup, adequate controls, and QA/QC practices.

3.4 Conclusions

Using methods similar to regulatory-approved standard acute toxicity test methods in other marine invertebrate species, a reproducible LC50 for BP-3 was determined for *G. fascicularis* in the mg/L range which is similar to observations by He et al. (2019a) on adults of two different coral species. Our results did not support bleaching as a mode of action of BP-3 toxicity, as observed chl-α loss correlated well

with mortality-driven tissue loss, which suggests BP-3 bleaching may be species or life-stage dependent. Given the robust methodology of these tests, the BP-3 LC50 achieved should be used as a reliable hazard estimation for risk assessments and, when combined with robust environmental concentrations near coral reefs (Mitchelmore et al., 2019) and an assessment factor, resulted in a RQ <1 (using conservative, uncorrected measured values) which indicates risk to *G. fascicularis* from BP-3 exposure is not expected.

Comparing this test to prior UV filter toxicological testing highlights that care and consideration should be made when selecting a coral species on which to perform tests. These results demonstrate that there appears to be substantial variability among coral species and life stage and emphasizes the need for standardized procedures to accurately determine the risk that these and other anthropogenic compounds may pose to a diverse reef environment. Corals are variable in their hardiness, growing conditions, and algal symbionts, and the distribution of sensitive species may require flexible management policies to address this variation.

Chapter 4: Chronic toxicity of the UV filter oxybenzone to the hard coral *Galaxea fascicularis*

4.1 Introduction

Although the LC50 information achieved in the prior chapter gives important insight into the toxicity of BP-3 to corals, mortality-driven risk assessments are only the first step as they introduce a higher amount of uncertainty than risk assessments using sublethal endpoints from chronic testing. Growth and reproduction are typically the focal endpoint in chronic toxicity tests (e.g. length, weight, fecundity, etc.) as well as the endpoints usually used for a formal acute risk assessment (ECHA, 2008). However, no chronic toxicity test performed thus far with BP-3 and hard corals has quantified significant growth impacts.

Furthermore, chronic studies on BP-3 thus far lack important standard toxicity test components including positive controls, basic water quality QA/QC, and appropriate analytical verification of the exposure solutions. Not a single study of BP-3 on hard corals utilized positive controls which makes it difficult to determine the reproducibility of the results or compare the sensitivity of the species and individual cultures of the species used. This study employs the use of a positive control, the pesticide diuron, due to its known mode of action as a photosystem II inhibitor and published hard coral data for this compound (e.g. Watanabe et al., 2007; 2006; Fel et al., 2019; Cantin et al., 2007; Sheikh et al., 2012; Negri et al., 2005; 2011; Jones and Kerswell, 2003; Jones et al., 2003; Råberg et al., 2003).

Another weakness of chronic UV filter studies conducted thus far are their dosing schemes and associated sampling of the exposure solutions for analytical verification. For

example, He et al. (2019a) dosed once at the beginning of 7- and 14-day exposures, allowing solution concentrations of the parent compound to diminish over that time period. These concentrations were measured at the beginning and the end of the exposures and although starting concentrations were close to nominal, the concentrations at the end of testing ranged from <LOD to 2% of nominal. Furthermore, Wijgerde et al. (2020) dosed the header tank for exposure every 48 hours during their 6-week study; however, their average measured test concentration was just 6% of nominal. These dosing and sampling schemes do not lead to a steady baseline for calculations of toxicological thresholds nor is it environmentally relevant. Assuming the input of BP-3 to the environment is primarily due to sunscreen, this compound would be introduced into the environment daily as people return to the beaches for swimming, snorkeling, and other recreational activities.

Therefore, we exposed BP-3 to *Galaxea fascicularis* in a chronic, 28-day exposure to determine its sublethal impacts to growth. This species was chosen because of its large polyps for visual observations, ease of culture and handling, and it being a shallow-water reef building species (Hoeksema and Cairns, 2020) meaning it lives in the zone of highest impact from sunscreen products. Furthermore, this species grows relatively quickly so growth impacts will be discernable in 28 days. Exposure solutions were renewed daily in all experiments and samples were taken for analytical verification from all concentrations twice a week. Paired, 24-hour aged samples were also analyzed to determine the decline of the compound over the exposure water-change interval. The results for growth impacts (i.e. buoyant weight) were used to calculate an effect

concentration where 10% of the maximal response is seen (EC10) which were used to determine a PNEC in order to calculate an RQ.

The objective of this study was to conduct a chronic (28 days) toxicity test with BP-3 using appropriate QA/QC procedures to provide the first evidence of significant growth impacts for the UV filter BP-3 in an adult intact hard coral species. Results from this work provide a reproducible and reliable estimation of the chronic toxicity of BP-3 in a representative hard coral species and are used along with concentrations from a well-controlled monitoring study to calculate a conservative RQ for BP-3 and *G. fascicularis*.

4.2 Materials and Methods

4.2.1 Test Species and Coral Culture Conditions

The test species chosen was *Galaxea fascicularis* due to its ability to be easily cultured in artificial seawater, its relatively fast growth rate, and its large polyps making visual observations simple. *G. fascicularis* were obtained from St. Mary's College of Maryland (SMCM) from a culture system described in Chapter 2 (see Text S1 for a complete summary of culture conditions and Table S1 for summary of culture water quality parameters). Conditioned ASW from this culture was used, unfiltered, as dilution water for all preliminary and definitive exposures and was kept at exposure temperature and aerated until use.

At least three different parent colonies provided polyps for toxicological testing. Individual polyps were fragmented from the parent colony and attached on their sides to a plain ceramic poker chip in the "4-star" pattern. Polyps were allowed to recover for 4 weeks after fragmentation at the coral culture facility at SMCM before moving them to

the Chesapeake Biological Laboratory (CBL). At CBL, the polyps were placed into the treatment vessels and acclimated to test conditions for 24 hours.

Corals were fed once daily during the exposure. Five days during the week they were fed Golden Pearl Reef and Larval Fish Food (300 to 500 µm; Brine Shrimp Direct, Ogden UT) and twice weekly they were fed approximately 0.5 mL *Artemia sp*. nauplii per exposure beaker that were hatched in-house and < 24 h old. All feedings occurred at least 2 hours before observations began.

4.2.2 Chemicals

BP-3 (CAS# 131-57-7, 99.96%, Certified Reference Material) and Diuron (CAS# 330-54-1, >98%) for exposure solutions and analytical standards were obtained from Sigma-Aldrich, St. Louis, MO. Deuterated d_5-BP-3 (CAS# 1219798-54-5, >98%) and d_6-diuron (1007536-67-5, >99.0%) used for internal standards for LC-qqq-MS quantification were obtained from Sigma-Aldrich, St. Louis, MO.

Dosing stocks for BP-3 and diuron were prepared for each exposure concentration at the beginning of each exposure in Optima LC/MS grade methanol (Fisher chemical, Pittsburg, PA) and stored at -25 °C. For chemical extraction, LC/MS grade methanol, Optima LC/MS 0.1% formic acid (Fisher chemical), and formic acid (>98%, Sigma-Aldrich) were used.

4.2.3 Test Setup

Information on dosing frequency determination and acute testing used for concentration selection can be found in Chapter 3 and Text S5. The use of diuron as a

positive control was also based off of testing from Chapter 3 and the results from multiple published studies (Fel et al., 2019; Cantin et al., 2007; Watanabe et al., 2006; 2007). A single, definitive 28-day chronic exposure with diuron as a positive control was carried out based on EPA guidelines for Mysid Acute and Chronic Toxicity Testing (US EPA, 2016b; US EPA, 1996) and EPA and OECD general guidelines for toxicity testing (OECD, 2019a; US EPA, 2016a) with modifications in parameters such as lighting scheme and salinity to reflect appropriate conditions to maintain coral health as well as the addition of the use of a positive control.

The exposure was carried out in a system identical to that of the acute testing: under full-spectrum illumination on a 12:12 light:dark cycle in aerated 2.0 L beakers, loosely covered to prevent evaporation. Photosynthetically active radiation (PAR, µmol m^{-2} s^{-1}) was measured daily to confirm spectral quantity. Vessels were kept in a water bath set to maintain an exposure temperature of 26 ± 1 °C which was monitored continually. Seawater samples of pooled replicates from new (i.e. immediately after solution preparation) and aged (i.e. after 24 h of coral exposure, before renewal) solutions were taken twice weekly for confirmation of exposure concentrations. Additional samples from these time points were filtered and the filter and filtrate were independently frozen at -80 °C for chlorophyll-α (chl- α)/phaeophytin analysis and additional water quality analyses (Table S16), respectively. Daily water quality (temperature, dissolved oxygen, salinity, pH) and light condition (PAR) are summarized in Table S15.

All polyp observations were conducted before water changes to avoid impacts due to handling stress. Weekly, new algal growth was removed from the ceramic poker chips using a soft toothbrush and organisms (e.g. worms and crustaceans) were removed with

forceps. Beakers were also wiped out at this time to discourage algal/biofilm growth and buildup. The corals were then weighed using the buoyant weight technique (Davies, 1989). At day 16, the order of observations was partially inverted (from controls, BP-3, diuron to diuron, BP-3, controls) to even out total feeding time throughout the test. On day 0, additional coral polyps that were not part of the exposure were photographed, observed, and then immediately frozen at -80 °C for tissue analyses. After observation on Day 28, all test corals were immediately frozen at -80 °C for the same purpose.

4.2.4 Chronic Toxicity Test

Before testing, a study was conducted to determine the growth rate of *Galaxea fascicularis* under control testing conditions. Using buoyant weight, coral chips were weighed periodically for 35 days and seen to have sufficient growth rates for a 28-day study (Text S7; Figure S10).

The definitive 28-day toxicity test was conducted in October, 2019. The test was a static renewal with a 24 h (daily) renewal interval including negative controls, solvent controls (50 μL/L), positive controls (diuron) and 6 exposure concentrations (0.009, 0.019, 0.038, 0.075, 0.15, and 0.30 mg/L nominal) with 4 replicates each (each replicate containing n = 3 coral chips with n = 4 individual polyps on each chip). The positive control diuron was conducted at the same time as the BP-3 exposure test and consisted of a 3 concentration (50, 10, and 2 μg/L nominal) by 3 replicate matrix.

4.2.5 Chemical Extraction and LC-qqq-MS Analysis

BP-3 and diuron exposure water samples and method recovery spikes were extracted with solid-phase extraction using modified protocols outlined in Mitchelmore et al. (2019) and Carabias-Martínez et al. (2004), respectively. The most notable change from the BP-3 extraction protocol was that MeOH was utilized instead of acetonitrile (ACN) because adequate and consistent recoveries for this concentration range were achieved.

Seawater samples (50 mL each) for BP-3 and diuron chemical analysis were filtered through a 25 mm 0.7 µm Whatman GF/F glass fiber filter (GF/F filter) which was stored at -20 °C until particulate analysis. All filtrate samples for dissolved phase analysis were acidified to pH 2 using 100% formic acid (FA), manually agitated, then run through a pre-conditioned HLB cartridge (Waters Oasis®, 6 cc, 150 mg sorbent; conditioned with 5 mL MeOH and 5 mL 0.1% FA). 5 mL 0.1% FA was added to the HLB to remove residual sample solution containing salts followed with 7 mL MeOH to elute BP-3 or diuron from the cartridge into 8 mL borosilicate glass amber vials which were stored at -20 °C until further analysis.

For particulate analysis, liquid-liquid extraction was used. Filters were allowed to thaw briefly before addition of 5 mL ACN. Samples were then shaken at 300 rpm for 24 h and 5 mL DI water, 0.6 g NaCl, and 1.5 g $MgSO_4$ were added to the sample to remove salt and enhance solvent partitioning. Samples were shaken for 1 h then centrifuged at 3000 rpm for 10 minutes before 2.5 mL of the top layer of supernatant was removed and run through a pre-conditioned HLB cartridge (rinsed with 3 mL ACN) and retained. An additional 3 mL ACN was run through the cartridge and retained to ensure full recovery of the sample. This sample was evaporated under N_2 gas and dissolved in 2 mL MeOH.

Before analysis, all samples were diluted with MeOH to levels that were within the calibration range and spiked with an internal standard of d_3-BP-3 or d_6-diuron, respectively.

Liquid-chromatography triple-quadrupole mass spectrometric (LC-qqq-MS) analyses were performed using an Agilent 6420A LC-qqq-MS (Agilent, Santa Clara, CA). To quantify samples, individual calibration curves for BP-3 and diuron were created in MRM mode using precursor ions (m/z) 229.09 and 233 respectively and product ions 151/105.1 and 160/72 respectively. LOQs for BP-3 and diuron were 0.015 mg/L and 0.074 mg/L respectively with the LODs calculated as LOQ/3 (0.005 mg/L and 0.025 mg/L respectively; see Text S6 for explanation).

4.2.6 Biological Endpoints

The main endpoints for this test were bleaching (quantified multiple ways) and buoyant weight. As true reproduction is difficult to achieve in laboratory setting for this species, it was not used. So, an attempt to quantify new budding polyps was made to use as a proxy for reproduction. Additional endpoints were also explored as described below.

Growth was calculated as weight differences over the course of the experiment. Weight was measured weekly using a buoyant weight technique (Davies, 1989). Briefly, a tared balance (Model SLF103; Fisher Science Education, Pittsburg, PA) was positioned above a beaker of control ASW using an expanded-polystyrene housing and the coral chip was hung from the bottom of the balance until fully submerged. The resulting weights were recorded.

Daily photographs were used to determine the degree of polyp retraction through measurements of tentacle length as well as to quantify the number of new polyps that appeared over the study period. Polyp length and width were also quantified using a similar protocol to tentacle length as is described in detail in Text S8.

Coral bleaching was assessed in a number of ways. First, it was quantified using an image analysis method modified from Siebeck et al. (2006) where images were standardized using the poker chip as a white standard and analyzed using Adobe Photoshop® to look at the brightness and saturation composition. Saturation of the polyp was shown to be a more sensitive value with higher variability over the spectrum of healthy to bleached coral (Figure S1) and so was used for statistical analyses.

Second, bleaching was quantified by the photosynthetic pigments from released algae in the exposure waters using the following protocol from NASL (2019). The retained GF/F filters were briefly thawed and placed into a centrifuge tube with 5 mL 90% acetone and allowed to sit overnight. The next day, the tubes were centrifuged and the acetone was removed, filtered, and placed into a cuvette where absorbance readings were made at the wavelengths 750, 665, 664, 663, 647 and 630 nm using a spectrophotometer (Spectra Max PLUS 384, Molecular Devices, San Jose, CA). Then, the extract was acidified with 1 N HCl and readings were again made at the aforementioned wavelengths. These readings were used in the equations outlined in the standard operating procedure referenced above to calculate uncorrected chl-α, phaeophytin, and chl-α corrected for phaeophytin.

Second, the algal pigments chl-α and phaeophytin in coral tissue were quantified following Yost and Mitchelmore (2010). Briefly, coral tissue was removed from the coral

skeleton by airbrushing with filtered ASW. For pigment analysis, this tissue slurry was extracted in acetone, centrifuged, and the absorbance of the supernatant was read using the protocol described above. To standardize coral tissue chl-α, both polyp skeleton surface area and total protein content of the coral tissue were quantified (Text S3).

Finally, although not a direct measure of bleaching, assessment of algal health was conducted using a Junior PAM (Pulse-Amplitude-Modulation) fluorometer (Heinz-Walz, Effeltrich, Germany) using a saturating pulse to measure light-adapted photosynthetic efficiency ($\Delta F/F_m'$) of each of the polyps twice weekly using the PAM software (WinControl-3 v 3.29). Detailed methods for all biological endpoints are provided in Text S3 and S8.

4.2.7 Statistical Analysis

Statistical analysis was performed using R (v. 3.4.2) and R Studio (v 1.2). Controls were pooled for analysis in the event that there was no statistically significant negative effect of the solvent control. Concentrations used in statistical analyses were presented both as nominal concentrations as well as averaged from analytical chemistry measurements of new solutions without a correction for method recovery (Text S6). To determine if there was a dose-dependent effect, the Jonckheere Test was employed for monotonic data that did not satisfy the assumption of normally distributed data as determined by a Shapiro-Wilks test and confirmed visually. For data that satisfied the assumption of normality, and were non-monotonic, Dunnett's Test was employed. To determine NOEC and LOEC the package "mixtox" was used (Zhu, 2017) and for LC/EC_x

determination and graphing of the dose response curve, the "drc" package was used (Ritz and Strebig, 2011). A dose-response curve was chosen using AIC.

4.3 Results and Discussion

Chemical analysis to verify exposure solution concentrations demonstrated no BP-3 in the controls with a single exception in the solvent controls (Table 7). However, this was in an "aged" sample. Because the "new" sample of this solution (i.e. 24-hours prior) showed BP-3 <LOQ, it is likely that this was a result of contamination during sample analysis. Overall, concentrations did not overlap and recovered from 0.007 to 1.1 mg/L which was significantly higher than expected (Table 7). No calculation or analysis problems were found. It should be noted that the sample from day 0 was only one to two times expected for all concentrations. By day 7, these concentrations had increased to approximately three to four times nominal and remained at that level for the remainder of the test. Therefore, these increased concentrations are likely from contamination or buildup on exposure vessels or solution preparation vessels. As has been noted prior, BP-3 adsorbs severely to surfaces (Saxe et al., 2020) and so even with multiple cleaning protocols, it is possible that some BP-3 remained in preparation vessels. Furthermore, exposure beakers were not cleaned during this chronic test so it is not surprising that buildup of the compound might have occurred. Particulate BP-3 was minimal (under 2% of nominal, Table S18) and so it was not included in total concentrations. Because measured concentrations were significantly higher than expected, averaged, new dissolved concentrations were utilized for statistical analysis alongside nominal

concentrations and both endpoint calculations are reported in Table 8. For simplicity, endpoints in the text are reported in nominal concentrations only except where noted.

Table 7. Dissolved concentrations of solutions for chronic BP-3 exposure (n = 5).

Concentration	**Avg. New (SD)**	**Avg. Aged (SD)**	**% Nominal (new samples)**
Control	<LOQ	<LOQ	NA
S. Control	<LOQ	0.001 (0.002)*	NA
0.009	0.024 (0.016)	0.007 (0.006)	267%
0.019	0.066 (0.029)	0.009 (0.007)	347%
0.038	0.131 (0.045)	0.064 (0.057)	345%
0.075	0.288 (0.117)	0.150 (0.114)	384%
0.15	0.579 (0.179)	0.194 (0.071)	386%
0.30	1.115 (0.399)	1.011 (0.811)	372%

*driven by a single sample

Table 8. Endpoints calculated for chronic exposure of corals to BP-3. Concentrations are given as nominal (measured) in mg/L.

Endpoint	Significant ($p < 0.05$)	NOEC	LOEC	EC10	EC20	EC50
Quant. Retraction	YES	0.075 (0.288)	0.15 (0.579)	0.0357 (0.085)	0.0815 (0.228)	0.334 (1.222)
Light-Adapted Photosynthetic Efficiency	YES	0.038 (0.131)	0.075 (0.288)	0.093 (0.325)	0.374 (1.299)	4.04 (13.864)
Newly Emerged Polyps	NO	0.30 (1.115)	NA	NA	NA	NA
Buoyant Weight (Day 7 to 28)	YES	0.019 (0.066)	0.038 (0.131)	0.0233 (0.083)	0.0242 (0.085)	0.0259 (0.090)
Width	YES	0.15 (0.579)	0.30 (1.115)	0.042 (0.142)	0.067 (0.223)	0.147 (0.478)
Length	NO	0.30 (1.115)	NA	NA	NA	NA
Brightness	YES	0.075 (0.288)	0.15 (0.579)	0.339 (1.261)	0.541 (2.007)	1.202 (4.445)
Saturation	NO	0.30 (1.115)	NA	NA	NA	NA
Tissue Chl-α	NO	0.30 (1.115)	NA	NA	NA	NA
Tissue Phaeophytin	NO	0.30 (1.115)	NA	NA	NA	NA

As expected based on the previous acute toxicity tests, no mortality was seen in any test concentration for BP-3 or the positive control, diuron. An extended table of endpoints for both compounds can be found in Table S17. Weights were quantified as the change in buoyant weight in an individual chip from day 7 to day 28. These were not calculated from day 0 because the weights were highly variable when quantified in this manner (Figure S11) likely due to the fact that many worms and crustaceans evacuated during the first few days of exposure. The results from day 7 to day 28 show a significant decrease in weight beginning at 0.038 mg/L (Figure 4; Table 8). Control corals, on average, grew 0.09375 g over 28 days showing a growth rate of 0.0033 g/day. Corals in the highest BP-3 condition (0.3 mg/L) grew 0.04825 g over 28 days or 0.0017 g/day which results in a 48.5% reduction in growth. Growth was quantified by McCoshum et al. (2016) in soft coral (*Xenia spp.*) and found a reduction in growth after 72 h exposure

of 0.26 mL/L nominal BP-3 followed by a 28-day recovery period. However, adults of *S. pistillata* and *A. tenuis* showed no differences in growth rate after a 6-week exposure to 1 µg/L BP-3 (0.06 µg/L measured; Wijgerde et al., 2020). This is similar to our results as no statistically significant growth impacts were seen at the lowest concentration (9 µg/L BP-3).

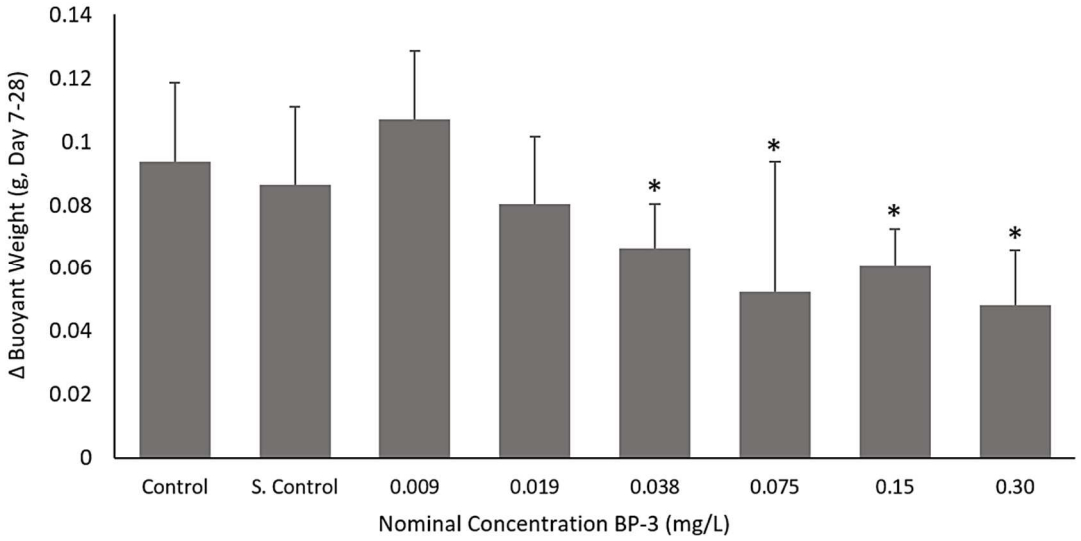

Figure 4. Change in buoyant weight from day 7 to 28 of chronic BP-3 exposure. Growth (increase in buoyant weight) was seen in *G. fascicularis* in all concentrations after 28 days. Significant negative impacts to growth ($p < 0.05$) from BP-3 exposure were seen at 0.038 mg/L nominal BP-3 (0.131 mg/L measured) and above.

This difference in weight seems to be at least partially driven by horizontal extension of the skeleton. Change in width of the polyp at the leading edge of the tissue in the highest concentration (0.3 mg/L; Table 8) was significantly less than in the control exposures. The difference in length from bottom to top of the skeleton (not including tentacles) was not statistically significant. These two trends make sense considering the colony structure of this species. They are generally found in wide, low-growing colonies, which suggests that the horizontal growth is likely much faster than vertical growth. However, this does not completely explain the mass effect which either means the tools

used to measure length and width are not as sensitive as the buoyant weight measurements or that there is another component being affected like the density of the skeleton or growth of the polyp in the other width direction (top to bottom on the poker chip).

There was no significant impact to the number of newly emerged polyps over 28 days in any concentration (Table 8). However, visual inspection of the data shows that the 0.15 and 0.30 mg/L concentrations had slightly lower numbers of new polyps than did both of the controls. This suggests that there may be a significant impact at higher concentrations or if the study was extended to a longer time period. This new polyp growth includes a skeletal component and may also have some impact on the differences in buoyant weight at the higher concentrations. However, newly emerged polyps and change in mass as measured using buoyant weight were not significantly correlated ($p = 0.367$).

Another endpoint often reported for corals is polyp retraction which has been shown to be one of the more sensitive whole-organism endpoints following UV filter and other chemical stressor exposures (He et al., 2019a; 2019b; Stien et al., 2019; May et al., 2020). Polyp retraction was significantly affected at 0.15 mg/L and higher concentrations (Table 8). Significant correlations were seen between quantitative retraction and weight, new polyps, and width ($p < 0.02$). It can be deduced that for heterotrophic species like *G. fascicularis*, long-term polyp retraction may reduce feeding which would in turn decrease nutrient intake (May et al., 2020). Polyp retraction is likely unsustainable because it requires energy and both increases the demand for and limits the availability of oxygen. Because of this, the cost of not retracting (e.g. toxicant exposure) is likely higher than the

cost of retraction (Swain et al., 2015). This suggests that although this endpoint may not lead directly to negative impacts, it may be a good indication of significant stressor exposure and an attempt for the coral polyp to protect against it. Furthermore, this energetically expensive behavior change may shade symbionts, decreasing their carbon fixation and leading to decreased growth if retraction is sustained over longer periods of time. These long-term effects could be exacerbated by negative impacts to the symbionts directly. Due to its sensitivity and potential energetic impacts, polyp retraction may be worth considering as a standard endpoint for sublethal effects to corals in chronic toxicity testing.

Photosynthetic efficiency was significantly impacted (LOEC) at 0.075 mg/L (Table 8). It would be reasonable to assume some negative impacts to the algal symbionts which resulted in decreased photosynthetic efficiency. This would likely explain the decreased growth seen at high BP-3 concentrations. Interestingly, photosynthetic efficiency did not correlate well with any endpoint except buoyant weight at 28 days ($p = 0.005$). This seems to indicate that there is a relationship between the two. However, saturation, tissue chl-α (Figure 5), and exposure water chl-α were not significantly impacted by BP-3 exposure suggesting that BP-3 does not cause sublethal bleaching to *G. fascicularis* over this time period. Hence, these impacts in photosynthetic efficiency are not directly manifesting as coral bleaching as was seen during acute BP-3 testing. In He et al. (2019a), exposures of BP-3 to both larvae (14 days) and adult (7 days) *S. caliendrum* resulted in a bleaching LOEC of 1 mg/L (nominal) while neither larvae nor adults of *P. damicornis* showed an impact up to the highest concentration tested (1 mg/L nominal). These bleaching observations did not correlate to any impacts to algal density

in adults of either species. Furthermore, Wijgerde et al. (2020) showed no impact to algal density in *A. tenuis* or *S. pistillata* adults over 6 weeks of 1 µg/L nominal (0.06 µg/L measured) but saw a 4-5% decrease in PSII yield in *S. pistillata* adults and 5% decrease for *A. tenuis* adults. Overall with the results presented in this study, this suggests that any bleaching impacts of BP-3 to these species are negligible over the testing periods (7 days to 6 weeks).

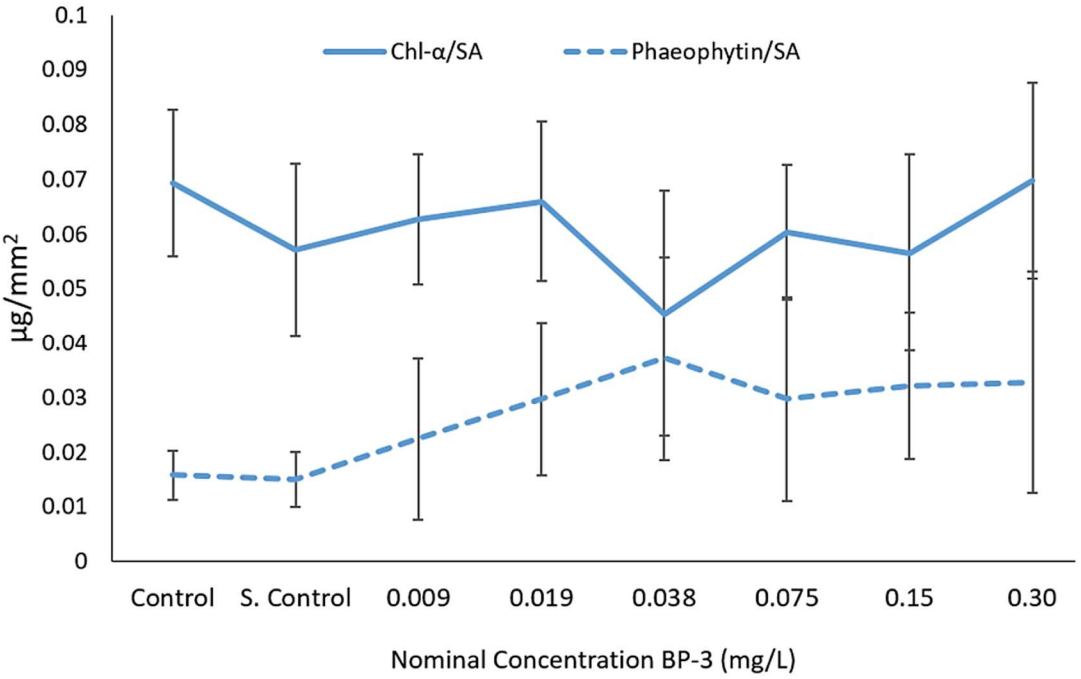

Figure 5. Surface-area standardized chlorophyll-α and phaeophytin on day 28 of chronic BP-3 exposure. No significant differences in chl-α or phaeophytin were seen over 28 days in response to BP-3 exposure to *G. fascicularis* ($p > 0.05$). Some slight negative impacts were seen from solvent control (S. Control) exposure and were steady throughout the increasing BP-3 exposure concentrations. This suggests chronic BP-3 exposure does not induce bleaching in this species.

In Mitchelmore et al. (2021), three tests calculated risk of BP-3 to corals (Tsui et al., 2014; 2017; He et al., 2019a) and were standardized to use the same assessment factors (i.e. 1000 for acute data and 100 for chronic data). To determine the risk of BP-3 to *G. fascicularis* using a sublethal endpoint, a RQ was deterministically derived as MEC/PNEC using the most conservative values of this study and an assessment factor of

100 for consistency with published data (Mitchelmore et al., 2021) combined with a monitoring study in Hawaii (Mitchelmore et al., 2019). The EC10 values used to calculate the PNEC were 0.013 mg/L nominal BP-3 and 0.043 mg/L measured BP-3 which were the growth EC10s as determined by buoyant weight. Mitchelmore et al. (2019) BP-3 concentrations ranged from <LOD to 142.7 ng/L so this highest measured value of 142.7 ng/L BP-3 was used as the MEC. This resulted in an RQworst of 1.10 (nominal) and 0.33 (measured) which are within the range of the studies referenced above. As an RQ > 1 is generally the threshold used to determine risk, this suggests that BP-3 exposure may pose a risk to G. fascicularis. These results do not take into account potential co-stressors (e.g. increased temperature, increased sedimentation, other chemical pollutants) or longer term exposures which may exacerbate negative impacts. For a discussion of results for the positive control diuron, please see Text S9.

4.4 Conclusions

Using methods similar to regulatory-approved standard chronic toxicity test methods in other marine invertebrate species, significant growth impacts for BP-3 were determined for *G. fascicularis* which are the first published growth impact of BP-3 on hard corals. Our results did not support bleaching as a mode of action of BP-3 toxicity, as no chl-α loss or BP-3-driven bleaching was seen, which suggests BP-3 bleaching may be species or life-stage dependent. Given the robust methodology of this test, the BP-3 growth EC10 achieved should be used as a reliable hazard estimation for risk assessments and, when combined with robust environmental concentrations near coral reefs (Mitchelmore et al., 2019) and an assessment factor, resulted in a RQ <1 using nominal

concentration and a RQ slightly greater than 1 using measured concentrations which indicates potential risk from BP-3 to *G. fascicularis*.

As these are the first growth impacts seen in a hard coral to BP-3, additional testing following similar protocols will better help determine the risk of BP-3 to other coral species. It is our hope that this experiment will allow policymakers to make more informed decisions that accurately reflect the risk of BP-3, UV filters, and other emerging compounds of concern to corals.

Conclusions

Summary of Findings

Repeated acute toxicity testing of copper with *Galaxea fascicularis* demonstrated it to be a good candidate for a standard coral toxicity testing species. Observations were straightforward, culturing this species using artificial seawater resulted in healthy organisms and, most importantly, the results of repeated testing were highly replicable. Copper was also found to be a good positive control for acute toxicity testing because it reliably elicited mortality in this species well below the limit of water solubility.

Using this standard toxicity testing protocol in triplicate acute (96-hour) toxicity tests exposing BP-3 to *G. fascicularis* demonstrated that mortality can occur at high concentrations; however, these levels are unlikely to exist in the natural environment. The results from our experiments did not support a bleaching mode of action that had been reported initial BP-3 coral toxicity studies like Downs et al. (2016). However, more recent studies corroborate our results so it is unclear if this difference between the initial and more recent studies is due to methodological differences, differences in species, life stage or other inconstancies among these studies. The risk quotient calculated using acute mortality data suggest BP-3 does not pose a risk to this species. It should be noted, however, that we do not know how representative this species is of corals as a whole and, until further research is complete, these conclusions should not be extrapolated to other species.

Continuing to explore the toxicity of BP-3 to *G. fascicularis*, a pilot growth study demonstrated sufficient growth in a toxicity testing time scale (i.e. 28 days or less) and no

significant signs of stress with daily handling which again supports its use as a standard testing organism. A single, 28-day exposure to BP-3 showed that the bleaching mode of action was, again, not supported for this species as no significant bleaching was seen with BP-3 exposure at any concentration over the duration of the study. However, significant growth impacts were observed in the µg/L range which resulted in two RQs calculated, one less than 1 and the other slightly greater than 1, suggesting possible risk of BP-3 to this species of coral. This chronic study also demonstrated that the pesticide diuron is a good positive control for chronic growth impacts as reducing photosynthesis in the symbiont significantly decreases growth.

Overall, these results suggest that *Galaxea fascicularis* is an appropriate species which can be used in any laboratory to conduct standard-style toxicity testing. Results using this species for testing with BP-3 do not support the bleaching mode of action on acute or chronic time scales. The risk quotients calculated using the data from chronic and acute testing suggested that BP-3 may pose a risk to *G. fascicularis* but as these calculations are conservative and the highest is close to the threshold for determining risk, should be considered as a "worst-case" scenario. These results agree with other studies suggesting risk is generally low, but increased risk may be present in some situations.

Gaps and Recommendation for Further Research

Because this testing focused on a single UV filter compound and a single species and life stage of hard coral, there are obviously many questions still to answer. First and foremost, additional strains and cultures of *G. fascicularis* still need to be tested to ensure

reproducibility of results as prior literature has suggested that the 96 h LC50 for copper and *G. fascicularis* may be an order of magnitude lower than what was presented in these experiments. Furthermore, copper toxicity tests using similar protocols on other species and life stages need to be completed in order to determine the sensitivity of *Galaxea spp.*, allowing risk calculations using this species to better reflect the sensitivity of other hard coral species. Copper may also have utility as a positive control for chronic exposures so its reproducibility over longer timescales should be explored.

This chronic testing, although robust, was not repeated to confirm results of no environmental risk of BP-3. Furthermore, as diuron growth impacts have not been quantified in this or any other hard coral species, a stand-alone chronic toxicity test using diuron as the compound of interest should be completed. However, it is suggested that lower concentrations be utilized to include a no-effect concentration as all three concentrations tested here reduced growth at least 98%.

Another facet to look more closely at is polyp retraction. In all studies it was noted to be one of the most sensitive endpoints. Therefore, deeper exploration into the ramifications of polyp retraction needs to occur. Once it is better understood, polyp retraction may also be useful as an early warning system for corals experiencing stress in the environment.

Finally, the larger question to be answered is which sunscreen UV filter compounds, if any, pose a significant risk to reef-building corals. In order for this to be determined, testing on all UV filters on hard corals needs to be completed on both adult and larval life stages. Using this information, any legislation necessary will truly reflect what is in the best interest for these essential organisms.

Appendices

Appendix A: Supplemental Text

Text S1. Coral Culture Conditions

Artificial seawater for the coral culture facility is made up in a tank which does not freely flow into the culture system. Deionized water (DI) water is mixed with Instant Ocean® to 35 ‰ and allowed to sit for a minimum of 48 h to allow salts to completely dissolve. The seawater make-up tank and the coral culture tank systems are interconnected with a circulating pump and during water changes, a certain amount of seawater is drained from the main system and replaced with the fresh sea water.

Six parent fragments of *Galaxea fascicularis* were collected from the Lexington Park, MD Marine Aquaria during its rebuild in 2002. Additional specimens were obtained from a local marine aquaria group as well as Roozen's Nursery (Fort Washington, MD). There is no reliable information on the geographic origin of these specimens, but at least 8 different individual colonies from various sources were collected to ensure some genetic diversity. These stocks were not kept separate so any colony in the system is a haphazard sampling of the 8+ original colonies. Additionally, the culture system in which these organisms were held concurrently housed multiple other species of coral, fish, and various invertebrates in separate tanks but sharing water flow. *Galaxea fascicularis* were not fed directly but the entire system was supplied with Golden Pearl Reef and Larval Fish Food (300 to 500 μm; Brine Shrimp Direct, Ogden UT). Nutrients levels were monitored biweekly and chemistry adjusted accordingly.

Text S2. Test Setup Additional Details

Exposures were carried out in 2.0 L glass beakers modified to contain a glass tube attached to the inner wall of the beaker with food-grade silicone (Figure S5). Into this tube, a glass pipette attached to an airline created a bubble lift to maintain dissolved oxygen levels and drive circulation in the vessel as appropriate water flow conditions are essential for the health of this species (Schutter et al., 2010). Vessels were loosely covered with plastic wrap to prevent evaporation and changes in salinity. Culture water was used as control water and to make dosing solutions. This water was shaken to ensure a homogenous mixture of any algae or solids before and after the addition of the chemical of interest and again before addition of the test organism.

EcoTech Marine Radion XR30W lights provided full-spectrum illumination for all exposures and programmed using EcoSmart Live to follow the "Shallow Reef (Natural)" 12:12 cycle. Photosynthetically active radiation (PAR, μmol m^{-2} s^{-1}) was measured with a handheld full-spectrum quantum meter (Apogee MQ-501) daily to confirm spectral quantity.

Vessels for all tests were placed indiscriminately in a water bath set to maintain a coral exposure temperature of 26 °C ± 1° C which was monitored using a HOBO Pendant® MX Water Temperature Data Logger (Onset, MA). Using a YSI Professional Plus Multi-Parameter Meter (YSI, Inc. Yellow Springs, OH), water quality was performed daily on pooled replicates of new (i.e. immediately after solution preparation) and aged (i.e. after 24 h of coral exposure, before renewal) solutions for each copper concentration.

Before daily water changes, images of each chip were taken using a Cannon EOS 60D camera with a macro lens for the analyses described below and light adapted

photosynthetic efficiency measured using a Junior PAM fluorometer (Heinz-Walz, Effeltrich, Germany). For acute testing, daily replicates of newly made up seawater for each exposure concentration were pooled as were samples of aged seawater from each treatment prior to the new addition. For chronic testing, these samples were taken biweekly. Unfiltered samples for analytical confirmation of new (n = 2 per concentration) and aged (n = 1) exposure concentrations were refrigerated until processing and analysis as described below. Additional daily new and aged seawater samples from each concentration were filtered through a 0.7 μm GF/F glass fiber filter. The filter and filtrate were independently frozen at -80 °C for chlorophyll-α/phaeophytin analysis and additional water quality analyses (nitrates, nitrites, ammonia, phosphates, and alkalinity), respectively.

Text S3. Biological Endpoints Additional Details

Daily photographs were used to determine the degree of polyp retraction by visually scoring them from 0 (no polyps visible, full retraction) to 4 (full extension, polyps appear relaxed and freely moving). This was compared to a quantification of polyp extension using Adobe Photoshop® where one tentacle from each polyp was measured using the "measure" tool from where it became visible above the skeleton cup to its tip and standardized against the diameter of the poker chip (see Figure S1 for an example). Because of the high correlation between visual and quantitative measures of polyp retraction, it is suggested that quantitative polyp retraction using image analysis should be used in the future if this endpoint is utilized, as this would decrease the bias and variation between individual observers.

Coral bleaching was assessed in a number of ways. First, it was qualified using the Coral Color Reference Card from Siebeck et al. (2006) then quantified using an image analysis method modified from the same study. Images were standardized using the white poker chip as a white standard and analyzed using Adobe Photoshop® to look at the brightness and saturation composition in an averaged 5x5 pixel selection. One selection was taken per polyp then averaged by total treatment replicates (i.e. n = 12 corals). Saturation of the polyp was shown to be a more sensitive with a larger range of values over the spectrum of healthy to bleached coral (Figure S1) and so is the quantitative bleaching endpoint used for statistical analysis. Visual scoring of the polyps correlated well with quantification of saturation (Figure S6). Therefore, to reduce observer bias, saturation was the bleaching endpoint used in statistical analysis.

Second, the algal pigments chl-α and phaeophytin in coral tissue were quantified following Yost and Mitchelmore (2010). After defrosting from -80 °C, coral tissue was removed from the skeleton by air-brushing with 0.2 µm filtered ASW (at 35‰ using Crystal Sea® Marinemix [Marine Enterprises, Baltimore, MD]) in a plastic bag using an Iwata-Media Eclipse HP BCS Dual action air brush. The resulting tissue slurry was rinse with seawater into a glass homogenizing tube on ice. With a Teflon pestle, this was then homogenized to obtain a consistent slurry, the total volume measured and recorded and the slurry divided into the appropriate tubes and either immediately processed for algal pigment (i.e. chl-α, phaeophytin) or frozen at -40 °C for later assessment of protein levels.

For pigment analysis, 1 mL of the homogenate was filtered using a GF/F filter. The filtrate was then placed into 4-5 mL of 90% acetone and allowed to extract at 4°C for

12-18 h. Samples were removed from 4°C, centrifuged, and the supernatant was removed, filtered with a 0.45 µm PTFE syringe filter (Milex, Duluth, GA), and placed into a cuvette where absorbance readings were made at the wavelengths 750, 665, 664, 663, 647 and 630 nm using a spectrophotometer (SpectraMax® PLUS 384 [Molecular Devices, San Jose, CA]). The extract was then acidified with 1 N HCl and readings were again made at the aforementioned wavelengths. These results were used in the equations outlined in NASL (2019) to calculate uncorrected chl-α, phaeophytin, and chl-α corrected for phaeophytin. To determine the best metric to standardize coral tissue chl-α, both polyp skeleton surface area and total protein content of the coral tissue were quantified.

Both protein and surface area were quantified as potential standardization methods for pigment content. This allowed us to investigate the relationship with total protein versus surface area of the polyp and help determine if the toxicant results in overall tissue loss. Total protein content was determined using the Pierce™ BCA method (Thermo Fisher Scientific, Waltham, MA). Briefly, the tissue homogenate was defrosted, re-homogenized, and 100 µL was added to 200 µL of 0.2 µm filtered ASW (i.e. a 1:3 dilution). Methods were conducted as outlined in the BCA protocol with 25 µL of the diluted tissue sample added to triplicate wells. Surface area was calculated in a manner similar to quantitative polyp retraction. Pictures of coral skeletons after tissue removal were taken. Using Adobe Photoshop®, each polyp skeleton was measured using the "measure" tool along the central length and width. These measurements were used to calculate the surface area of a cylinder, an approximation of the surface area of the coral polyp skeleton.

For light-adapted photosynthetic efficiency determination, the last 2 inches of the filament were covered in black tape to decrease additional light input from the sides. If ΔF/Fm' was below 0.200, the reading was re-measured to confirm. On Day 0 for all definitive tests, PAM was done on all polyps of the representative corals and also done on one polyp of each poker chip of the exposure concentrations and controls. For BP-3 tests, on day 0 and 2, one polyp on each chip was measured (n = 3 per treatment vessel). On day 4, measurements were taken for all polyps (n = 12 per treatment vessel). For copper tests, all polyps were measured daily.

Text S4. Justification of Solvent Choice

Some of the studies examining coral toxicity to UV filters have also utilized dimethyl sulfoxide (DMSO; Downs et al., 2016; Stien et al., 2019; Wijgerde et al., 2020) which is not advised due to some potential confounding impacts of the solvent. DMSO is an antioxidant (Sunda et al., 2002) so any toxicity of BP-3 due to oxidative stress may be counteracted by this solvent. Furthermore, DMSO is known to aid in transport across biological membranes which may increase the uptake of BP-3 (Rammler and Zaffaroni, 1967; Yu and Quinn, 1994). Contrastingly, methanol is not known to have any such properties.

Text S5. Preliminary Acute BP-3 Investigations

To determine the appropriate dosing frequency and exposure water volume/organism mass ratios for the static tests, a preliminary investigation was conducted using four conditions of combinations of exposure water volume, biomass

(coral) presence, and BP-3 concentration. Abiotic vessels contained dilution water spiked with two concentrations of BP-3 (1 mg/L and 100 ng/L) with the biotic vessels containing 3 poker chips of the 4-star polyps. Five 100 mL samples were removed from each vessel at various time points over 48 h and these samples were extracted and analyzed for BP-3 concentration as described in the main text. The BP-3 concentration steeply dropped by approximately 50% in the first 24 hours but then stabilized in all conditions up to the 48-hour time point. Coral health and water quality conditions were not different in any of the conditions. Therefore, a 24-hour renewal was decided with 1 L of exposure water to maintain BP-3 concentrations as close to nominal as possible while using minimal exposure water.

Preliminary toxicity tests were then carried out with BP-3 and G. fascicularis. There were 5 to 8 treatment concentrations for each range-finding test with no replicates and 3 chips of coral in each vessel. Photographs of the polyps were taken daily as were visual observations of mortality and polyp retraction. Two sets of range-finding exposures to BP-3 were conducted. The first test covered a range of concentrations from 10 mg/L to 1 ng/L with a factor of 10 between each (i.e. 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10 mg/L) as well as a negative control and high solvent control (500 µL/L). The second rangefinder narrowed in the range and decreased the solvent load so the concentrations spanned from 100 ng/L to 10 mg/L with a factor of 10 between with a negative control and low and high solvent load control (100 and 200 µL/L, respectively). One preliminary range-finding exposure was done for diuron, exposing the organisms to the concentrations of 0.0001, 0.001, 0.01, 0.1, 1, and 10 mg/L. Before the third BP-3 test using copper as a positive control, copper was used as the primary compound for three

preliminary and two definitive acute tests to confirm reproducibility and the threshold of copper toxicity for use as a positive control in this coral species (manuscript in preparation).

Definitive testing differed from preliminary, range-finding tests in the increased number of organisms and replicates, narrowed concentration range, chemical analysis, and full endpoint characterization that is performed in definitive testing, but not in preliminary testing. The first BP-3 definitive test did not include a positive control, the second test utilized diuron as a positive control, and the third test used copper as a positive control.

Text S6. Method Recovery Determination and Additional Analytical parameters

The most notable change from the BP-3 extraction protocol was that MeOH was utilized instead of acetonitrile (ACN) in the latter two exposures because adequate and consistent recoveries for this concentration range (58 ± 2% [SD] with MeOH versus 89 ± 3% [SD] recovery with ACN) were achieved with this eluent. Furthermore, it allowed for the omission of drying samples under N_2 gas and reconstituting them in methanol which ensured more timely sample analysis.

The following recovery spikes were performed to determine acute method recoveries. On days 0, 2 and 4 of the third BP-3 test, the lowest BP-3 exposure solution (0.63 mg/L) was spiked with 1.13 mg/L BP-3 in MeOH. Diuron exposure solutions of 2.0 µg/L from the chronic exposure (described below) were spiked with an additional 2.0 µg/L Diuron in MeOH. Deuterated internal standard concentrations of 4.975 µg/L d3-BP-

3 and 0.9388 µg/L d6-diuron were spiked into diluted samples before analysis. Recoveries of diuron in acute exposure solutions were 72 ± 16% SD.

For chronic testing, on days 0, 7, 14, 21 and 27, the highest BP-3 exposure solution (0.30 mg/L) was spiked with 1.13 mg/L BP-3 in MeOH and diuron exposure solutions of 2.0 µg/L were spiked with an additional 2.0 µg/L Diuron in MeOH. Deuterated internal standard concentrations of and 4.975 µg/L d3-BP-3 and 0.9388 µg/L d6-Diuron were spiked into diluted samples before analysis. Recoveries of diuron were 97 ± 7% SD. BP-3 recoveries for both acute and chronic tests were not accounted for as a recovery experiment demonstrated severe loss during storage. However, at this time, this loss cannot be quantified. Analytical concentration in the acute test were likely low due to adsorption and fallout issues. Chronic concentrations were higher than expected likely due to buildup. This was evidenced in the concentrations of BP-3 on day 0 of the chronic exposure which were significantly lower than the subsequent samples at all concentrations.

Limit of quantification (LOQ) was determined by the lowest concentration which was quantifiable at the sample dilution used to quantify all exposure concentrations. Then, the limit of detection was calculated as LOQ/3. Generally, the limit of detection (LOD) is quantified using the lowest detection limit of the analysis method and the LOQ is determined as LOD*3. However, the overall quantification method used had a much lower limit that was required for this test (i.e. in the ng/L range) and the ASW used in this study had low but quantifiable concentration of BP-3 which has been seen in other methods as well (Saxe et al., 2020). Therefore, the manner of reporting is only meant to

reflect that the BP-3 in the control conditions was significantly lower than any treatment and not in any way contaminated with the toxicant.

For LC-qqq-MS analysis of BP-3 and diuron respectively, 8 and 5 µL of diluted sample was injected via the autosampler onto an ACE C18 column (Advanced Chromatography Technologies Ltd, Aberdeen, Scotland). The mobile phase gradient used started at 75% methanol and 25% 0.1% formic acid, then ramped to 97% methanol within 30 seconds and kept at this mobile phase for the remainder of the run. After completion, the initial mobile phase was allowed to equilibrate for 8 minutes prior to the next injection. Sampling needle wash occurred for 10 s with 50% MeOH in DI water for diuron samples and pure isopropyl alcohol (Fisher chemical, Pittsburg, PA) for BP-3 analyses. Positive ESI mode was employed for analysis of both compounds with a gas temperature of 300 °C with a flow rate of 7 L/min and a nebulizer pressure of 32 psi for BP-3 analysis, and 350 °C with a flow rate of 10 L/min and a nebulizer pressure of 40 psi for diuron analysis.

Text S7. Pilot Growth Study

A growth study was conducted to see the growth rate of Galaxea fascicularis under testing conditions. 3 of the 4-star poker chips were placed in each of 5 2-L beakers filled to 1.0 L with control seawater. This seawater was changed daily.

Using buoyant weight, each chip was weighed at minimum every 48 h for the first 10 days. Then, corals were weighed weekly. After 21 days, growth was not apparent so corals were fed daily (with dry pellets only) from that point on. Feeding was done approximately 1-3 hours before the water change to allow corals to feed. After 35 days,

growth was seen to be increasing at approximately 0.004 g/day (Figure SX). This would result in an expected 0.112 g increase at the end of a 28-day test.

Text S8. Chronic-Specific Test Setup and Biological Endpoint Details

To make up chronic test solutions, exposure water was split into 5 L glass bottles with 4.5 L coral culture water in each bottle for BP-3 concentrations and 3.5 L culture water for Diuron concentrations. For both compounds, each bottle was then spiked with its own methanol stock for each concentration at 50 uL/L (i.e. 225 uL stock solution per 4.5 L bottle), or in the case of the solvent stock, plain methanol. Solvent stocks were made up weekly and an aliquot was set aside for chemical verification. All bottles were thoroughly shaken before being split into their replicate vessels.

Weight was measured weekly using the buoyant weight technique (REF). A balance (Model SLF103; Fisher Science Education, Pittsburg, PA) was positioned above a beaker of control culture seawater using a Styrofoam housing and the coral chip was hung from the bottom of the balance until fully submerged. The balance was tared before each weight. Weights were recorded to the 0.001 decimal. Each coral was labeled with a number so individual change in mass could be tracked. Any polyps that were broken off were recorded separately.

Polyp width and length for growth measurements were completed in similar way where length was measured using the "measure" tool down the center of the polyp from the base of the polyp to the tip of the skeleton, not including tentacle length. Width was measured as the widest part of the coral near where the tissue cover ends. This location was seen to be the leading edge of growth with both tissue and skeleton extension along

with the majority of newly budded polyps occurring there. These measurements were also standardized using chip diameter.

Images were also used to quantify the number of new polyps that occurred over the study period. New polyps were any small polyps that were not counted as one of the major 4. This scoring was done weekly on Thursday images. Total counts per replicate were summed and Day 0 values were subtracted from each week to determine the total number of new polyps to that point. Total new polyps per replicate after 28 days were used to in calculations.

On Day 0, PAM was done on all polyps of the representative corals which were then immediately frozen and also done on one polyp of each poker chip of the exposure concentrations and controls. Twice weekly, measurements were taken for all polyps (i.e. n=12 per treatment vessel).

Text S9. Diuron positive control

The interaction between photosynthetic efficiency and buoyant weight was clear in the diuron positive control. The photosynthetic efficiency was effectively knocked out in all tested diuron concentrations (because diuron is a PS II inhibitor) which resulted in significant differences in weight and number of new polyps at all concentrations (2 µg/L and up). Furthermore, the change in width of these polyps, brightness, saturation, and polyp retraction were significantly impacted at 10 µg/L and up. This clearly shows the significant negative impacts of diuron and its usefulness as a positive control for chronic testing. Interestingly, the length of the polyps in the diuron concentrations was the only endpoint not impacted, again suggesting that it is not a parameter with sufficient variation

over this time period to use to chronic testing of this species. Diuron concentration recovered similar to expected with high recoveries (Table S19).

Text S10. Additional References

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Appendix B: Supplemental Tables

Table S1. Water quality parameters for coral culture during time of testing.

	Phosphorus (mg/L)	Nitrate (mg/L)	Iodine (mg/L)	pH	Temperature (°C)	Salinity (ppt)
Average	0.306	0.020	0.115	7.82	25.37	36.00
SD	0.124	0.049	0.121	0.05	0.54	0.72

Table S2. Summary of water quality parameters and light quantity measured daily for acute copper testing. Parameters given as a range (average) during each test.

Test	Light ($\mu mol\ m^{-2}\ s^{-1}$)	Temp. (°C)	Salinity (‰)	DO (mg/L)	DO (%)	pH
Copper #1	108 - 210 (155)	24.2 - 26.8 (25.2)	33.98 - 36.08 (34.95)	5.64 - 6.93 (6.10)	77.0 - 89.8 (85.9)	7.64 - 8.09 (7.89)
Copper #2	89 - 209 (135)	24.7 - 26.1 (25.6)	33.06 - 34.33 (33.80)	5.27 - 6.15 (5.68)	78.1 - 87.0 (83.4)	7.63 - 8.08 (7.88)
Copper #3	94 – 171 (128)	24.6 - 25.5 (25.1)	31.91 – 32.51 (32.21)	6.00 - 7.02 (6.68)	81.1 - 102.4 (95.6)	7.72 - 8.44 (8.20)
Copper Pos. Control	97 - 197 (136)	24.5 - 26.3 (25.8)	31.49 - 33.08 (32.66)	6.37 - 7.18 (6.96)	82.1 - 103.6 (100.1)	7.67 - 8.02 (7.81)

Table S3. Summary of additional water quality parameters measured during acute copper testing. Parameters given as average ± standard deviation during each test.

Test	NH_4^+ (mg/L)	NO_2^- (mg/L)	PO_4^{3-} (mg/L)	NO_3^- (mg/L)	Alkalinity (dKH)
Copper #1	0.13 ± 0.11	0.005 ± 0.002	1.04 ± 0.53	0.014 ± 0.011	4.98 ± 0.40
Copper #2	0.05 ± 0.07	0.005 ± 0.002	1.36 ± 0.21	0.048 ± 0.081	4.20 ± 0.39
Copper #3	0.02 ± 0.03	0.004 ± 0.001	0.31 ± 0.13	0.030 ± 0.017	4.77 ± 0.56
Copper Pos. Control	0.17 ± 0.2	0.007 ± 0.001	0.65 ± 0.07	0.015 ± 0.010	4.78 ± 0.49

Table S4. Concentrations of dissolved copper from all acute exposures. Dissolved exposure concentrations ranged from 67-108% of nominal.

Test	Nominal Concentration (µg/L)	Average New (µg/L)	Average Aged (µg/L)	% 24 h reduction	Overall Average (µg/L)	Average Expected (µg/L)	Average % Exposed
Test 1	**Control**	7	4	NA	5.5	0	NA
	63	52.7	45.5	14%	49.1	62.8	78%
	125	105.9	87.5	17%	96.7	125.5	77%
	250	198	159.8	19%	178.9	251.1	71%
	500	387.3	322.6	17%	355	502.1	71%
	1000	768.6	661.7	14%	715.2	1004.2	71%
Test 2	**Control**	13	9.4	NA	11.2	0	NA
	63	65.3	57.9	11%	61.6	62.6	98%
	125	123.5	103.9	16%	113.7	125.1	91%
	250	228.7	174.3	24%	201.5	250.3	81%
	500	473.6	331.4	30%	402.5	500.5	80%
	1000	838.7	617.2	26%	743.8	1001	74%
Test 3	**Control**	9	6.5	NA	8	NA	NA
	63	54	51.6	4%	53	63	84%
	125	118	110.3	6%	114	125	91%
	250	213	190.9	10%	202	250	81%
	500	419	372.9	11%	396	501	79%
	1000	828	514.1	38%	671	1002	67%
Pos. Control	**Control**	1	NA	NA	NA	NA	NA
	250	227	NA	NA	NA	256	89%
	500	553	NA	NA	NA	511	108%
	1000	969	NA	NA	NA	1022	95%

Table S5. Particulate copper concentrations and total copper concentrations (i.e. dissolved and particulate copper) for copper test 2.

Concentration (mg/L)	Dissolved (mg/L)	Particulate (mg/L)	Total Copper (mg/L)	Particulate Fraction (%)	Total Recovery (%)
Control	13.5 ± 2.8	0.5 ± 0.5	13.9 ± 3.3	3.0 ± 2.9	NA
0.063	65.3 ± 2.3	1.2 ± 0.6	66.5 ± 2.4	1.8 ± 0.9	106.3 ± 3.1
0.13	123.5 ± 9.9	1.5 ± 1.2	125.0 ± 9.3	1.3 ± 1.0	99.8 ± 5.4
0.25	228.7 ± 25.2	2.2 ± 0.5	230.9 ± 25.3	0.9 ± 0.2	92.2 ± 8.7
0.5	473.6 ± 67.2	3.7 ± 0.3	477.3 ± 67.4	0.8 ± 0.1	95.2 ± 12.1
1.0	838.7 ± 91.8	10.6 ± 2.4	849.3 ± 93.7	1.2 ± 0.2	84.8 ± 7.8

Table S6. Calculated copper LC50s for coral species of all life stages.

Reference	Species	Life Stage	Duration (h)	LC50
Esquivel, 1986	*P. damicornis*	larva	2	0.556
Esquivel, 1986	*P. damicornis*	larva	2	0.552
Esquivel, 1986	*P. damicornis*	larva	4	0.44
Esquivel, 1986	*P. damicornis*	larva	4	0.438
Esquivel, 1986	*P. damicornis*	larva	6	0.286
Esquivel, 1986	*P. damicornis*	larva	6	0.29
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	6	0.248
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	6	0.26
Esquivel, 1986	*P. damicornis*	larva	8	0.195
Esquivel, 1986	*P. damicornis*	larva	8	0.19
Esquivel, 1986	*P. damicornis*	larva	12	0.123
Esquivel, 1986	*P. damicornis*	larva	12	0.12
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	24	0.121
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	24	0.137
Esquivel, 1986	*P. damicornis*	larva	24	0.115
Esquivel, 1986	*P. damicornis*	larva	24	0.114
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	48	0.04
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	48	0.087
Esquivel, 1986	*P. damicornis*	larva	48	0.09
Esquivel, 1986	*P. damicornis*	larva	48	0.087
Kwok et al., 2016	*P. acuta*	Larva	48	0.11
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	72	0.034
Reichelt-Brushett and Harrison, 2004	*G. aspera*	Larva	72	0.082
Esquivel, 1986	*P. damicornis*	larva	72	0.069
Esquivel, 1986	*P. damicornis*	larva	72	0.07
Esquivel, 1986	*P. damicornis*	larva	96	0.057
Esquivel, 1986	*P. damicornis*	larva	96	0.063
Kwok et al., 2016	*P. acuta*	Larva	96	0.107
Sabdono, 2009	*G. fascicularis*	Adult	96	0.032
This Study	*G. fascicularis*	Adult	96	0.436

Table S7. Summary of endpoints for repeated copper acute tests to *Galaxea fascicularis*. The lowest-observed effect concentration (LOEC) is the first concentration that was significantly different ($p < 0.05$) from the controls while the no-observed effect concentration (NOEC) is the highest concentration not showing a significant difference from the controls. A NOEC of NA (not applicable) designates a significant difference of all testing concentrations from the controls. LCx: Lethal concentration causing X% mortality; ECx: Concentration causing and X% decline in a given measurement; DRC: Dose-response curve; J: Jonckheere Test; D: Dunnett's test; S: Steel's Many-to-One Rank Test

	Endpoint	NOEC/ LOEC Test	P-value	NOEC	LOEC	DRC Function	LC/EC10	LC/EC20	LC/EC50
Test 1	Mortality	J	<0.001	0.25	0.5	LL.2	0.369	0.394	0.441
	$\Delta F/F_m'$	S	<0.05	0.50	1.0	LN.3	0.378	0.433	0.563
	Saturation	J	<0.001	0.25	0.5	LL.4	NA	NA	4.010
	Polyp Retraction	J	<0.001	NA	0.063	LL.5	0.0135	0.052	0.121
	Tissue Chl-α	D	<0.05	0.25	0.50	LL.4	0.140	0.189	0.314
Test 2	Mortality	J	<0.001	0.25	0.5	LL.2	0.472	0.499	0.547
	$\Delta F/F_m'$	S	<0.05	0.50	1.0	LN.3	0.470	0.517	0.618
	Saturation	J	<0.001	0.063	0.13	LL.4	0.124	0.164	0.265
	Polyp Retraction	J	<0.001	NA	0.063	LL.3	0.029	0.045	0.096
	Tissue Chl-α	D	<0.05	0.25	0.50	LL.3	0.253	0.344	0.580
Test 3	Mortality	J	<0.001	0.25	0.50	LL.3	0.334	0.362	0.415
	$\Delta F/F_m'$	J	<0.001	NA	0.063	LL.4	0.0478	0.0707	0.138
	Saturation	S	<0.001	0.063	0.13	LL.4	0.0848	0.133	0.289
	Polyp Retraction	J	<0.001	NA	0.063	LL.5	0.0114	0.0259	0.0894
	Tissue Chl-α	S	<0.05	0.13	0.25	LL.3	0.033	0.0871	0.457

Table S8. Summary of water quality parameters measured daily for acute BP-3 testing. Parameters given as a range (average) during each test.

Test	Light (µmol m^{-2} s^{-1})	Temp. (°C)	Salinity (ppt)	DO (mg/L)	DO (%)	pH
BP-3 #1	150 - 351 (229)	25.5 - 28.2 (26.4)	34.76 - 36.69 (35.40)	3.12 - 6.35 (5.86)	46.0 - 96.4 (89.1)	8.06 - 8.65 (8.53)
BP-3 #2	131 - 359 (209)	25.7 - 29.2 (26.4)	34.91 - 36.51 (35.60)	4.00 - 6.91 (6.06)	60.2 - 93.7 (90.2)	8.18 - 8.70 (8.46)
Diuron Pos. Control	113 - 315 (175)	25.7 - 29.2 (26.4)	34.79 - 36.52 (35.57)	5.46 - 6.15 (5.88)	83.2 - 91.9 (88.5)	8.32 - 8.57 (8.42)
BP-3 #3	103 - 242 (143)	24.5 – 26.3 (25.8)	31.73 - 33.15 (32.76)	4.20 - 8.89 (7.08)	61.1 - 105.3 (99.7)	7.28 - 8.14 (7.79)
Copper Pos. Control	97 - 197 (136)	24.5 – 26.3 (25.8)	31.49 - 33.08 (32.66)	6.37 - 7.18 (6.96)	82.1 - 103.6 (100.1)	7.67 - 8.02 (7.81)

Table S9. Summary of additional water quality parameters measured during acute BP-3 testing. Parameters given as average ± standard deviation during each test.

Test	NH_4^+ (mg/L)	NO_2^- (mg/L)	PO_4^{3-} (mg/L)	NO_3^- (mg/L)	Alkalinity (dKH)
BP-3 #1	0.01 ± 0.02	0.003 ± 0.002	0.40 ± 0.15	0.028 ± 0.018	4.67 ± 0.39
BP-3 #2	0.01 ± 0.03	0.006 ± 0.002	1.01 ± 0.40	0.012 ± 0.004	4.71 ± 0.31
Diuron Pos. Control	<0.01 ± 0.01	0.006 ± 0.002	1.17 ± 0.36	0.015 ± 0.012	4.58 ± 0.43
BP-3 #3	0.04 ± 0.10	0.005 ± 0.001	0.63 ± 0.45	0.019 ± 0.013	4.73 ± 0.44
Copper Pos. Control	0.17 ± 0.2	0.007 ± 0.001	0.65 ± 0.07	0.015 ± 0.010	4.78 ± 0.49

Table S10. Dissolved concentrations of newly prepared solutions of BP-3 (n = 1) in Acute Test 2. Considerable background concentrations as well as contamination over time is evident, likely due to BP-3 adhesion to the extraction manifold. Calculations are done using only the first 2 time points (0 and 24 h) as contamination is minimal to exhibit similar exposure solutions to Test 3. Contamination of aged samples is severe at all time points. LSC: low solvent control; HSC: high solvent control

Nominal (mg/L)	0 h	24 h	48 h*	72 h*	Avg. 0-24 h	SD	Avg. - Control	% Nominal
Control	0.08	0.06	0.06	0.50	0.07	0.01	NA	NA
LSC	0.04	0.05	0.15	3.45	0.04	0.00	NA	NA
HSC	0.09	0.04	0.15	2.91	0.07	0.04	NA	NA
0.31	0.07	0.08	0.99	9.15	0.07	0.01	0.00	1%
0.63	0.12	0.14	0.15	1.89	0.13	0.01	0.06	10%
1.3	0.16	0.17	0.25	1.29	0.17	0.01	0.10	8%
2.5	0.24	0.30	0.50	2.94	0.27	0.05	0.20	8%
5.0	0.52	0.46	0.59	1.05	0.49	0.04	0.42	8%
10	0.70	0.88	0.92	1.22	0.79	0.12	0.72	7%

*Time points omitted from calculations due to contamination

Table S11. Summary of endpoints for repeated BP-3 acute tests to *Galaxea fascicularis*. The lowest-observed effect concentration (LOEC) is the first concentration that was significantly different ($p < 0.05$) from the controls while the no-observed effect concentration (NOEC) is the highest concentration not showing a significant difference from the controls. A NOEC of NA (not applicable) designates a significant difference of all testing concentrations from the controls. LCx: Lethal concentration causing X% mortality; ECx: Concentration causing and X% decline in a given measurement; DRC: Dose-response curve; J: Jonckheere Test; D: Dunnett's test; S: Steel's Many-to-One Rank Test

	Endpoint	NOEC/ LOEC Test	P-value	NOEC	LOEC	DRC Function	LC/EC10	LC/EC20	LC/EC50
Test 1	Mortality	J	<0.001	5.0	10	LL.3	5.20	5.53	6.15
	$\Delta F/F_m'$	D	<0.05	2.5	5.0	LL.3	0.81	2.57	18.63
	Saturation	S	<0.05	1.3	2.5	LL.4	2.42	3.02	4.40
	Polyp Retraction	J	<0.001	0.63	1.3	LL.4	0.95	1.70	4.59
	Tissue Chl-α	D	<0.05	0.63	1.3	LL.4	0.46	1.27	7.12
Test 2	Mortality	J	<0.001	5.0	10	LL.3	5.55	5.84	6.37
	$\Delta F/F_m'$	S	<0.05	5.0	10	LL.3	5.25	6.89	10.94
	Saturation	S	<0.05	5.0	10	LL.3	4.32	4.67	5.35
	Polyp Retraction	S	<0.05	1.3	2.5	LL.4	1.43	2.16	4.40
	Tissue Chl-α	D	<0.05	2.5	5.0	LL.3	2.22	3.01	5.08
Test 3	Mortality	J	<0.001	5.0	10	LL.5	6.31	6.58	7.06
	$\Delta F/F_m'$	S	>0.05	NA	0.63	LL.3	5.41	5.97	7.06
	Saturation	S	>0.05	NA	0.63	LL.4	0.015	0.086	1.64
	Polyp Retraction	J	<0.001	0.63	1.3	LL.3	0.59	0.95	2.16
	Tissue Chl-α	D	<0.05	2.5	5.0	LL.3	0.96	1.66	4.23

Table S12. Dissolved concentrations of newly prepared solutions of BP-3 (n = 2) in Acute Test 3. LSC: low solvent control; HSC: high solvent control

Nominal (mg/L)	0 h	24 h	48 h	72 h	Avg.	SD	% Nominal
Control	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA	NA
LSC	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA	NA
HSC	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA	NA
0.63	0.02	0.04	0.04	0.03	0.03	0.01	5%
1.3	0.17	0.15	0.15	0.16	0.16	0.01	12%
2.5	0.23	0.38	0.36	0.36	0.33	0.07	13%
5.0	0.68	0.77	0.94	0.89	0.82	0.11	16%
10	1.04	1.02	0.56	0.74	0.84	0.23	8%

Table S13. Dissolved concentrations of aged exposure water solutions of BP-3 (n = 1) in Acute Test 3. LSC: low solvent control; HSC: high solvent control

Nominal (mg/L)	24 h	48 h	72 h	96 h	Avg.	SD	% Nominal
Control	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA	NA
LSC	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA	NA
HSC	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA	NA
0.63	0.01	0.01	0.04	0.01	0.02	0.01	3%
1.3	0.08	0.09	0.09	0.10	0.09	0.01	7%
2.5	0.16	0.13	0.44	0.51	0.31	0.19	12%
5.0	0.80	0.76	0.84	1.06	0.87	0.13	17%
10	0.24	0.42	0.56	0.56	0.44	0.15	4%

Table S14. Concentrations of particulate BP-3 (n = 1) in Acute Test 3. LSC: low solvent control; HSC: high solvent control. LSC and HSC not measured as Control and 0.63 mg/L showed negligible particulate BP-3.

Nominal (mg/L)	0 h new	48 h new	48 h aged	96 h aged	Avg.	SD	% Nominal	% Total Measured
Control	<0.001	0.006	0.005	<0.001	0.003	0.003	NA	NA
LSC	NA	NA	NA	NA	NA	NA	NA	NA
HSC	NA	NA	NA	NA	NA	NA	NA	NA
0.63	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	0.1%	2.7%
1.3	0.004	0.379	<0.001	0.002	0.096	0.188	15.1%	54.4%
2.5	0.012	0.227	<0.001	0.003	0.061	0.111	4.4%	24.9%
5.0	0.274	0.317	0.002	0.028	0.155	0.163	3.3%	16.6%
10	0.5	1.037	0.001	0.197	0.434	0.451	4.5%	35.0%

Table S15. Summary of water quality parameters measured daily during BP-3 chronic testing. Parameters given as a range (average) during each test.

Compound	Light (µmol m^{-2} s^{-1})	Temp. (°C)	Salinity (ppt)	DO (mg/L)	DO (%)	pH
BP-3	79 - 280 (167)	24.0 - 27.6 (25.4)	33.37 - 37.31 (35.23)	5.48 - 7.43 (6.26)	82.8 - 105.0 (91.9)	8.24 - 8.81 (8.50)
Diuron	61 - 240 (134)	24.3 - 27.5 (25.6)	33.40 - 37-27 (35.21)	5.21 - 7.46 (6.03)	78.4 - 93.6 (88.3)	8.29 - 8.63 (8.45)

Table S16. Summary of additional water quality parameters measured during BP-3 chronic testing. Parameters given as average ± standard deviation during each test.

Compound	NH_4^+ (mg/L)	NO_2^- (mg/L)	PO_4^{3-} (mg/L)	NO_3^- (mg/L)	Alkalinity (dKH)
BP-3	0.001 (± 0.005)	0.006 (± 0.003)	0.75 (± 0.65)	0.01 (± 0.01)	4.5 (± 0.4)
Diuron	0.001 (± 0.003)	0.004 (± 0.002)	0.81 (± 0.67)	0.01 (± 0.005)	4.7 (± 0.5)

Table S17. Summary of endpoints for BP-3 and diuron positive control chronic testing to *G. fascicularis*. Endpoints are given as Measured (Nominal) in mg/L.

Toxicant	Endpoint	LOEC Test	P	NOEC	LOEC	DRC Function	EC10	EC20	EC50
BP-3 (mg/L)	Quant. Retraction	D	<0.05	0.288 (0.075)	0.579 (0.15)	LL.3	0.085 (0.0357)	0.228 (0.0815)	1.222 (0.334)
	$\Delta F/F_m$'	D	<0.05	0.131 (0.038)	0.288 (0.075)	LL.3	0.325 (0.093)	1.299 (0.374)	13.864 (4.04)
	New Polyps	D	0.17	1.115 (0.30)	NA	NA	NA	NA	NA
	Weight (D7-28)	D	<0.05	0.066 (0.019)	0.131 (0.038)	LL.4	0.083 (0.023)	0.085 (0.024)	0.090 (0.026)
	Width	D	<0.05	0.579 (0.15)	1.115 (0.30)	LL.3	0.142 (0.042)	0.223 (0.067)	0.478 (0.147)
	Length	D	0.21	1.115 (0.30)	NA	NA	NA	NA	NA
	Brightness	D	<0.05	0.288 (0.075)	0.579 (0.15)	LL.3	1.261 (0.339)	2.007 (0.541)	4.445 (1.202)
	Saturation	D	0.20	1.115 (0.30)	NA	NA	NA	NA	NA
	Tissue Chl-α	D	0.30	1.115 (0.30)	NA	NA	NA	NA	NA
	Tissue Phaeo.	D	0.28	1.115 (0.30)	NA	NA	NA	NA	NA
Diuron (µg/L)	Quant. Retraction	J	<0.001	2.9 (2.0)	15.8 (10)	LL.3	0.189 (0.104)	0.756 (0.447)	8.065 (5.409)
	$\Delta F/F_m$'	J	<0.001	NA	2.9 (2.0)	LL.3	0.013 (0.009)	0.057 (0.040)	0.675 (0.467)
	New Polyps	D	<0.05	NA	2.9 (2.0)	LL.4	2.177 (1.452)	2.450 (1.657)	3.000 (2.077)
	Weight (D7-28)	J	<0.001	NA	2.9 (2.0)	LL.3	<0.0001 (<0.0001)	0.00100 (0.00052)	0.0698 (0.0385)
	Width	D	<0.05	2.9 (2.0)	15.8 (10)	LL.4	1.921 (1.415)	2.216 (1.589)	2.793 (1.938)
	Length	D	0.22	NA	NA	NA	NA	NA	NA
	Brightness	D	<0.05	2.9 (2.0)	15.8 (10)	LL.4	10.529 (6.744)	11.813 (7.541)	14.379 (9.127)
	Saturation	J	<0.001	2.9 (2.0)	15.8 (10)	LL.4	3.079 (2.116)	4.185 (2.830)	7.072 (4.648)
	Tissue Chl-α	D	0.018	NA	2.9 (2.0)	LL.4	10.557 (6.767)	11.654 (7.471)	13.799 (8.789)

Table S18. Concentrations of particulate BP-3 during chronic exposure (n = 1).

Nominal Concentration (mg/L)	Avg. particulate (mg/L)	SD	% nominal	Avg. new Dissolved (mg/L)	% of new measured solutions
Control	0.000422	0.000333	NA	<LOQ	NA
S. Control	0.000324	0.000218	NA	<LOQ	NA
0.009	0.000429	0.000212	5%	0.046	0.5%
0.019	0.000365	0.000229	2%	0.127	0.2%
0.038	0.000506	0.00013	1%	0.252	0.1%
0.075	0.001826	0.001275	2%	0.556	0.2%
0.15	0.008661	0.013651	6%	0.797	1.7%
0.30	0.01028	0.014366	3%	2.148	0.7%

Table S19. Concentrations of new solutions of dissolved diuron in positive control (n = 10).

Nominal Concentration (µg/L)	Average Dissolved Concentration (µg/L)[a]	SD	% Nominal
2	2.9	0.2	145%
10	15.8	3.3	158%
50	63.0	4.8	126%

[a]Method recovery was 97%

Appendix C: Supplemental Figures

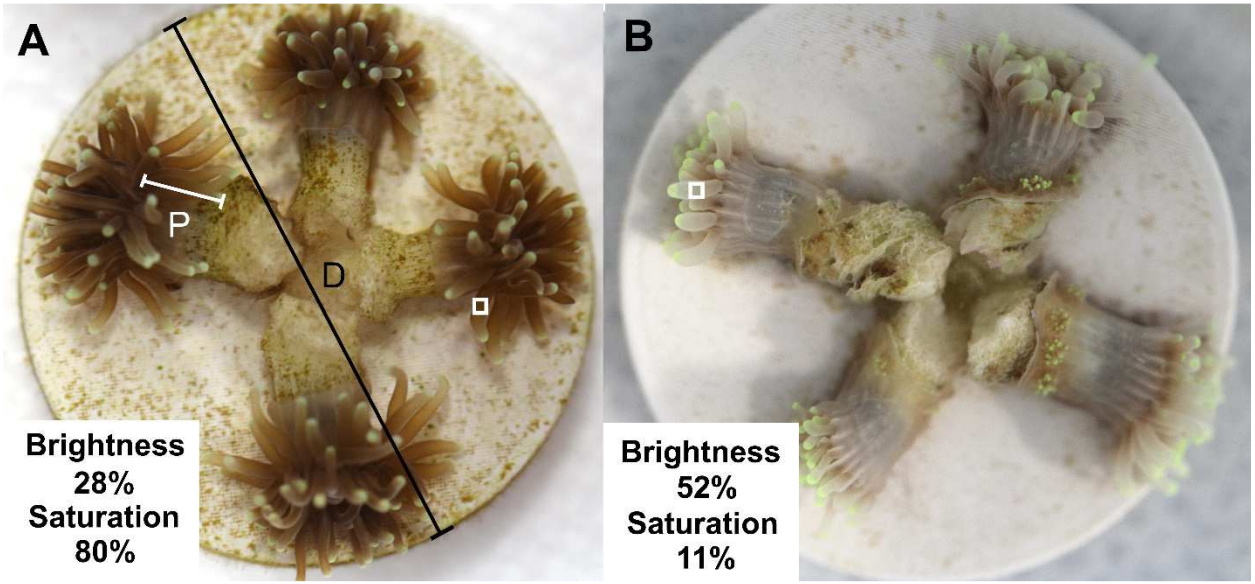

Figure S1. Diagram of image-based measurements using Adobe Photoshop using images of A; Day 0 control chip and B; Day 4 diuron concentration 2.5 mg/L chip. An image of the 4-star polyp on each poker chip was photographed daily. For polyp retraction measurements, panel A shows lengths of "P" (white line) or the tentacle length from origination to tip and "D" (black line) or the diameter of the poker chip (standard length) which were measured and recorded. Polyp extension/retraction was expressed as a ratio of P/D then multiplied by the poker chip diameter (39 mm) to determine the length of the polyp tentacles. Both panels A and B show selections used for bleaching determination (brightness and saturation, white boxes). As noted in the lower left hand box of each panel, a bleached polyp (panel B) has a higher brightness and lower saturation than an unbleached polyp (panel A). This also shows the lager variation in saturation between bleached and non-bleached (69%) than for brightness (24%) making saturation the more sensitive of the two measurements for bleaching determination.

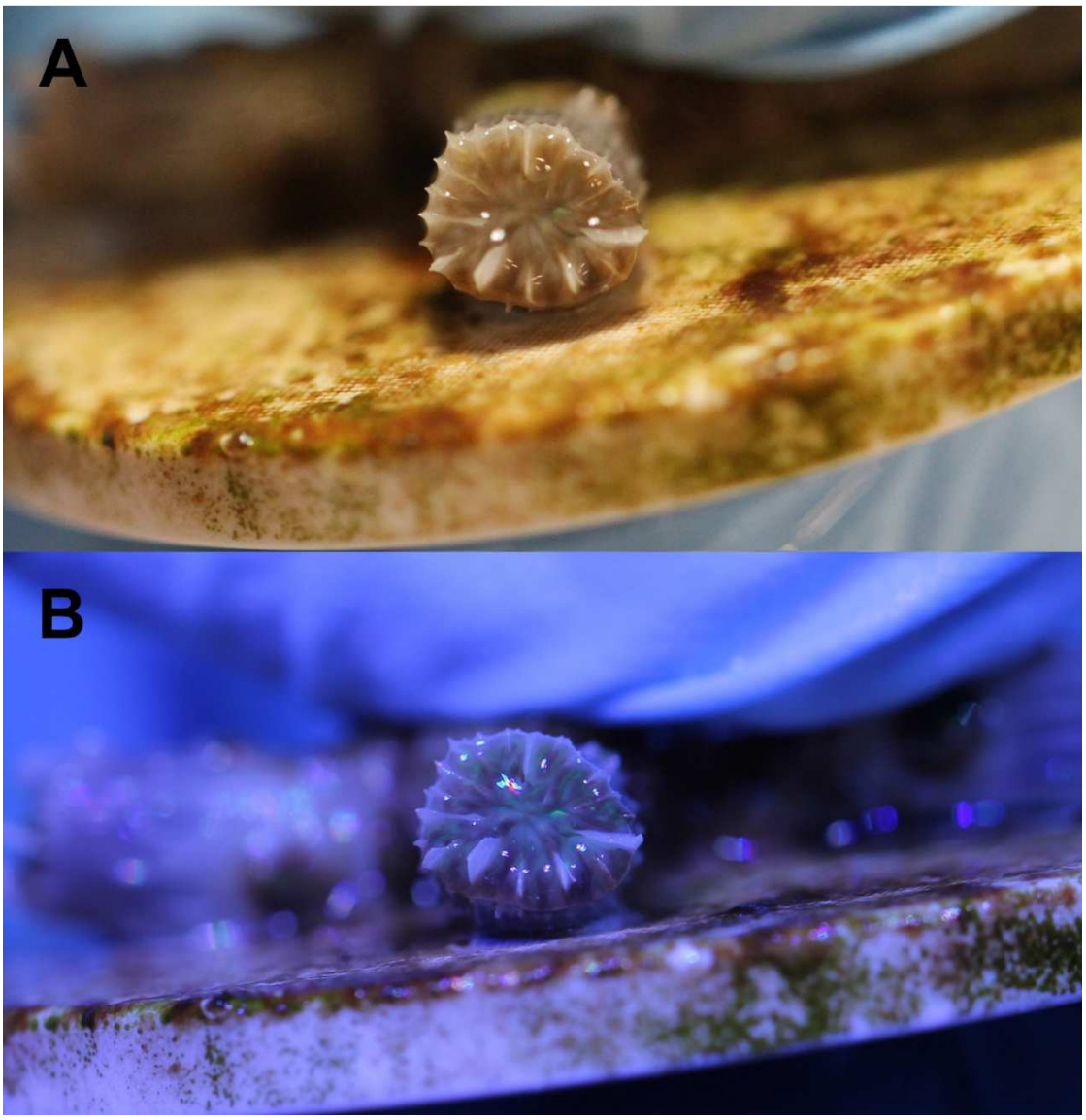

Figure S2. Comparison between polyp close to death under (A) ambient laboratory light and (B) full spectrum culture lighting. Holding the corals under full spectrum light allows for easier identification of dead coals. As can be seen in panel B, live corals that appear dead under ambient lighting show fluorescent green tips under full-spectrum (UV) lighting making intact tentacles more clearly identified.

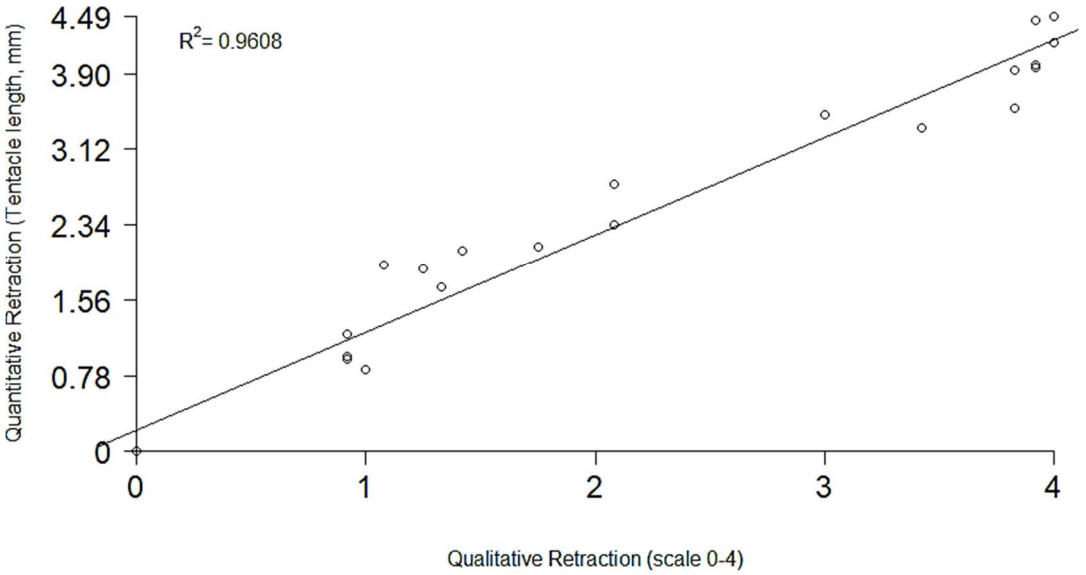

Figure S3. Correlation between qualitative retraction observations and quantification through polyp tentacle length measurement for BP-3 acute 3. Observed and measured polyp retraction were highly correlation (R^2 = 0.9608, p <0.001). Therefore, quantitative retraction was used for statistics moving forward to avoid any bias of the observer.

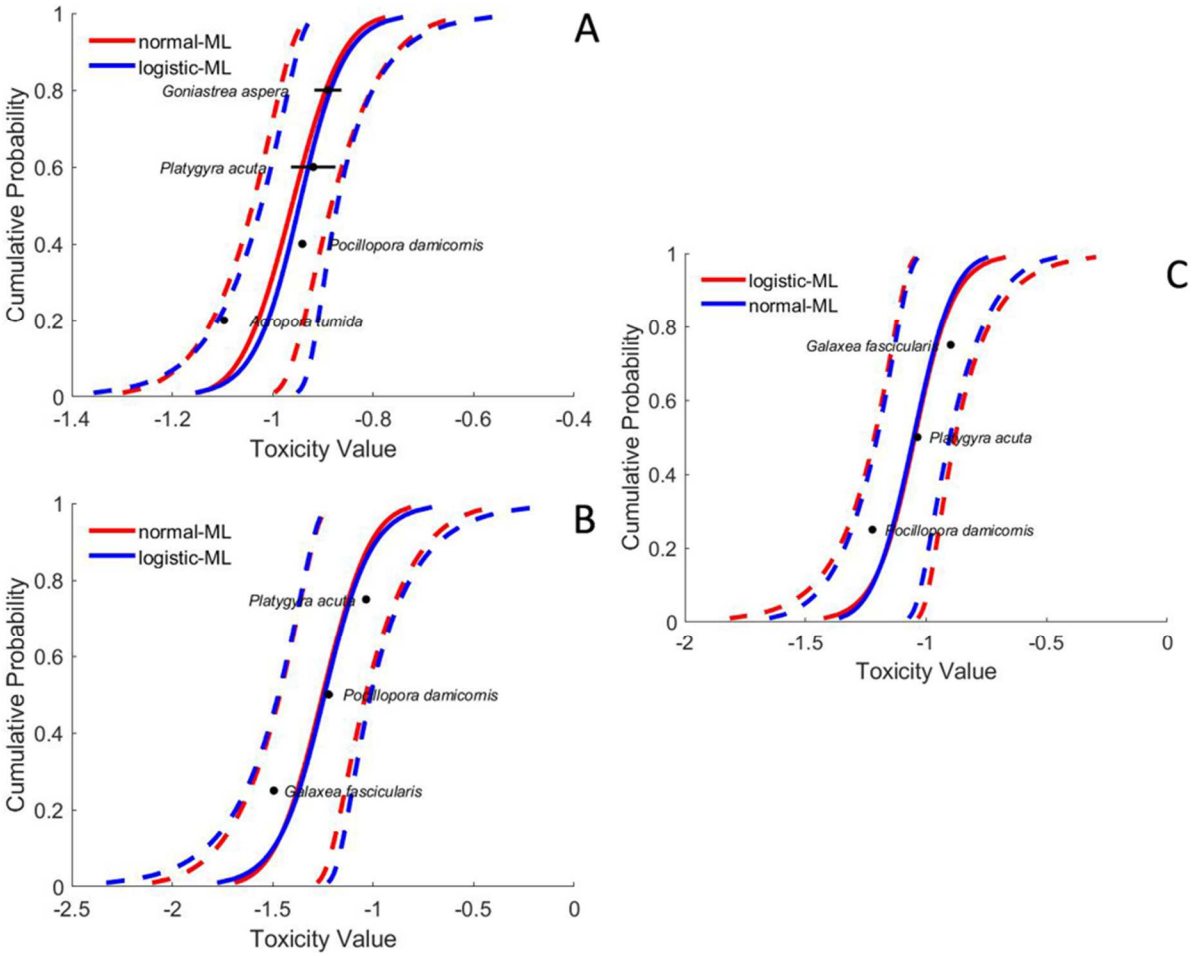

Figure S4. Species sensitivity distribution (SSD) for copper modeled using a normal and logistic model. A: SSD using copper results for 24h LC50. B: SSD using 96 h LC50s not including the results from these tests. This generally agrees with the 24 h LC50 SSD as to the relative position of *P. damicornis* and *P. acuta*. C: SSD using 96 h LC50 with our results. Including our results flips the sensitivity of *G. fascicularis* from most sensitivt to least sensistive species.

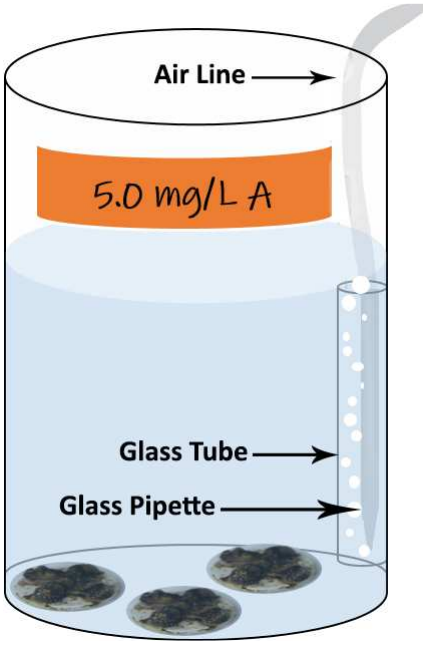

Figure S5. Diagram of beaker modified for bubble lift. A glass 2-liter beaker was modified for exposures by attaching a glass tube to the inner wall using food-grade silicone. Then, a glass pipette attached to an airline was inserted into the tube.

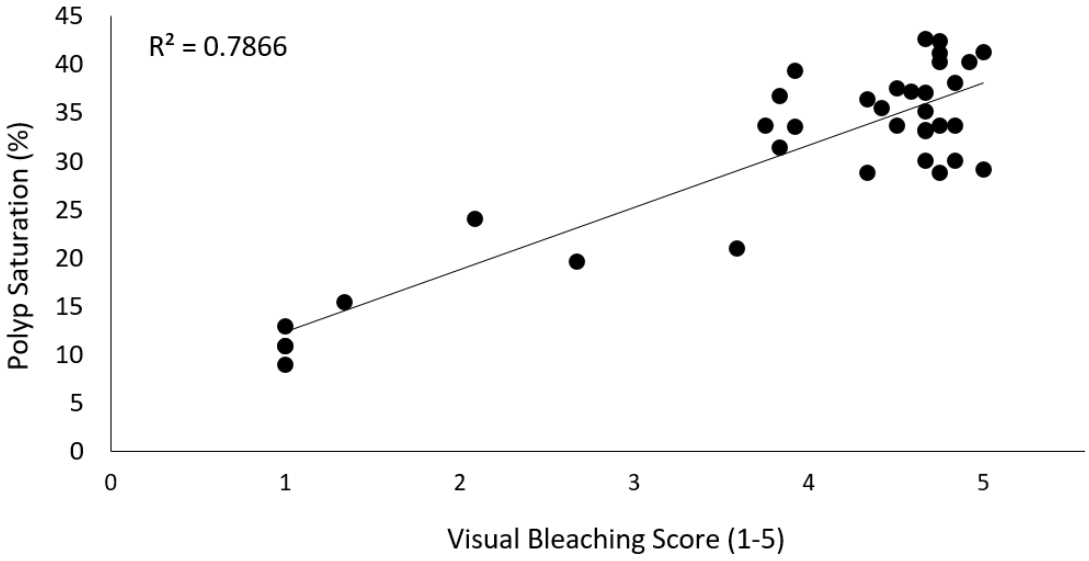

Figure S6. Correlation between visible and saturation bleaching in acute BP-3 testing. Visual bleaching and saturation correlated well ($R^2 = 0.7866$). Saturation was used as the mean bleaching endpoint to reduce observer bias.

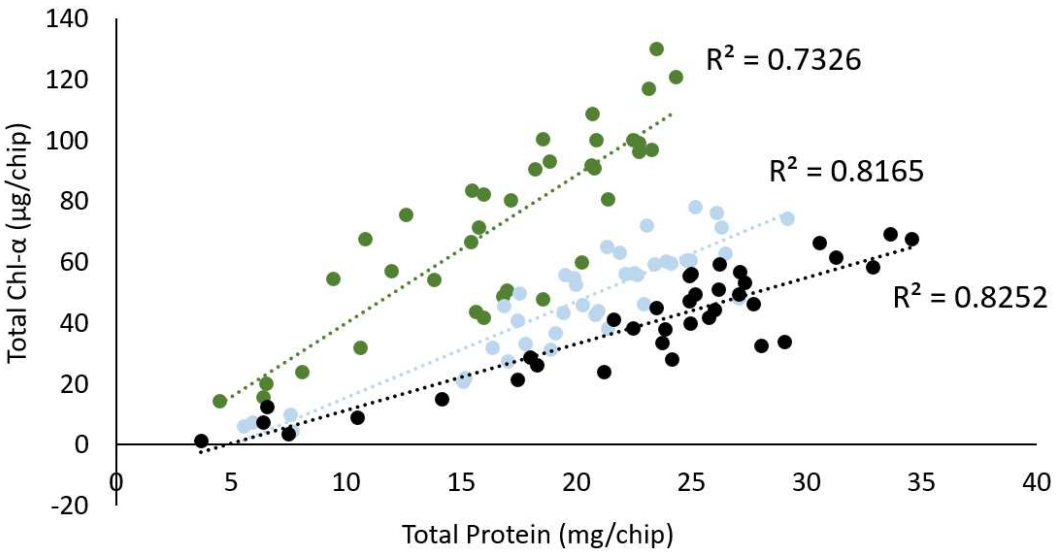

Figure S7. Correlation between total protein and total chlorophyll in coral tissue samples in acute BP-3 testing. All three acute BP-3 exposures demonstrated a high level of correlation between total protein and total chlorophyll-α. This suggests that most of the variation in chlorophyll content of these corals is dependent on the amount of tissue present and therefore the majority of bleaching seen is not due to symbionts leaving the organism, but from mortality-related tissue sloughing.

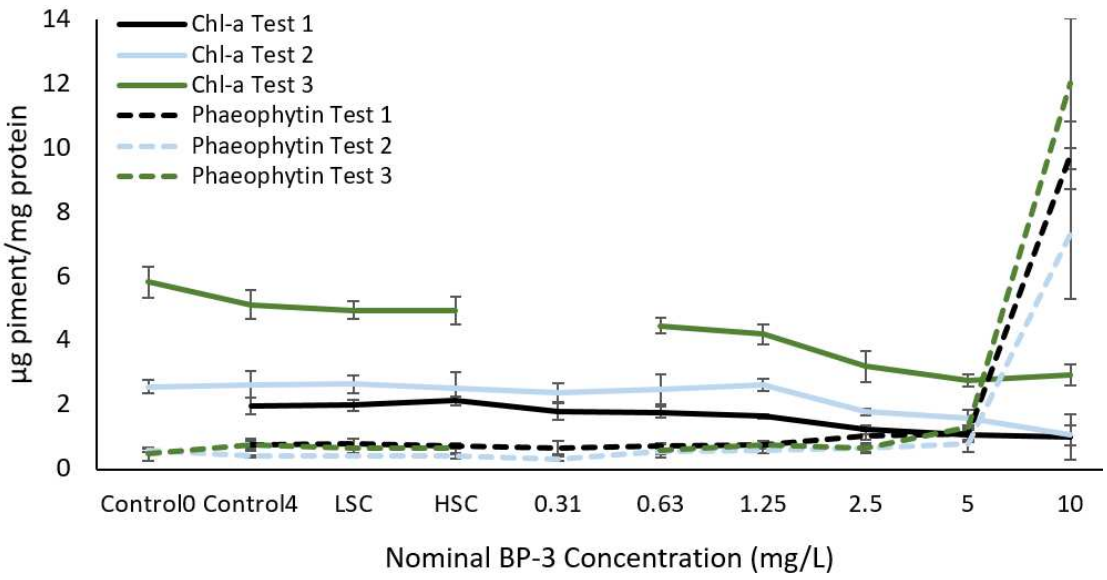

Figure S8. Protein-standardized photosynthetic pigments in coral tissue on day 4 of acute BP-3 testing. Standardizing pigments by protein removed much of the variation in a dataset and also lessened the impact of concentration on chl-α concentration. This, combined with the high correlation between protein and chl-α suggest chl- α loss is primarily driven by mortality-driven tissue loss. This also makes obvious the impact of BP-3 on the algal symbionts at 10 mg/L. It would seem due to the high concentration of phaeophytin that there is a large amount of damage to these symbionts and supports the observation of mortality at that concentration.

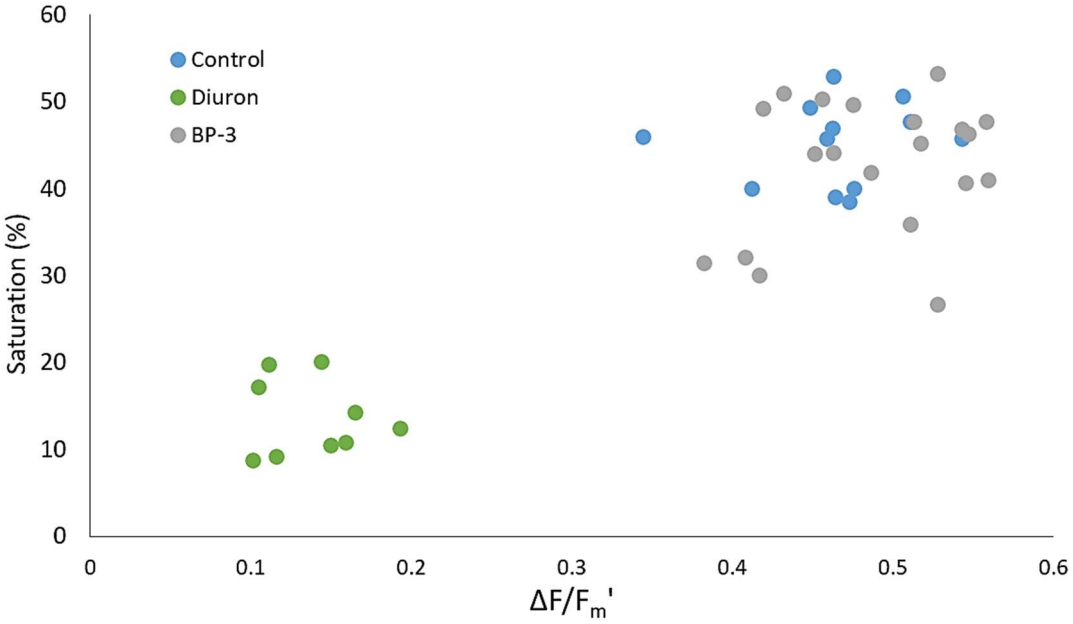

Figure S9. Association between $\Delta F/F_m$' and Saturation (Bleaching) in acute BP-3 testing. Control polyps (blue) generally have both high $\Delta F/F_m$' and saturation (%) signifying low bleaching. Exposure to BP-3 (gray) results in some low levels of bleaching at higher concentrations (saturation <40%) however the $\Delta F/F_m$' values remain at approximately 0.4 to 0.6. However, exposure to diuron, a photosystem II inhibitor, results in both severe bleaching (saturation <25%) and reduced $\Delta F/F_m$' (0.1 to 0.2).

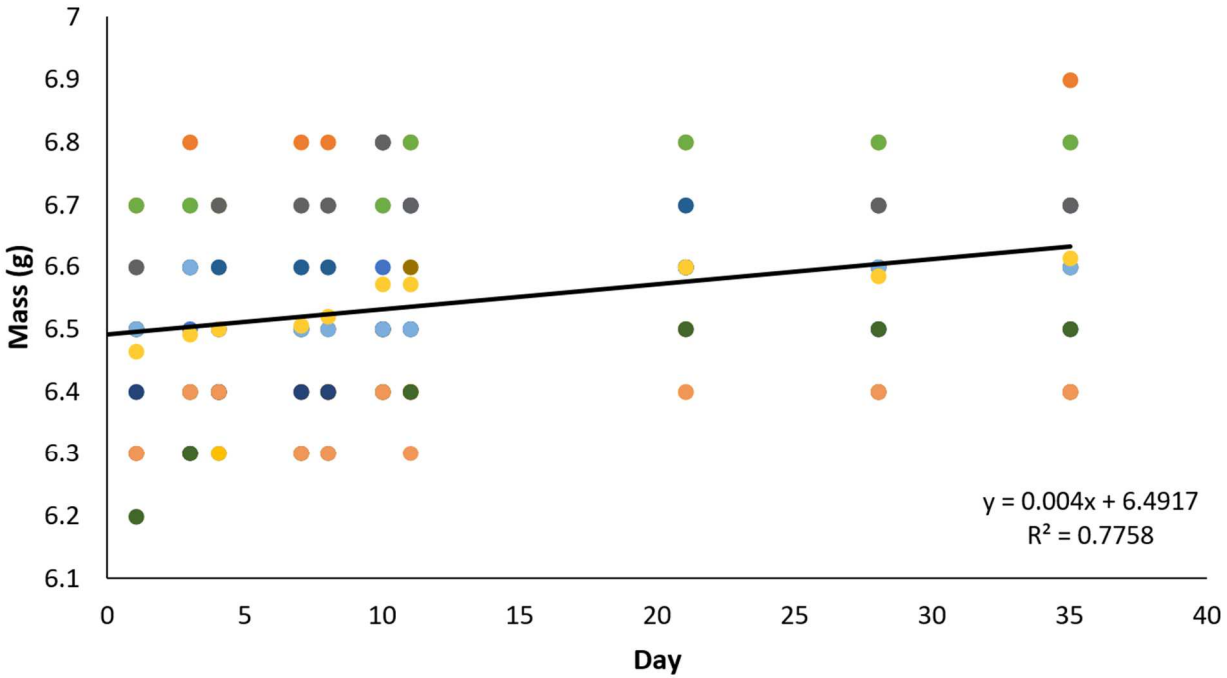

Figure S10. Results of growth rate study. Individual points are individual coral chip weights. Each chip was tracked individually over time therefore each color is a unique chip. Growth rate was seen to increase, on average, 0.004 g/day after 35 days of monitoring.

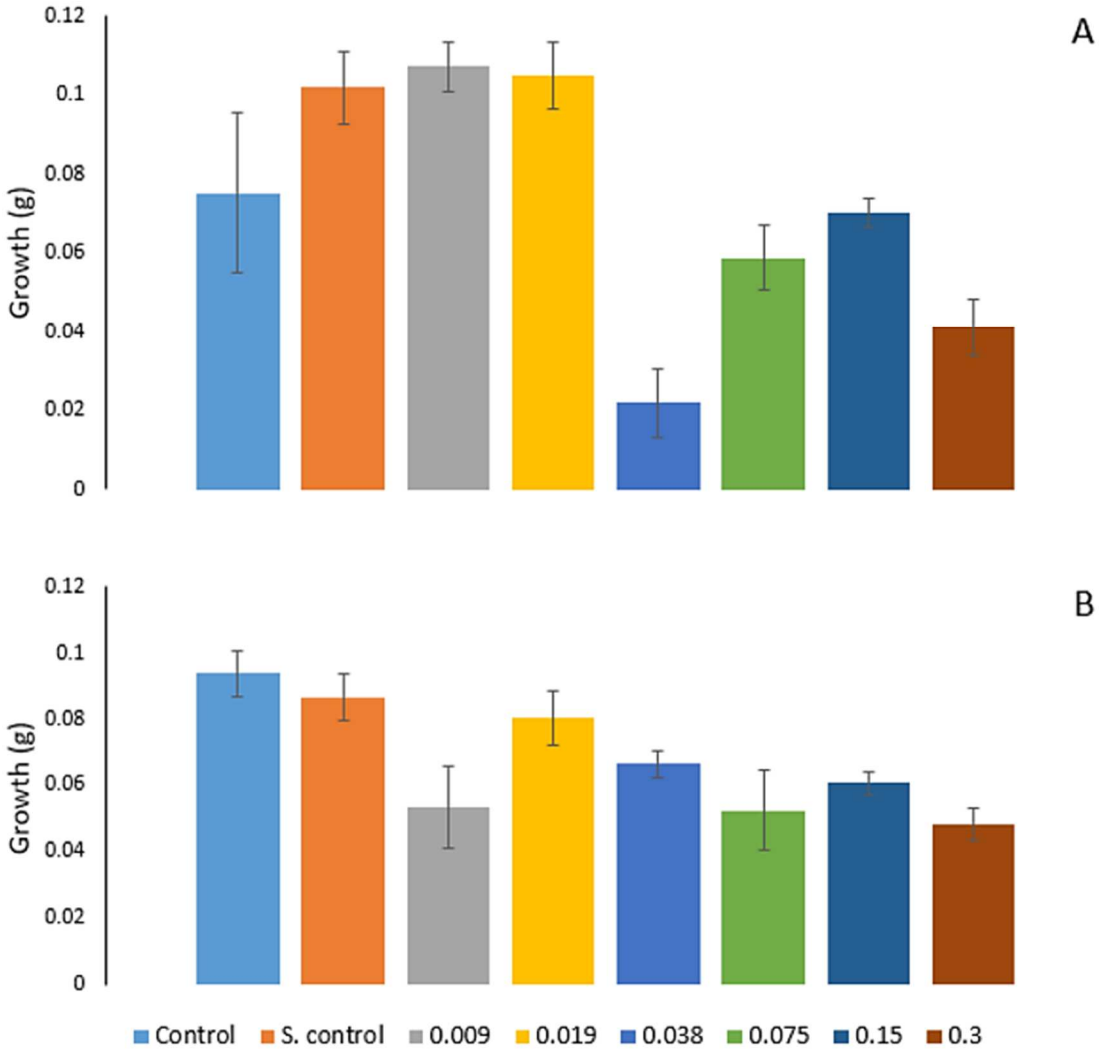

Figure S11. Growth Change at 28 Days from Day 0 (A) vs. Day 7 (B). Average growth per concentration is shown +/- standard deviation. Average growth in controls approaches 0.1 g/day in concentrations to 0.019 mg/L which was as expected from growth study. Variability assumed to be from hidden non-target organisms (worms, shrimp, etc.) was likely a factor on day 0 for many concentrations (A). Calculating growth from Day 7 shows a fairly clear dose-dependent response. Preliminary statistics shows the LOEC at either 0.038 or 0.075. Additional investigation is needed to determine the discrepancy between day 0 and day 7-derived growth for the 0.009 mg/L concentration.

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