UNIVERSITY OF CALIFORNIA SAN DIEGO

# Impacts of Organic and Inorganic Sunscreen Active Ingredients on the Photobiology of Sea Anemone *Anthopleura elegantissima*

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in

Marine Biology

by

Alana Rosen

Committee in charge:

Professor Martin Tresguerres, Chair
Professor James Day
Professor Jennifer Taylor

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The thesis of Alana Rosen is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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2022

# DEDICATION

This thesis is dedicated to my friends and family who have supported me through this journey. I dedicate this work to the dreamers and to those who dare to think of the possibilities. Always pursue your passion and explore your curiosity.

# EPIGRAPH

Don’t let anyone rob you of your imagination, your creativity, or your curiosity. It’s your place in the world; it’s your life. Go on and do all you can with it, and make it the life you want to live.

Mae Jemison

Every time I slip into the ocean, it’s like going home.

Sylvia Earle

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# LIST OF ABBREVIATIONS

PSII: photosystem II

$F_v/F_m$: maximum photosynthetic efficiency

$F_v$: variable fluorescence

$F_m$: maximum fluorescence

$F_o$: minimum fluorescence

$\Delta F$: variable fluorescence of EQY

$F_m'$: maximum fluorescence of EQY

GFP: green fluorescent protein

BP-3: benzophenone-1 3; oxybenzone

ROS: reactive oxygen species

ZnO: zinc oxide

$TiO_2$: titanium dioxide

PAM: pulse amplitude modulation

N: replicate number

DMSO: dimethyl sulfide

MQY: dark acclimated maximum quantum yield

EQY: light acclimated effective quantum yield

$Q_m$: pressure over photosystem II

ICP-MS: inductively coupled plasma-mass spectrometry

GC-MS: gas chromatography-mass spectrometry

SPE: solid phase extraction

SIM: scanning ion mode

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ABSTRACT OF THE THESIS

Impacts of Organic and Inorganic Sunscreen Active Ingredients on the Photobiology of Sea Anemone *Anthopleura elegantissima*

by

Alana Rosen

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Professor Martin Tresguerres, Chair

Sunscreen products contain chemicals such as oxybenzone or minerals such as titanium dioxide ($TiO_2$) and zinc oxide (ZnO), which are a threat to marine biota and especially to coral reefs. Mineral sunscreens are being marketed as “reef-safe”; however, their impacts on marine organisms are not well understood. The aim of my thesis was to evaluate the effects of oxybenzone, $TiO_2$, and ZnO on photophysiological properties of sea anemone *Anthopleura elegantissima*. This invertebrate belongs to the phylum Cnidaria and is a close relative to reef-building corals. Anemones were exposed to three environmentally relevant concentrations of oxybenzone, $TiO_2$, and ZnO (nominally, 0.01 μg/L, 0.1 μg/L, and 1 μg/L) for 21 days, followed

by seven days of recovery in the absence of sunscreen ingredients. Throughout this 28-day period, measurements of fluorescence intensity and photosynthetic efficiency were taken as proxies for anemone photophysiology. The sunscreen ingredients did not seem to affect either parameter over the course of the experiment. However, analyses of seawater samples using inductively coupled plasma-mass spectrometry (ICP-MS) revealed prominent mismatches in the concentrations of $TiO_2$ and ZnO compared to the target values, including much higher concentrations in the controls than in treatment samples. Furthermore, gas chromatography-mass spectrometry (GC-MS) analysis failed to detect oxybenzone. Overall, the unexpectedly high $TiO_2$ and ZnO concentrations measured in the experimental aquarium coupled with oxybenzone analysis issues prevented reaching conclusions about their potential toxicity on anemone photophysiology. These factors must be considered for future studies aiming to assess potential toxic effects of sunscreen compounds on marine life.

## Introduction

### *Sunscreen active ingredients*

Productive ecosystems such as coral reefs are facing severe threats from environmental and anthropogenic stress. A primary concern for seawater chemistry is the potential detrimental impacts of sunscreen pollutants on marine life. Coastal and marine tourism are a growing and expanding industry with the development of water activities in highly productive ecosystem areas. It is estimated that 25% of sunscreen applied on skin washes off during aquatic activities (McCoshum et al. 2016). Chemical sunscreens are commonly manufactured with organic UV filters such as oxybenzone (benzophenone-1 3 (BP-3)), octinoxate, homosalate, and avobenzone (Barone et al. 2019). These filters absorb UV rays and convert them into heat energy that can be released out from the skin (Solish et al. 2020). Chemical sunscreen active ingredients have been studied and publicized for their potentially harmful impacts on coral species.

As alternatives to chemical sunscreens, so-called "eco-friendly" mineral sunscreens are becoming more widely manufactured using inorganic zinc oxide (ZnO) and titanium dioxide ($TiO_2$) nanoparticles that scatter away and block UV rays (Solish et al. 2020). These inorganic minerals are labeled as "reef friendly" because they reside on the skin and physically deflect the UV rays, and therefore are hypothetically less likely to absorb into organisms and damage them. There are two types of mineral formulations used in sunscreens: nano form, particles smaller than 100 nanometers, and non-nano form, particles larger than 100 nanometers. The smaller particles get absorbed into the skin more easily, whereas the larger particles sit on the skin blocking the potentially harmful rays (Solish et al. 2020). Nano zinc oxide is used more commonly in sunscreen products because it leaves less of a white cast on the skin (Wong et al. 2010). However, these minerals were proposed to have potential harmful impacts as well.

There are few studies on the impacts of sunscreen UV filters on marine life, most of which are corals (Danovaro et al. 2008; Downs et al. 2016; Wijgerde et al. 2019; McCoshum et al. 2016). Like reef-building corals, sea anemones belong to the phylum Cnidaria. However, anemones are easier to keep in the laboratory and do not have a calcium carbonate skeleton that complicates many experimental techniques. As a result, anemones are often used as the cnidarian model organism of choice for studies about symbiosis, photophysiology, and toxicology (Howe et al. 2012). Sea anemones play important roles in coral reef communities as they act as homes for various marine organisms. Anemones are commonly found in coastal and tourist areas, and it is necessary to understand the potential consequences of pollution in these environments (Howe et al. 2012).

***Biology of sea anemones***

*Anthopleura elegantissima*, also known as the clonal anemone (originally described by Brandt in 1835 as *Actina xanthogrammica*), is an aggregating sea anemone commonly found on rocky substrates in the intertidal zone along the west coast of North America, from Alaska to Baja California (Morris et al. 1981). *A. elegantissima* can reproduce both asexually through longitudinal binary fission that creates aggregating clones, and sexually through broadcast spawning (Hossfeld et al. 2020). This species typically reaches adult size two years after settlement and grows to an average diameter of 2.5-4 cm (Piazzola et al. 2015).

Sea anemones have an oral disc with tentacles sprawled out and a pedal disc that attaches to its substrate. *A. elegantissima's* tentacles feature variable-colored tips that normally contain a green colored column. The green color mostly comes from pigment cells the anemone produces for protection against UV rays (Piazzola et al. 2015) (Figure 1). In addition, *A. elegantissima*

hosts endosymbiotic dinoflagellate algae that provide a golden-brown color. The anemones ingest the algae from the seawater into their endodermal cells and provides them with inorganic nutrients and shelter; meanwhile, the algae provide products of photosynthesis to the anemone host. The algal species most commonly found establishing endosymbiotic relationships with cnidarians, including *A. elegantissima*, belongs to the family Symbiodiniaceae (Schwarz et al. 2002).

***Cnidarian-Symbiodiniaceae symbiosis***

The Cnidarian-Symbiodiniaceae symbiosis is vulnerable to environmental changes and exposure to stressful conditions can result in the expulsion of the algae causing the animal to turn a white color. This phenomenon is known as "bleaching," and it has been reported to happen in response to changes in sea surface temperature, light intensity and salinity, as well as increased sedimentation and presence of pollutants (Fitt et al. 1982; Hoepner et al. 2019; Brown 1997). Extreme changes in light intensity can generate photodamage in the algae and oxidative damage in the coral host cells resulting in bleaching as well (Roth et al. 2010). Similarly, sea anemones, when exposed to high temperature or increased light conditions may egest their symbionts and lose their color (Norin et al. 2018). Since the symbiotic algae provide a significant source of nutrients to the cnidarian host, bleaching can have devastating consequences including death. Previous experiments involving light manipulations resulted in organisms releasing symbiotic algae through expulsion. For example, exposure of *A. elegantissima* to darkness for nine weeks resulted in a decrease in the number of endosymbiotic algae, and by 15 weeks, some anemones were completely devoid of symbiotic algae (Buchsbaum 1968). This experiment provides

evidence that sea anemones may expel their symbiotic algae when exposed to extreme changes in light intensity.

Changes in seawater temperature can also negatively impact the cnidarian-algae symbiosis. When exposed to extreme increases in temperatures 10-20ºC above normal (13-15ºC), sea anemones had little reduction in symbiotic algae after 4 weeks at 25ºC, however those at 30-32ºC egested all symbiotic algae (Buchsbaum 1968).

Damage to the symbiotic relationship can also impact photosynthetic efficiency of the symbiont. Symbiotic algae carry out photosynthesis in photosystem I and II (PSI and PSII) (Roth et al. 2010). Under normal conditions, the symbionts carry out photosynthesis efficiently, however, under extreme changes in conditions, this PSII efficiency may be reduced due to the expulsion of symbiotic algae (Brown 1997). Roth et al. (2012) found that both cold (21ºC) and heat (31ºC) compared to control (26ºC) resulted in a decrease in maximum photosynthetic efficiency of PSII in corals, suggesting stress on the symbiotic algae photosynthetic system. These results support the use of changes in photosynthetic efficiency of photosystem II as a practical proxy of symbiont health.

When sea anemone symbionts are under extreme changes in environmental conditions, their PSII becomes less efficient at the process of utilizing light energy (Wijgerde et al. 2019). Endosymbionts acclimate to changing light levels through either photoacclimation or photoprotection (Warner et al. 2002). Photoacclimation refers to the physiological response to changes in the light environment, and can include increases in photosynthetic pigment concentrations, photosynthetic efficiency of PSII, and dinoflagellate density.

***Fluorescent proteins***

There are many mechanisms that evolved to protect the photosystem from elevated conditions. Green fluorescent proteins (GFP) were first found in jellyfish and subsequently other cnidarians, including corals and sea anemones (Roth et al. 2010). Although the physiological functions of FPs are not well understood, hypotheses include photoprotection, photosynthesis enhancement, and camouflage (Roth et al. 2010). The most commonly accepted hypothesis is that FPs protect the organism from excess light by dissipation of energy (Leutenegger et al. 2007).

FPs absorb high energy UV/blue light and re-emit light at a lower energy, typically green fluorescence (Roth et al. 2013). This phenomenon of biofluorescence has scarcely been studied in sea anemones, however there are numerous studies on corals. Roth et al. (2010) exposed the coral *Acropora yongei* to changes in light intensity to examine the impacts on green fluorescence intensity and GFP concentration. Corals exposed to either low light (30 μmol quanta $m^{-2}s^{-1}$), medium control light (300 μmol quanta $m^{-2}s^{-1}$), or high light (900 μmol quanta $m^{-2}s^{-1}$) exhibited increased GFP concentration (~1.6x) and green fluorescence intensity (~1.9x) with increased light intensity compared to control light, and decreased GFP concentration (~4x) and green fluorescence intensity (~1.9x) with reduced light intensity compared to control light. The positive correlation between light intensity and both GFP concentration and fluorescence intensity suggests that GFPs may have a photoprotective function.

A similar study was performed with increased and decreased temperature treatments to further explore whether other sources of stress may impact physiological function in corals. Roth et al. (2013) examined the effects of both cooling and warming temperatures on the coral *Acropora yongei* and found that the GFP concentration and fluorescence intensity decreased

under both conditions. GFP concentration in the cold treatment (-5ºC) decreased by 91% compared to the control, while green fluorescence decreased by 79%. Corals showed signs of GFP concentration recovery in both treatments towards the end of the experiment, however, the heat-treated corals eventually bleached. Green fluorescence was positively correlated with dinoflagellate photosynthetic efficiency, and they concluded that green fluorescence could be used as a proxy for coral health.

***Sunscreen effects on marine organisms***

In addition to temperature and light stress, one of the greatest threats to productive ecosystems is pollution and contaminants in the ocean, including sunscreen. One study on the impacts of sunscreen ingredients on *Acropora* corals found that exposure to oxybenzone at concentrations of 10, 33, 50 and 100 μL/L resulted in increased viral infections and bleaching (Danovaro et al. 2008). Viral abundance surrounding the coral branches increased by a factor of 15 compared to the controls. The study also established that higher oxybenzone concentrations (50 μL/L and 30 μL/L) resulted in faster bleaching rates. They concluded that sunscreen UV filters stimulate viral infections and have a rapid bleaching effect on hard corals and also postulated that UV filters like oxybenzone can have potentially damaging impacts even at lower concentrations than those tested (Danovaro et al. 2008). Downs et al. (2016) exposed coral planula larvae to oxybenzone at concentrations of 0.01 μmol/L, 0.1 μmol/L, 1 μmol/L, 10 μmol/L, 100 μmol/L, and 1000 μmol/L to determine its toxicological effects. They reported that oxybenzone disrupted skeletal development and induced deformations in the planula in a dose-response fashion (Downs et al. 2016). Additionally, McCoshum et al. (2016) observed the effect of sunscreen containing multiple ingredients (homosalate, oxybenzone, octocrylene, octisalate,

and avobenzone) at concentrations of either 0 or 1.0 mL on various organisms, including flatworms, diatoms, anemones and corals. All organisms displayed lower growth rates when exposed to sunscreen compared to controls. The authors concluded that organisms near populated tourist areas are at risk of population decline, and that these sunscreen active ingredients negatively impact all of the studied species.

Reactive oxygen species (ROS) are highly reactive compounds that are hypothesized to be produced due to damaged photosynthetic systems in the symbiont, and cause damage to the host cell as well. The accumulation of ROS can lead to oxidative damage in cells and result in bleaching of corals (Smith et al. 2005). ROS are produced naturally during the photosynthesis process, however, when the production of ROS is higher than the rate of detoxification by enzymes through the antioxidant system, ROS can lead to cellular damage including membrane damage, protein oxidation, and DNA degeneration (Nielsen et al. 2018). Sunscreen ingredients, like oxybenzone, may also increase the accumulation of reactive oxygen species (ROS) in the presence of UV radiation (Wijgerde et al. 2019). However, literature on the production and role of ROS is limited.

### *Alternative sunscreen impacts*

Nano forms of both $TiO_2$ and ZnO may produce harmful ROS when exposed to UV radiation (Maipas & Nicolopoulou-Stamati 2015), however, many $TiO_2$ nanoparticles are often coated with silica, magnesium, or aluminum and may produce less ROS (Lewicka et al. 2013). Corinaldesi et al. (2018) hypothesized that uncoated ZnO and two types of $TiO_2$ nanoparticles would negatively affect *Acropora* spp. through its symbiotic algae. They tested this hypothesis by exposing the coral to a concentration of 6.3 mg/L of different UV filters and found that ZnO

exposed corals produced the greatest number of symbiotic algae released; however, $TiO_2$ exposure similarly caused a higher symbiont release rate than the control. All UV filter exposure treatments changed the interaction between coral and symbiont and induced immediate coral bleaching with ZnO, whereas the $TiO_2$ exposure did not cause bleaching. Thus, both mineral filters could have damaging impacts, though when $TiO_2$ is used alone, those effect could be reduced.

Few studies have distinguished between the effects of chemical and mineral sunscreens on marine organisms. Barone et al. (2019) compared the effects of a non-nano-$TiO_2$ based sunscreen to an oxybenzone based sunscreen on clownfish mortality, feeding behavior, and swimming behavior. Following exposure to a series of sunscreen concentrations (0 mg/L, 1 mg/L, 3 mg/L, 10 mg/L, 30 mg/L, and 100 mg/L) clownfish experienced a 25% increase in mortality at the highest concentration (100 mg/L) of oxybenzone and an increase of 6.7% mortality at the highest concentration (100 mg/L) of the $TiO_2$ along with abnormal feeding behavior. The oxybenzone-based sunscreen at 100 mg/L also resulted in 100% feeding failure and 100% abnormal swimming behavior, whereas the $TiO_2$ sunscreen resulted in 26.7% abnormal swimming movement (Barone et al. 2019). This study provided evidence that oxybenzone-based sunscreen has a more negative effect on crucial fish behaviors than the $TiO_2$ sunscreen.

Chemical and mineral filters may also directly affect symbiotic algae photosynthesis to different extents. Fel et al. (2019) examined the effects of both ZnO and inorganic filters at concentrations ranging from 10-5000 μg/L on the photosynthetic efficiency of coral symbionts to test for chronic effects of sunscreen ingredients. ZnO had the greatest damage on the photosynthetic efficiency at a concentration of 90 μg/L, decreasing maximum photosynthetic

efficiency by 38% compared to controls. The chemical UV filters octocrylene and avobenzone exhibited a significant decrease in maximum photosynthetic efficiency at concentrations of 1300 μg/L and 87 μg/L, respectively. It was concluded that chemical filters have either no effect or only impact the symbiotic algae PSII at higher concentrations, and that mineral UV filters, such as ZnO, may present toxic consequences to marine organisms.

***Challenges to environmental sunscreen research***

There is a consensus among the limited research thus far that sunscreen contaminants have overall negative impacts on cnidarians. Oxybenzone is regarded as the most harmful chemical compound to cnidarian physiology, however, $TiO_2$ and ZnO may also have harmful impacts. Although previous studies provide examples of sunscreen ingredients causing changes to the overall health of organisms, many of these studies were not repeated to establish consistent results and did not follow a standard protocol or experimental design, making it particularly challenging to compare results (Wijgerde et al. 2019; Corinaldesi et al. 2018; Fel et al. 2019). Corals and sea anemones are nonstandard ecotoxicological test species, meaning there are no standardized guidelines for toxicity tests (Mitchelmore et al. 2021). Length of studies, exposure levels (acute vs chronic), concentrations used, species used, and endpoints measured vary considerably between studies (Mitchelmore et al. 2021). Downs et al. (2016) focused on the larval form of *Stylophora pistillata*, whereas Wijgerde et al. (2019) used the adult form of *Stylophora*, and Danovaro et al. (2008) tested *Acropora* species. Additionally, most of the previous literature focused on corals as a test species. The lack of previous studies and variability of experimental designs makes it challenging to support results.

Measuring environmental concentrations of sunscreens in seawater is particularly challenging because water conditions are highly variable due to sampling location, time, depth, sample collection method, and analysis method (Mitchelmore et al. 2021). Yung et al. (2015) compiled data sets from various sources to describe environmentally relevant concentrations of ZnO nano particles, and found the concentrations varied through surface water between 0.0001 μg/L-76 μg/L. Bratkovics et al. (2015) measured annual average oxybenzone concentrations for six sites along South Carolina, USA beaches and found that concentrations varied by site between 0.07 μg/L- 0.6 μg/L. Surface waters around Majorca Island in the Mediterranean have been measured having concentrations of $TiO_2$ in the range of 0.7-38 μg/L (Tovar-Sánchez et al. 2013). There is high variability of these UV filters depending on sampling location. There may be different concentrations of certain active ingredients in areas depending on sampling depth, time of sampling, or distance from shore (Mitchelmore et al. 2021). Therefore, the concentration of sunscreen compounds in the environment is bound to be highly variable, and consequently, have different impacts on organisms based on location.

***Research objectives and hypotheses***

The objective of my thesis was to investigate the impacts of sunscreen chemical and mineral active ingredients on sea anemone photobiological properties. My overarching hypothesis was that exposure to oxybenzone, $TiO_2$ and ZnO will induce decreases in both fluorescence intensity and photosynthetic efficiency.

There is an increasing amount of pollutants in the seawater, however, their impacts on important ecosystems are poorly understood. Both green and red fluorescence intensity and photosynthetic efficiency were used as metrics to compare the effects of various concentrations

of sunscreen active ingredients on the photophysiology of the anemone *Anthopleura elegantissima*. I was particularly interested in comparing the effects of the chemical UV filter oxybenzone to those of the "eco-friendly" mineral filters, $TiO_2$ and ZnO.

My specific hypotheses were: 1) sunscreen active ingredients will negatively impact photosynthetic efficiency and fluorescence intensity of sea anemones and symbiotic algae in a dose-response manner, 2) these parameters will be most affected by oxybenzone compared to other ingredients, 3) these parameters will negatively correlate with concentration of each of the three compounds, 4) the negative effects will progressively become worse under continuous exposure to sunscreen ingredients, and 5) once the anemones are placed in clean seawater without sunscreen active ingredients, fluorescence intensity and photosynthetic efficiency will recover to control levels within one week.

Aside from the fundamental understanding of the toxicological pathways sunscreen compounds may have on biofluorescence, an important question to explore is whether or not changes in this property over time can be used as a proxy for anemone and coral reef health.


Figure 1. Image of *A. elegantissima* from the fluorescent imaging stereoscope taken with an excitation of 470 nm (A) and under white light (B) with exposure times of 600 ms and 500 ms.

## Methods

### *Sea anemone maintenance*

Sea anemones *A. elegantissima* were obtained from a laboratory at the University of California Santa Barbara and kept in a tank with a flow through system of seawater coming from the Scripps Pier in the Scripps Institution of Oceanography Hubbs Hall experimental aquarium for six months prior to the experiment. The sea anemones were fed one scoop of AP100 Dry Larval Diet mixed into the seawater twice a week before the experiment began.

### *Experimental set-up*

Individual sea anemones (N= 55) were attached to separate ceramic tiles (1.5 x 1.5 inch) and placed in glass jars (3.5 x 3.5 inch) filled with 400 mL of seawater flowing in from the Scripps Pier to the Hubbs Hall experimental aquarium. The glass jars were distributed by treatment among three plastic trays and placed on an enclosed water table to keep the seawater at the ambient temperature range of 12-21ºC. Each jar had an aerator tube to maintain oxygen levels, and the seawater in each jar was changed daily to sustain a closed system with no flow through. Photoperiod was a 12h light:12h dark system to imitate a natural photoperiod in an enclosing box using a T5-HO Fluorescence Light with two 6000K bulbs and two Aqua Blue bulbs.

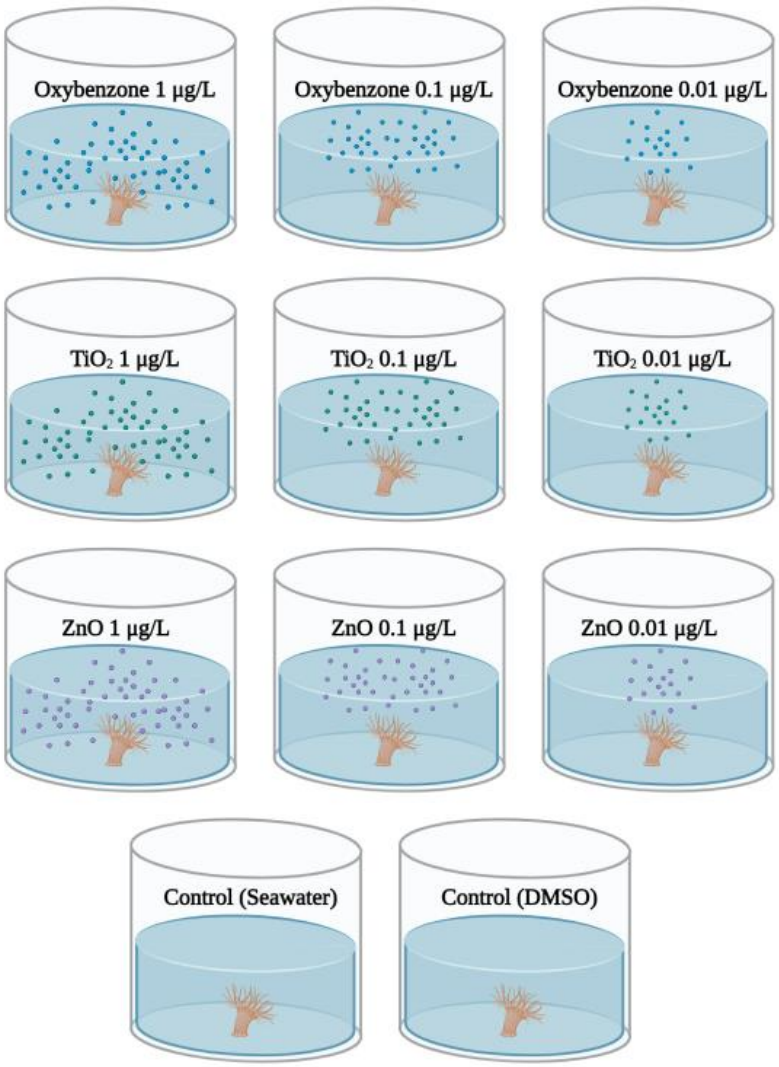


Figure 2. Experimental set up of eleven treatments. Each jar represents one of five replicates of the treatment. Each jar contains 1 anemone, for a total of 55 anemones.

***Exposure to sunscreen active ingredients***

The experiment entailed a 21-day experimental exposure period and a 7-day recovery period. Anemones were exposed to nine sunscreen treatments, with five replicates each (n=5) (Figure 2). The sunscreen ingredients were oxybenzone, $TiO_2$ and ZnO, and the concentrations were chosen to reflect previous levels measured in the environment (0.01 µg/L, 0.1 µg/L, and 1 µg/L) (Bratkovics et al. 2015; Sánchez Rodriguez et al. 2015, Sang et al. 2016; Tsui et al. 2014;

Wijgerde et al. 2019). The experiment also included two control treatments that did not receive any sunscreen ingredients; however, one of the two controls was dosed with 0.005% dimethyl sulfide (DMSO) to match the amount of DMSO used to dissolve the oxybenzone.

$TiO_2$ and ZnO were obtained from Millipore Sigma in the form of <25 nm and <50 nm particle size nanopowders, respectively. Oxybenzone (2-hydroxy-4-methoxybenzophenone) was obtained from Fisher Scientific. Stock solutions were made weekly by adding 50 mg of each sunscreen active ingredient to 100 mL of MilliQ water ($TiO_2$ and ZnO) or DMSO (oxybenzone) to achieve a 5 x $10^5$ μg/L dilution. A Millex-hv 0.45 μm filter and syringe were used to take out 20 mL of the dilution and combine with 80 mL of fresh seawater for a 1 x $10^5$ μg/L dilution. Seawater for each experimental treatment was prepared daily by serial dilutions of the stocks to achieve final concentrations of 0.01, 0.1, and 1 μg/L. The $TiO_2$ and ZnO stock solutions were placed in a sonicator water bath for 30 minutes during initial preparation and for 10 minutes prior to serial dilutions to ensure $TiO_2$ and ZnO dissolution. The stock solutions and serial dilutions were covered in aluminum foil to avoid photodegradation and kept in room temperature.

***Photosynthetic efficiency***

An Underwater Diving-PAM-II Chlorophyll Fluorometer (Heinz Walz GmbH Germany 2018) was used to measure PSII dependent photosynthetic efficiency of the algal symbionts on days 0, 3, 7, 10, 14, 17, 21 (during exposure to sunscreen ingredients), and on day 25 (recovery) (Figure 3). These measurements were made under dark and light conditions to obtain the dark-acclimated maximum quantum yield (MQY) and the light-acclimated effective quantum yield (EQY). Under dark conditions, the minimum fluorescence ($F_0$) and maximum fluorescence ($F_m$) were measured. Under light conditions, the variable fluorescence (ΔF) and maximum

fluorescence ($F_m$') were measured to calculate the effective and maximum quantum PSII yield. The MQY ($F_v/F_m$) was measured pre-dawn during the dark phase of the photoperiod to prevent light exposure on symbiotic algae. The EQY ($\Delta F/F_m$') was measured mid-day during the light phase of the photoperiod with the aquarium lights on. The pressure over PSII was measured to compare the MQY and EQY: $Q_m = 1-[(\Delta F/F_m')/ F_v/F_m)]$.

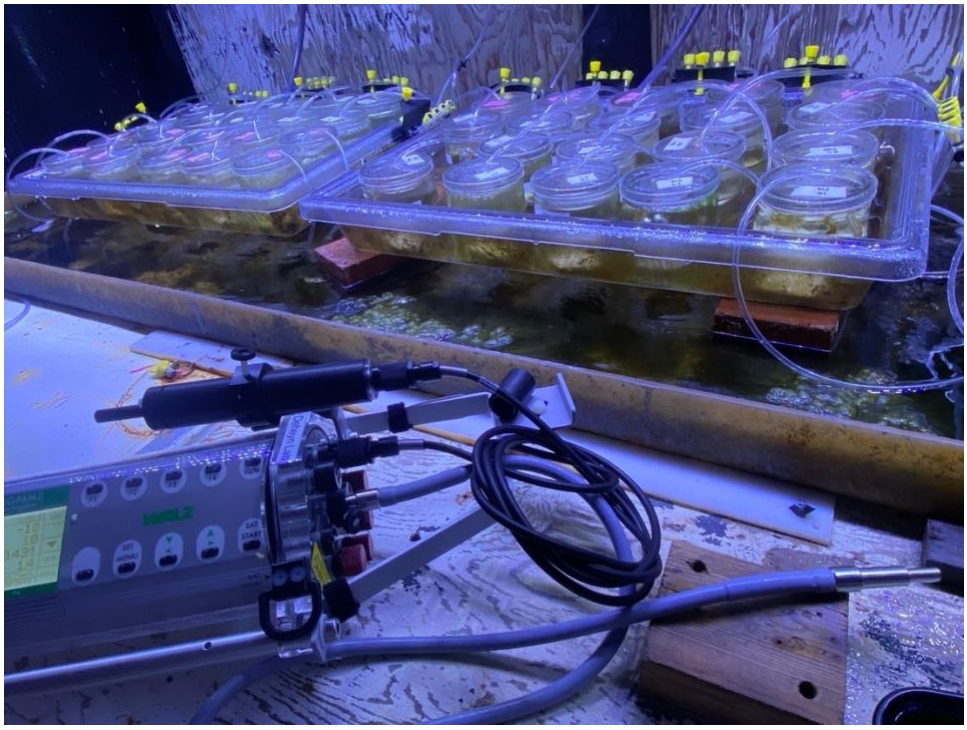

Figure 3. Image displaying the experimental set up measuring photosynthetic efficiency with the PAM instrument.

***Fluorescence Intensity***

Fluorescence was measured using a fluorescent imaging stereoscope (Nikon SMZ 1500 with 100 W mercury lamp and filter cube with excitation 390 nm and 470 nm) on days 0, 2, 9, 16 (exposure), and day 25 (recovery). Green and red fluorescence intensity were measured by exposing the sea anemone to a flash of light at a wavelength of either 470 nm or 390 nm and

imaging the produced fluorescence. Sea anemone GFPs emit fluorescence with an excitation peak of 460 nm from blue light and an emission peak of green fluorescence between 500-520 nm (Roth et al. 2013). Sea anemones were taken from the aquarium and brought to a separate laboratory for imaging. The anemones were kept in their jars in a dark enclosed container until they were taken out of their jars and placed in a small tray of seawater under the stereoscope for imaging. Seawater in the tray was changed after each anemone was imaged and was replaced with seawater from the Hubbs Hall experimental aquarium. Lights in the laboratory were kept off during the entire imaging process. Sea anemones were imaged individually under the same settings, including exposure times of 500 ms, 900 ms, 2 s, 5 s, and 7 s for the excitation of 390 nm; exposures of 100 ms, 300 ms, 600 ms, 900 ms, and 1 s for the excitation of 470 nm; and exposures of 80 ms, 200 ms, 500 ms, 700 ms, and 2 s under brightfield white light.

Fluorescent images were analyzed using ImageJ2 2.3.0 software. The images with an exposure of 600 ms for excitation 470 nm and an exposure of 5 s for excitation of 390 nm were processed in ImageJ to obtain the average green and red fluorescence intensity as previously seen in Roth et al. (2013). These exposure times were chosen due to consistent fluorescence and medium brightness. Ten points of each image were measured and averaged to obtain green and red fluorescence intensity (Figure 4). Each point had an area of 1264 pixels, and points were chosen at the same x and y coordinates for each image to maintain consistency. Points were selected around the tentacles of the anemones where fluorescence was strongest. Biofluorescence intensity levels estimated from these images were used as a proxy of GFP and symbiotic algae chlorophyll concentration. The 470 nm wavelength was used to determine anemone GFP intensity, and the 390 nm wavelength was used to determine algal chlorophyll intensity. The images were split into three different color channels: red, green, and blue, however, only red and

green were analyzed to establish red and green fluorescence intensity of algae and sea anemone, respectively.

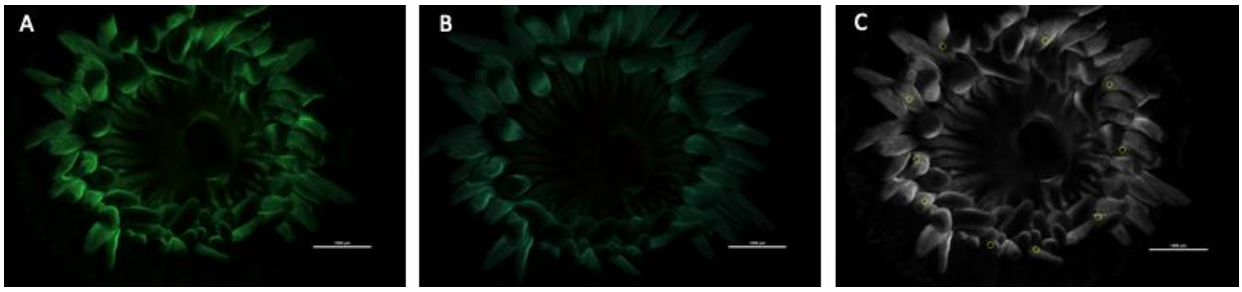


Figure 4. Images of *A.elegantissima* from the fluorescent imaging stereoscope taken with an excitation of 470 nm (A), 390 nm (B), and the green channel of the color split image (C) with exposure times of 600 ms, 5 s, and 600 ms. Ten points on (C) represent the points measured to obtain average fluorescence intensity.

***Seawater Sampling Analysis***

Sunscreen ingredient concentrations were determined from samples of experimental seawater taken from each experimental jar with an anemone. Water samples were taken twice weekly throughout the experiment, on days 8, 10, 16, and 18 for a total of 66 samples. Samples from days 8 and 16 were analyzed. Additional water samples were taken once per treatment at 0 and 24 hours between days 3-4 to determine if concentrations remained the same over the course of a day, as this was the duration between water changes. One sample per replicate was taken randomly, for a total of 22 samples.

Concentrations of $TiO_2$ and ZnO were determined in seawater samples using inductively coupled plasma-mass spectrometry (ICP-MS) to establish if concentrations were consistent over the course of the experiment. ICP-MS is a technique used to measure trace amounts of elements in a solution. Ti and Zn element concentrations in samples can be measured through this instrument. ICP-MS was conducted at the Scripps Isotope Geochemistry Laboratory (SIGL)

using a Thermo Scientific iCAP Qc ICP-MS (Thermo Fisher Scientific GmbH, Bremen, Germany). The samples were diluted 1:10 of 2% nitric acid ($HNO_3$). $^{46}Ti$, $^{47}Ti$, $^{49}Ti$, $^{50}Ti$, $^{66}Zn$ and $^{68}Zn$ isotopes were measured, and $^{49}Ti$ and $^{66}Zn$ were analyzed based on limited interference. The raw data was first corrected for diluents and then used to create a linear calibration curve. The equation from the standard curve graphs was used to obtain the concentrations of each element, which was then corrected for the dilution factor of x10.

Concentrations of oxybenzone were determined using gas chromatography-mass spectrometry (GC-MS) to establish consistent concentrations over the course of the experiment. GC-MS is a technique used for separating organic compounds, like oxybenzone, to measure the concentration in the sample. Solid phase extraction (SPE) was used to extract the organic compound from the seawater sample before injection into the GC-MS instrument. C18E cartridges are column chromatography cartridges used to absorb analytes from seawater solutions. These cartridges were placed on a vacuum manifold for SPE extraction and were first conditioned with 3 mL methanol (MeOH) followed by 3 mL MilliQ water. After loading the cartridges with 10 mL samples, they were washed with 6 mL of MilliQ water. The cartridges were dried thoroughly on a vacuum for 20 minutes and were then eluted with 2.5 mL ethyl acetate to obtain the compound of interest. The samples were dried down under compressed air and then redissolved in 100 μL of ethyl acetate. The GC-MS test was performed at the University of California San Diego Environmental and Complex Analysis Laboratory (ECAL) using a Thermo Scientific Trace 1310/TSQ 8000 Evo Triple Quadrupole GC-MS. Parameters for the GC-MS included scanning ion mode (SIM) to increase sensitivity and a spitless injection of 1 μL. The oven temperature began at 80ºC for 1 minute, increased by 25ºC increments for 3 minutes until reaching 330ºC, and held for 10 minutes. A standard curve of 0.1 μg/L, 1 μg/L, 10

μg/L, and 100 μg/L of oxybenzone in ethyl acetate was produced to create a calibration curve for analysis.

### *Statistical Analysis*

Statistical analyses were performed on all endpoints: maximum quantum yield (MQY), effective quantum yield (EQY), pressure over PSII ($Q_m$), red fluorescence intensity, and green fluorescence intensity, using Rstudio version 1.4.1106 software. Normality of data were checked using the Shapiro-Wilk test and homoscedasticity using Bartletts tests. Maximum quantum yield data was transformed by squaring, while effective quantum yield, pressure over PSII ($Q_m$), red fluorescence, and green fluorescence were each transformed by a square root transformation. A two-way ANOVA was completed to determine significant differences and interactions between treatment and time. The significant differences between the active ingredients and controls are summarized in Table 2. The 1 μg/L concentration of active ingredients were compared against the other treatments as well as controls from the initial exposure at days 0-3, and the recovery period at days 21-25 for the MQY, EQY, and Qm, and days 16-25 for green and red fluorescence intensity (Table 3). Due to interaction effects between treatment and time, end points were compared across treatments (ingredient and concentration) using the transformed data one-way ANOVAs, and a one-way ANOVA was also used to compare differences in treatments with respect to time (days) over the course of the experiment. A detailed summary of the one-way ANOVAs are summarized in the Appendix. A Tukey's multiple comparison post-hoc test was used when the ANOVAs resulted in significant differences between treatments (Tables 2 & 3). Outliers were determined using a Rosner test in Rstudio and were removed if they were true outliers. One outlier was removed from the maximum quantum yield data, four outliers were

removed from the red fluorescence data, and one outlier was removed from the green fluorescence data to sustain normality.

## Results

### *Seawater analysis*

The exposure concentrations throughout the experiment were different from expected (Table 1). The ICP-MS analysis of titanium and zinc samples in experimental treatments at days 3, 4, 8, and 16 revealed values ranging from ~5-17 μg/L and ~0-5 μg/L, respectively, instead of the target concentrations of 0.01-1 μg/L. These high levels indicate that the seawater itself may have had high amounts of these compounds even before adding any experimental $TiO_2$ or ZnO. The controls also had high amounts of titanium, ranging from ~7-55 μg/L. The controls exhibited zinc values similar to, or slightly higher than, those of the experimental jars, ranging from ~2-4 μg/L.

The GC-MS was unable to detect the oxybenzone in the samples due to the limited sensitivity of the machine coupled to low volume of the samples. Thus, the concentrations of oxybenzone in seawater could not be quantified.

Table 1. Seawater sample concentrations (μg/L) of $^{49}Ti$ and $^{66}Zn$ isotopes measured by ICP-MS from 0h to 24h and Week 2 (Day 8) to Week 3 (Day 16). Empty values represent concentrations below the limit of detection restricted by blank measurement.

| Concentration | $TiO_2$ | | $TiO_2$ | | ZnO | | ZnO | |
|---|---|---|---|---|---|---|---|---|
| | **0h** | **24h** | **Day 8** | **Day 16** | **0h** | **24h** | **Day 8** | **Day 16** |
| **Control** | 55.714 | 39.300 | 33.648 | 7.599 | 3.469 | 3.069 | 2.800 | 2.747 |
| **0.01 μg/L** | 6.828 | 9.654 | 5.294 | 5.334 | - | - | 2.719 | 0.674 |
| **0.1 μg/L** | 4.289 | 11.320 | 12.781 | 2.932 | 2.862 | 4.586 | 3.137 | 4.025 |
| **1.0 μg/L** | 16.892 | 7.599 | 5.136 | 5.729 | 0.965 | - | 2.174 | 1.521 |

***Photosynthetic efficiency of PSII***

*Dark acclimated maximum quantum yield (MQY)*

Average PAM values ranged from ~0.3-0.6. Over the course of the experimental phase (days 0-21), there were fluctuations in MQY for all the treatments and controls, but no meaningful statistically significant differences were detected. A similar lack of pattern was observed during the recovery period (days 21-25) (Figures 5-7; Tables 2-3).

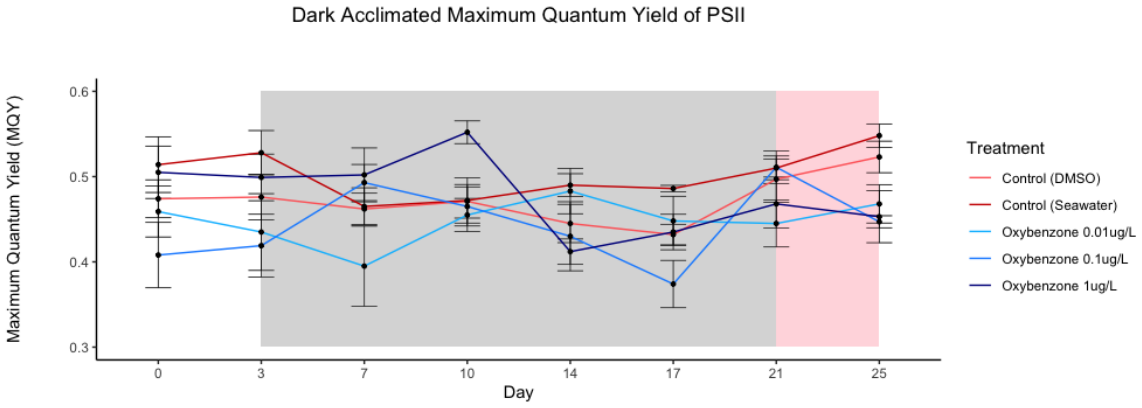


Figure 5. Dark acclimated maximum quantum yield of PSII (MQY) for the active ingredient oxybenzone at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

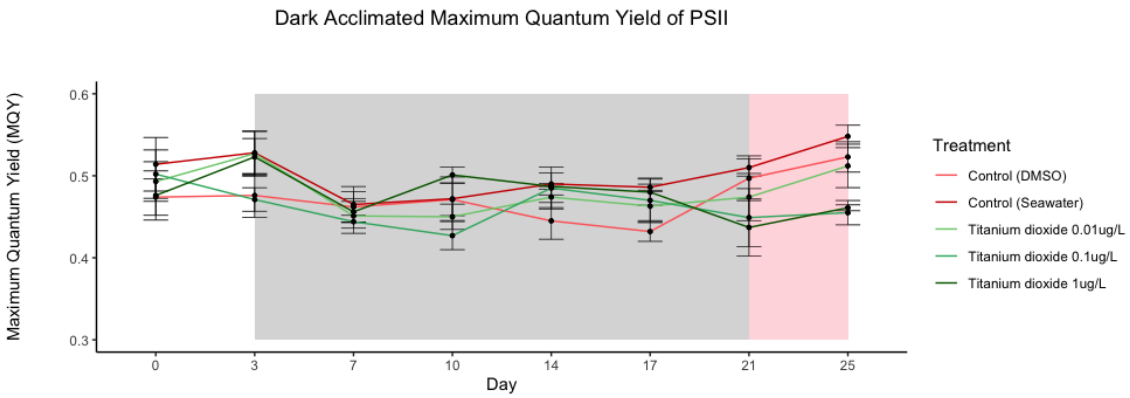


Figure 6. Dark acclimated maximum quantum yield of PSII (MQY) for the active ingredient $TiO_2$ at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

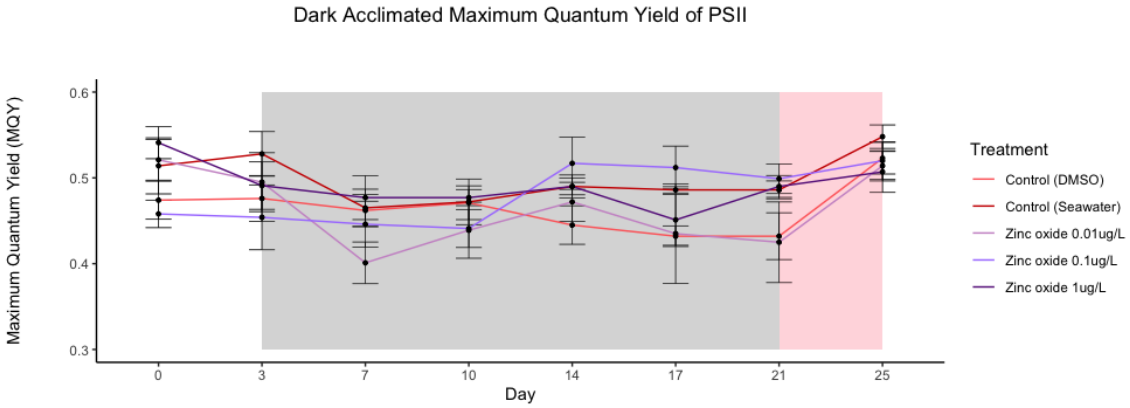


Figure 7. Dark acclimated maximum quantum yield of PSII (MQY) for the active ingredient ZnO at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

*Light acclimated effective quantum yield (EQY)*

Throughout the exposure phase of the experiment (days 0-21) as well as the recovery period (days 21-25), there was variation in EQY for all the treatments and controls, but no statistically significant differences occurred (Figures 8-10; Tables 2-3).

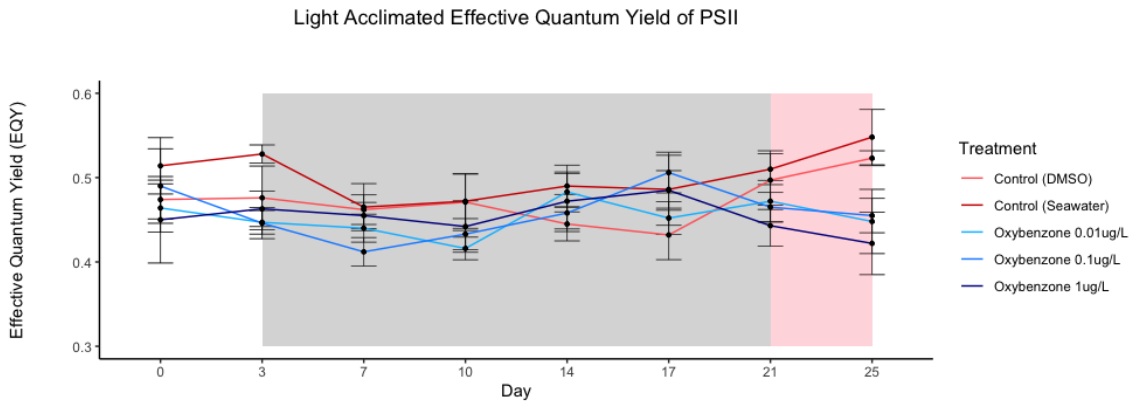


Figure 8. Light acclimated effective quantum yield of PSII (EQY) for the active ingredient oxybenzone at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

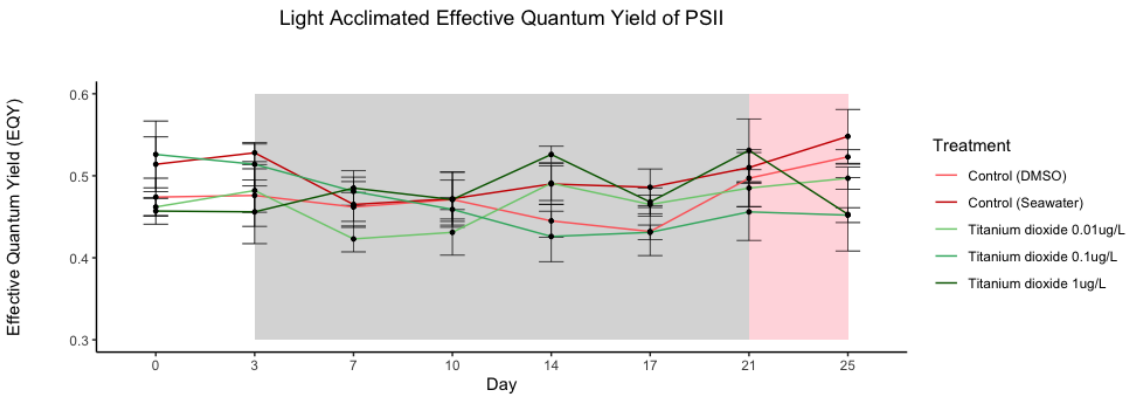


Figure 9. Light acclimated effective quantum yield of PSII (EQY) for the active ingredient $TiO_2$ at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

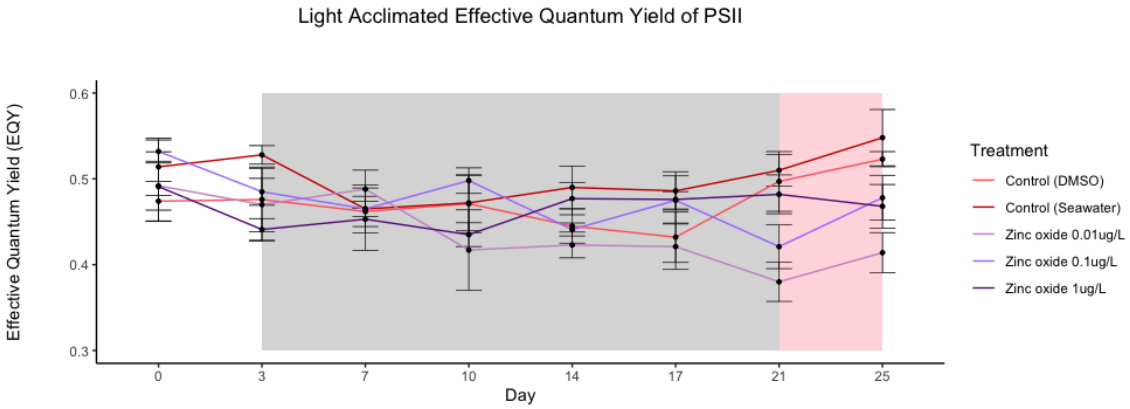


Figure 10. Light acclimated effective quantum yield of PSII (EQY) for the active ingredient ZnO at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

*Pressure over PSII ($Q_m$)*

During the exposure phase of the experiment (days 0-21) as well as the recovery period (days 21-25), there were slight fluctuations seen in $Q_m$ for all the treatments and controls, however there were no statistically significant differences observed (Figures 11-13; Tables 2-3).

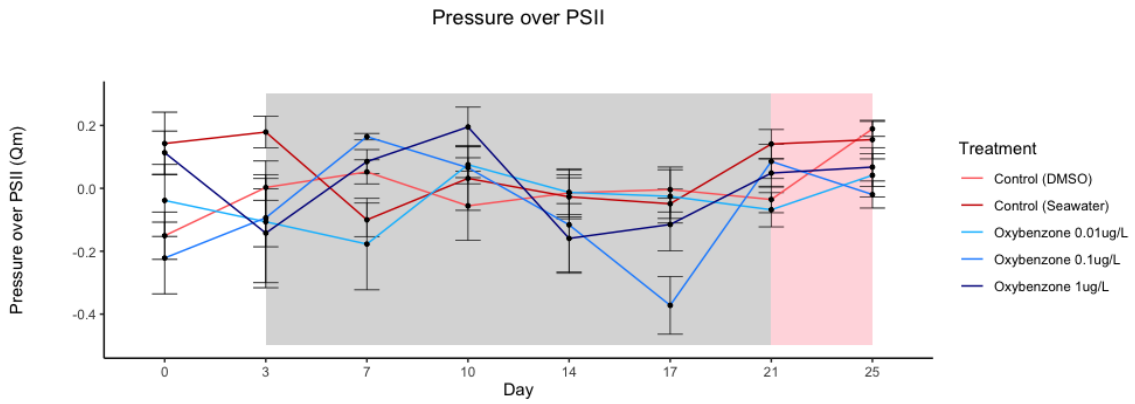


Figure 11. Pressure over PSII ($Q_m$) for the active ingredient oxybenzone at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

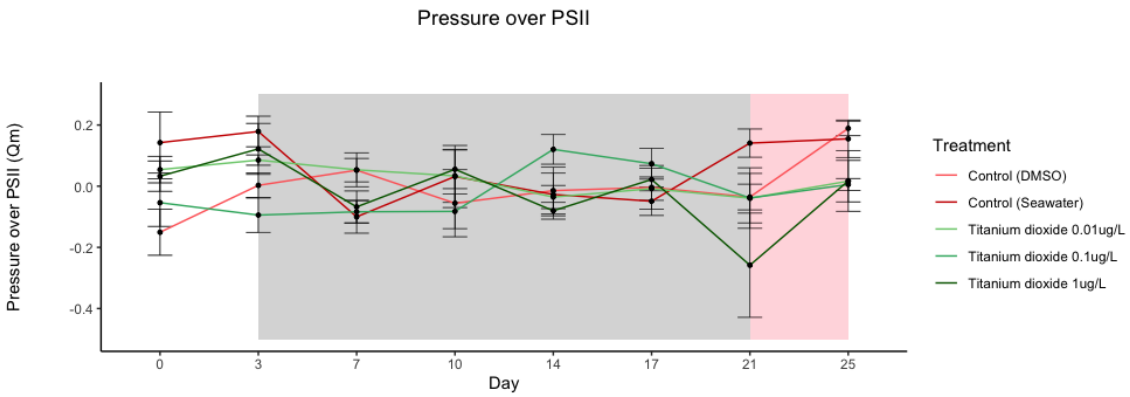


Figure 12. Pressure over PSII ($Q_m$) for the active ingredient $TiO_2$ at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

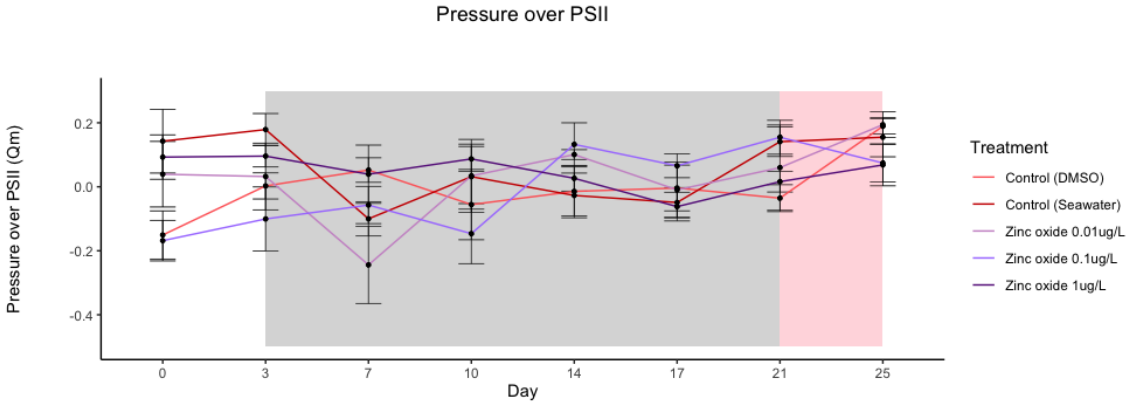


Figure 13. Pressure over PSII ($Q_m$) for the active ingredient ZnO at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 3-21 represents the experimental measurement period, and the pink shading between days 21-25 represents the recovery period. Error bars represent the standard error of the means.

*Green Fluorescence Intensity*

Throughout the experimental phase, days 0-16, and the recovery period (days 16-25), there was variation in green fluorescence intensity for the treatments as well as the controls, however there were no statistically significant differences (Figures 14-16; Tables 2-3).

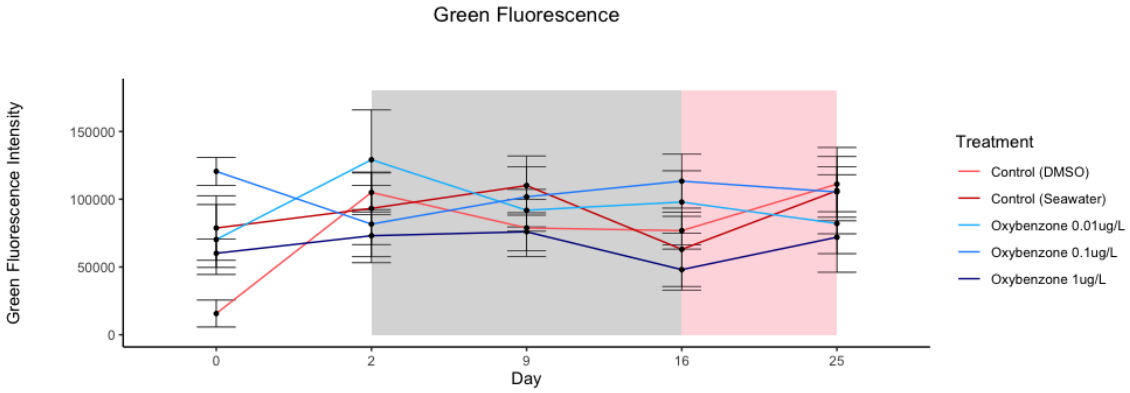


Figure 14. Green fluorescence intensity for the active ingredient oxybenzone at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 2-16 represents the experimental measurement period, and the pink shading between days 16-25 represents the recovery period. Error bars represent the standard error of the means.

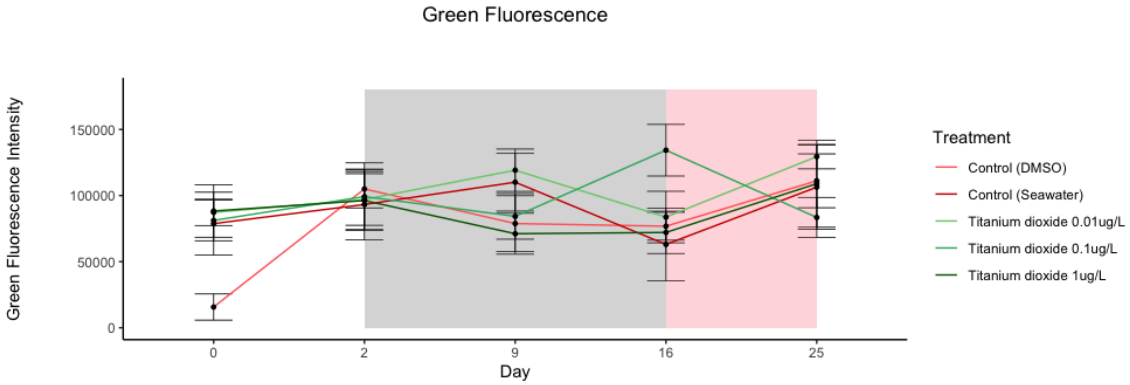


Figure 15. Green fluorescence intensity for the active ingredient $TiO_2$ at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 2-16 represents the experimental measurement period, and the pink shading between days 16-25 represents the recovery period. Error bars represent the standard error of the means.

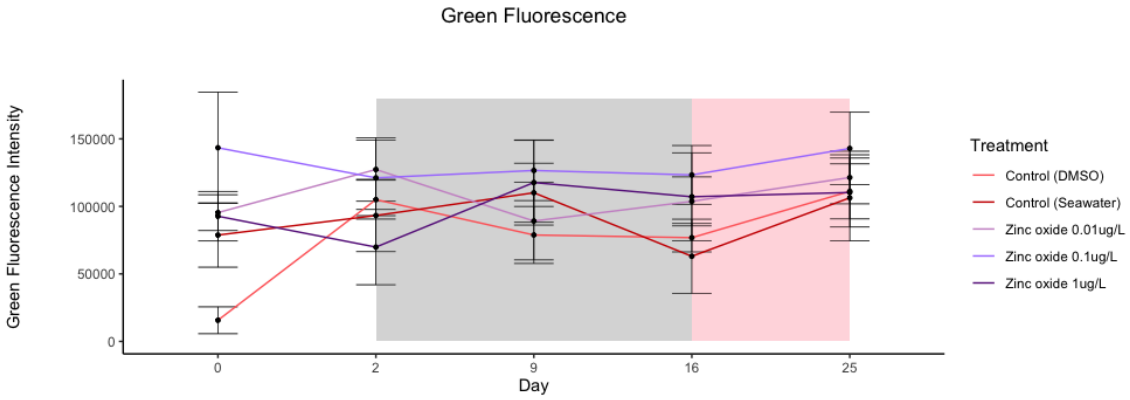


Figure 16. Green fluorescence intensity for the active ingredient ZnO at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 2-16 represents the experimental measurement period, and the pink shading between days 16-25 represents the recovery period. Error bars represent the standard error of the means.

*Red Fluorescence Intensity*

Throughout the experimental phase, days 0-16, as well as the recovery period (days 16-25), there were fluctuations in red fluorescence intensity for the treatments and controls, but no meaningful statistically significant differences were detected (Figures 17-19; Tables 2-3).

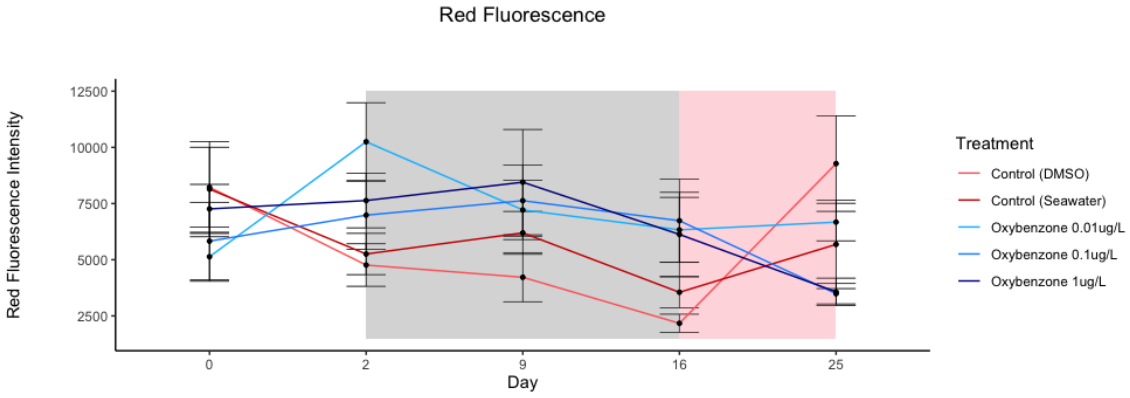


Figure 17. Red fluorescence intensity for the active ingredient oxybenzone at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 2-16 represents the experimental measurement period, and the pink shading between days 16-25 represents the recovery period. Error bars represent the standard error of the means.

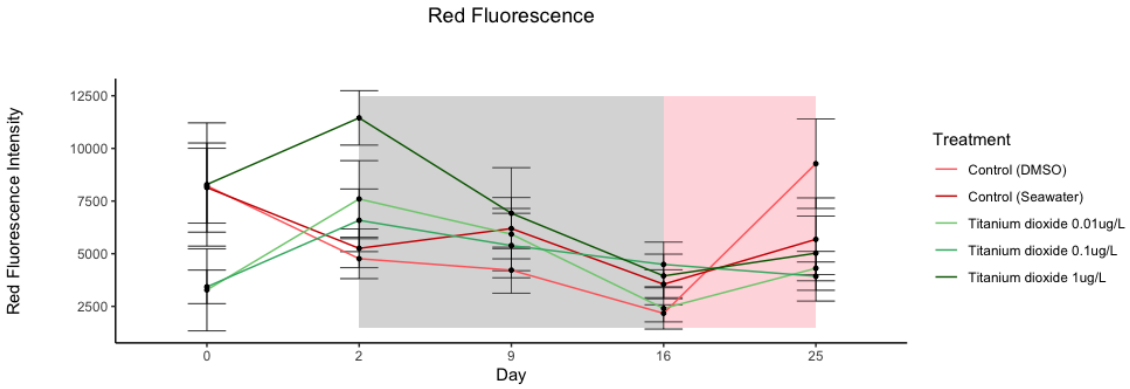


Figure 18. Red fluorescence intensity for the active ingredient $TiO_2$ at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 2-16 represents the experimental measurement period, and the pink shading between days 16-25 represents the recovery period. Error bars represent the standard error of the means.

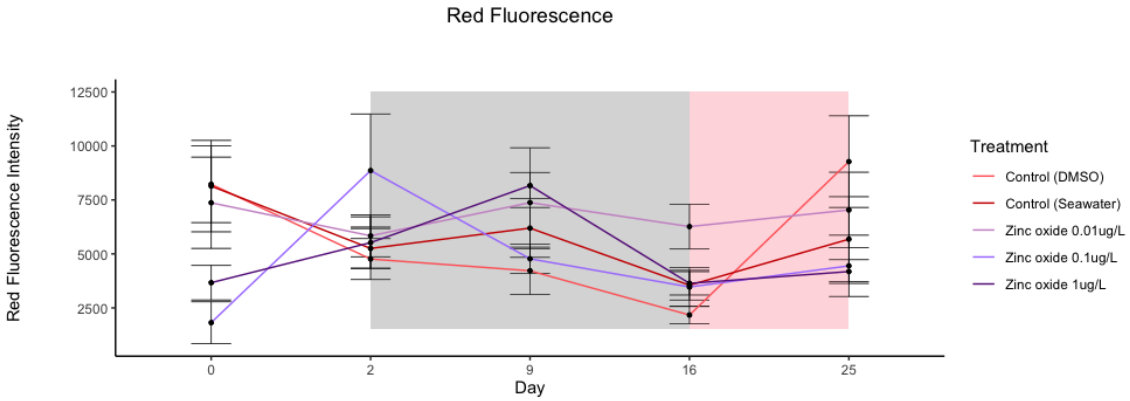


Figure 19. Red fluorescence intensity for the active ingredient ZnO at concentrations 0.01 µg/L, 0.1 µg/L, and 1 µg/L and controls. The gray shading between days 2-16 represents the experimental measurement period, and the pink shading between days 16-25 represents the recovery period. Error bars represent the standard error of the means.

Table 2. Summary of MQY, EQY, $Q_m$, Green fluorescence intensity and Red fluorescence intensity two-way analysis of variance (ANOVA) statistics to test for significant differences between exposure treatments and controls. Tukey's Multiple Comparison Post-Hoc test results are displayed when ANOVA: $p < 0.05$ for time, treatment, and interactions between time and treatment.

| | | **ANOVA** | | **Tukey's Post-Hoc Test** | | |
|---|---|---|---|---|---|---|
| Parameter | Treatment | Fixed effects | P value | Day | Treatment | P value |
| Maximum quantum yield | Oxybenzone | Time | **0.0155** | 10-17 | No clear pattern | 0.0112 |
| | | Treatment | 0.0963 | | | |
| | | Time x Treatment | **0.0070** | | | |
| | $TiO_2$ | Time | 0.1912 | | | |
| | | Treatment | 0.0813 | | | |
| | | Time x Treatment | 0.4932 | | | |
| | ZnO | Time | **0.0282** | 0-7 | | 0.0122 |
| | | Treatment | 0.0529 | | | |
| | | Time x Treatment | 0.3979 | | | |
| Effective quantum yield | Oxybenzone | Time | 0.3787 | | | |
| | | Treatment | 0.2320 | | | |
| | | Time x Treatment | 0.3205 | | | |
| | $TiO_2$ | Time | 0.4153 | | | |
| | | Treatment | 0.4304 | | | |
| | | Time x Treatment | 0.1635 | | | |
| | ZnO | Time | 0.1072 | | | |
| | | Treatment | 0.1973 | | | |
| | | Time x Treatment | 0.1919 | | | |
| Pressure over PSII | Oxybenzone | Time | **0.0377** | 7 | Oxybenzone 0.1 μg/L – Control (DMSO) | 0.0323 |
| | | Treatment | 0.4762 | | | |
| | | Time x Treatment | **0.0018** | | | |
| | $TiO_2$ | Time | 0.3614 | | | |
| | | Treatment | 0.1117 | | | |
| | | Time x Treatment | **0.0206** | 3 | $TiO_2$ 0.1 μg/L – Control (DMSO) | 0.0220 |
| | ZnO | Time | 0.1644 | | | |
| | | Treatment | 0.1133 | | | |
| | | Time x Treatment | **0.0372** | | | |
| Green fluorescence intensity | Oxybenzone | Time | | | | |
| | | Treatment | | | | |
| | | Time x Treatment | | | | |
| | $TiO_2$ | Time | | | | |
| | | Treatment | | | | |
| | | Time x Treatment | | | | |
| | ZnO | Time | | | | |
| | | Treatment | | | | |
| | | Time x Treatment | | | | |
| Red fluorescence intensity | Oxybenzone | Time | 0.1063 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Treatment | 0.1365 | | | |
| | | Time x Treatment | 0.2364 | | | |
| | $TiO_2$ | Time | **0.0015** | 2 | Control (DMSO) – $TiO_2$ 1 μg/L | 0.0171 |
| | | Treatment | 0.1626 | | | |
| | | Time x Treatment | 0.2098 | | | |
| | ZnO | Time | **0.0045** | 0-16 | | 0.0033 |
| | | Treatment | 0.4552 | | | |
| | | Time x Treatment | 0.0805 | | | |

Table 3. Summary of MQY, EQY, $Q_m$, Green fluorescence intensity and Red fluorescence intensity two-way analysis of variance (ANOVA) statistics to test for significant differences between treatments at 1 μg/L concentration during the initial time point of days 0-3 and the recovery time point at days 21-25 for MQY, EQY, and Qm, and days 16-21 for Green and Red fluorescence intensity. Tukey's Multiple Comparison Post-Hoc test results are displayed when ANOVA: $p < 0.05$.

| | | **ANOVA** | | **Tukey's Post-Hoc Test** | | |
|---|---|---|---|---|---|---|
| Parameter | Days | Fixed effects | P value | Day | Treatment | P value |
| Maximum quantum yield | 0-3 | Time | 0.9984 | | | |
| | | Treatment | 0.1040 | | | |
| | | Time x Treatment | 0.8425 | | | |
| | 21-25 | Time | **0.0191** | | | |
| | | Treatment | 0.0600 | | | |
| | | Time x Treatment | 0.7389 | | | |
| Effective quantum yield | 0-3 | Time | 9.3622 | | | |
| | | Treatment | 0.0754 | | | |
| | | Time x Treatment | 0.6841 | | | |
| | 21-25 | Time | 0.5734 | | | |
| | | Treatment | **0.0315** | | $TiO_2$ - ZnO | 0.0252 |
| | | Time x Treatment | 0.0882 | | | |
| Pressure over PSII | 0-3 | Time | 0.6453 | | | |
| | | Treatment | 0.1061 | | | |
| | | Time x Treatment | 0.8493 | | | |
| | 21-25 | Time | **0.0356** | | Control (DMSO) – Oxybenzone | 0.0141 |
| | | Treatment | **0.0358** | | Control (DMSO) – $TiO_2$ | 0.0152 |
| | | Time x Treatment | 0.6895 | | | |
| Green fluorescence intensity | 0-3 | Time | **0.0022** | 0-3 | Oxybenzone | 0.0178 |
| | | Treatment | 0.9162 | | | |
| | | Time x Treatment | 0.3526 | | | |
| | 16-25 | Time | **0.0002** | 25-16 | Oxybenzone | 0.0281 |
| | | Treatment | **0.0017** | | ZnO | 0.0403 |
| | | Time x Treatment | 0.3098 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Red fluorescence intensity | 0-3 | Time | 0.8647 | | | |
| | | Treatment | 0.9818 | | | |
| | | Time x Treatment | 0.0828 | | | |
| | 16-25 | Time | **0.0021** | 16-25 | Control (Seawater) | 0.0009 |
| | | Treatment | 0.1797 | | | |
| | | Time x Treatment | **0.0407** | | | |

## Discussion

This study aimed to evaluate the impact of the common sunscreen compounds oxybenzone, $TiO_2$, and ZnO on the fluorescence intensity of sea anemones and the photosynthetic efficiency of photosystem II (PSII) of their symbiotic algae. None of the compounds had any measurable effects on either fluorescence intensity or photosynthetic efficiency of PSII. However, there were several confounding factors and suboptimal experimental conditions, thus additional studies are needed to draw informative conclusions.

### *Challenges to Seawater Sample Analysis*

Achieving stable concentrations of a sunscreen compound in seawater is a challenge that proved to be the most confounding factor in this study. Many previous studies considered the nominal concentrations (i.e. the concentration that theoretically should be achieved after diluting the compound) when analyzing their results, yet they measured either lower or higher values, or did not measure the concentration of the compound at all. It is difficult to calculate accurate exposure concentrations due to the contaminant loss over time (Mitchelmore et al. 2021). For example, in the experiment conducted by Fel et al. (2019) on the photochemical response of corals to various organic and mineral UV filters, the measured concentrations of avobenzone and octocrylene were 2-22 times lower than the nominal concentrations. Additionally, there was a 91% and 48% loss in avobenzone and octocrylene, respectively, from a 1000 μg/L exposure over

the five-week period (Fel et al. 2019). In another experiment, He et al. (2019) exposed corals to octinoxate and octocrylene, common organic UV filters, and found that on day 1 the concentrations of octinoxate were only 50-87% of the nominal concentrations. Similarly, the concentrations of $TiO_2$ and ZnO that the sea anemones were exposed to in the present study differed from the target concentrations. The exposed treatments as well as the seawater controls presented higher amounts of $TiO_2$ and ZnO than expected.

The discrepancy between expected and actual compound concentrations in this study was most likely due to issues of solubility in seawater. Titanium dioxide particles aggregate together in seawater and fall in the water column, resulting in a gradual decrease in the concentration of dissolved particles; the higher the concentration of the $TiO_2$ particles, the more they aggregate (Jovanović et al. 2014). The ICP-MS analysis revealed that over the course of 24 hours, the higher concentration of $TiO_2$ dropped by 55%, whereas the lower concentration increased by 41%. The initial drop in concentration from the highest expected concentration is consistent with aggregations of these titanium particles (Wang et al. 2011). ZnO particles in the marine environment tend to aggregate as well and may attach to deposits or glass walls (Yung et al. 2015). Titanium is found independently in seawater, and there may have been trace amounts of titanium already in the seawater. Consequently, the seawater concentrations of both $TiO_2$ and ZnO were too disparate from targets to provide meaningful treatment conditions.

Additional challenges arose with the oxybenzone compound as it was not detected in the seawater samples by GC-MS, and thus could not be quantified. Therefore, the actual concentration in the jars is unknown. The target oxybenzone concentration was on the nanogram range, which is below the limit of detection of the instrument. Oxybenzone is relatively lipophilic, and therefore may have adhered to the glass jars or the aeration tubing. Oxybenzone

also can be taken up by the sea anemones (Wijgerde et al. 2019) and was previously detected in coral tissues (Mitchelmore et al. 2019). DMSO is a solvent widely used to dissolve compounds and has been utilized in existing literature as a viable way to dissolve oxybenzone (Kais et al. 2013). Conversely, DMSO can enhance biological uptake of substances, and was found to increase the uptake of the substance fluorescein in fish embryos (Kais et al. 2013). That experiment used concentrations of >0.1% DMSO, and the recommended maximum use of DMSO in fish embryo tests is 0.01%, as suggested by the Organization for Economic Co-operation and Development (Mitchelmore et al. 2021 & OECD 2014). However, the final DMSO concentrations in my study was 0.005% DMSO, which is well below the recommended maximum. Additionally, there were no significant differences between the DMSO and Seawater controls, so effects of DMSO can be ruled out.

As a result of the problems with maintaining and measuring sunscreen compounds in seawater, the treatments used in the present study cannot provide clear results and conclusions. Therefore, these results are based on the exposure of variable amounts of the compounds and cannot be accurately compared across treatments or time.

### *Photosynthetic efficiency*

Photosynthetic efficiency is a parameter utilized for identifying photosynthetic properties and overall health of an organism. Measurements from the PAM can provide insight into the capability of an organism to tolerate stressors that may have damaged the photosynthetic system. While there is some observed variation in photosynthetic efficiency across treatments and days, there is no definitive pattern between treatments. This is sensible considering the variability of the treatment conditions, as described above. Variability in photosynthetic efficiency can also

stem from the process itself. When light energy is absorbed, it can be utilized in various ways including: driving photosynthesis, dissipating of excess energy as heat (non-photochemical quenching), or reemitting as light (fluorescence) (Maxwell & Johnson 2000). When the PSII reaction center absorbs light and an electron is accepted, it cannot accept another until it has passed the first one onto an electron carrier. During this time, it is suggested that the reaction center is "closed", which leads to an overall reduction in photosynthetic efficiency (Maxwell & Johnson 2000). When there are high levels of light, the PSII center begins to breakdown, known as photoinhibition, due to the excess energy that cannot be used (Maxwell & Johnson 2000). Changes in maximum quantum yield (MQY) can show if the photosynthetic system recovered throughout the night, and changes in effective quantum yield (EQY) may suggest an imbalance between the amount of light energy absorbed and processed in the PSII reaction centers (Maxwell & Johnson 2000). However, in my experiment both MQY and EQY remained relatively constant and did not have any significant differences between treatments and controls. While there were few variations between specific treatments or days, this cannot be used as an indicator of overall changes in photosynthetic efficiency.

PAM measurements vary by organism and by the environment they inhabit. Bedgood et al. (2020) conducted an experiment on food availability for sea anemones, providing different feeding treatments. They evaluated photosynthetic efficiency over time and measured PAM values ranging from ~0.5-0.7. Another study observed the impacts of copper and copper oxide on sea anemones and measured PAM values with an average of 0.56 (Siddiqui et al. 2015). In this present experiment, the measured average PAM values were compatible, ranging from ~0.3-0.6.

There were no statistically significant differences or declines in the MQY and EQY between all of the treatments and controls, implying there was no photodamage to the symbiotic algae or sea anemones. The $Q_m$ values for all treatments fluctuated between negative and positive values. As $Q_m$ approaches 1.0, higher percentages of PSII reaction centers are closed (Frade et al. 2008). Negative values indicate the EQY is larger than the MQY. High levels of $Q_m$ can reflect the potential of photoinhibition in the organism (Frade et al. 2008). Considering that values varied for each treatment between days, it can be assumed that there are no trends or patterns in the pressure over PSII. This result is supported by other studies that found no significant impacts of sunscreen contaminants on photosynthetic efficiency. In an experiment testing the effects of various common sunscreen UV filter exposure on corals, it was found that octocrylene had no impact on the maximum photosynthetic efficiency (Fel et al. 2019). Another common filter, avobenzone, had a significant decrease in the maximum photosynthetic efficiency, yet only at a high concentration of 1000 μg/L, which is above concentrations found in-situ. It was concluded that these organic filters either have no impact on coral PSII or only have an impact at high concentrations. The results of these previous studies were only significant at concentrations higher than what is found in the environment and were higher than those used in the present study. These results also support the findings of the present study that presented no negative impacts of sunscreen active ingredients.

***Fluorescence intensity***

Green fluorescence intensity and red fluorescence intensity convey information regarding the sea anemone and symbiont chlorophyll fluorescent capabilities, respectively. There were no significant differences in green fluorescence intensity over the course of the experiment, and the

sea anemones did not adjust their fluorescence intensity. Since green fluorescence is proposed to have antioxidative properties, an increase in green fluorescence could help an organism against oxidative stress. Wijgerde et al. (2019) found an increase in green fluorescence after exposure of coral to a combination of oxybenzone and elevated heat and thus proposed that increased GFP production helped mitigate the impacts of stressful conditions. In my study, the lack of differences in green fluorescence suggest that the sunscreen active ingredients did not have stressful effects, or that normal sea anemone GFP production is enough to combat any effects. Haryanti & Hidaka (2019) exposed juvenile coral *Pocillopora damicornis* to elevated temperature (32ºC compared to normal 27-28ºC) and found that bleaching was associated with an increase in GFP fluorescence. They suggested that green fluorescence intensity in juvenile corals may be influenced by extreme condition changes, and that GFPs could present different functions at different developmental stages. Another study by Aihara et al. (2019) found that algal symbionts *Symbiodinium* were attracted to the green fluorescence emission of coral fragments and suggest that green fluorescence could help corals acquire symbiotic algae during early life stages. These studies all indicate the need for further research on potential GFP function and ecological role.

In anemones, the red fluorescence is due to the algae chlorophyll and therefore can be used as a proxy for algal density. Since red fluorescence intensity was relatively constant in my experiment, this indicates that there were not any significant changes in the density of algal symbionts. The excitation cube I used in the imaging microscope provided a 390 nm wavelength excitation in order to measure the red fluorescence emission of the symbiotic algae *Symbiodinium*. This may have been a low excitation, as *Symbiodinium* was observed to have a

peak absorption at 465 nm (Wangpraseurt et al. 2014). Therefore, the measured red fluorescence intensity could have been lower than at its peak absorption and may have skewed the results.

The present study did not find negative impacts on the fluorescence intensity of both sea anemones and their symbiotic algae. There are limited studies on the impact of sunscreen on fluorescence, as well as on the overall role of GFPs in cnidarians, which indicates the need for further research on this role and how it might change depending on environmental stressors.

**Future directions**

The present study provided some preliminary insights into how photosynthetic efficiency and fluorescence of sea anemones and their symbionts may be affected by seawater contaminated with selected sunscreen compounds. However, additional experiments are necessary to assess the impacts of these UV filters. In particular, the sunscreen in seawater exposure concentrations should be measured both before and during the experimental period, to confirm the exposure concentrations are remaining consistent throughout the study. $TiO_2$ and ZnO are insoluble in seawater, and further tests are needed to find alternative ways to dissolve the compounds prior to exposure. Furthermore, a standard protocol for measuring exposure concentrations must be developed for accurate analysis and comparison of data. In the present study, seawater samples were measured throughout the experiment and it was found that the exposure concentrations were not as expected.

The nominal concentrations of $TiO_2$, ZnO, and oxybenzone used in this study were based on previous studies on the environmentally relevant concentrations, which are variable and may be too low to use in an experimental setup designed to observe impacts of stress on the organisms. Titanium and zinc may be found at high concentrations in ocean habitats where sea

anemones live, and therefore low concentrations of $TiO_2$ and ZnO used in experiments may not induce impacts on sea anemones. For example, Fel et al. (2019) found that only a high exposure of 100 μg/L of ZnO resulted in a significant decrease in the dark acclimated maximum quantum yield ($F_v/F_m$) in the coral *Stylophora pistillata*. Future studies could employ higher concentrations to model the potential impacts of the addition of these compounds from recreational activities, and to better understand whether anemones become stressed after exposure to these mineral compounds.

Under stressful conditions, sea anemones may close their tentacles for protection. The sea anemones did not remain fully open throughout the fluorescence intensity measurements. This could have skewed results since it was not possible to keep the anemones' tentacles fully open for imaging. Previous studies used solutions to anesthetize the anemones to keep them open, which could be utilized in a future study. Due to the large number of anemones in the present study, the imaging was split across two days. This made it difficult to compare the intensity levels, and therefore may have affected the results. Future experiments could consider the length of time it may take to image large numbers of experimental anemones to ensure that all imaging is completed on the same day.

Additionally, green fluorescence intensity was measured in the present study, but not the GFP concentration changes over the course of the experiment. Changes in GFP concentration would provide information regarding the response of the organism to extreme conditions. GFP production may increase or decrease in response to changes in environmental conditions, like temperature, light, or contaminant stress (Roth et al. 2010). However, while it was proposed that cnidarians may regulate their GFP concentrations in order to acclimate to changing conditions

(Roth et al. 2010), actual functions, mechanisms, and effects are lacking. This must be studied further to understand the role of GFPs in cnidarians.

In-situ experiments could provide more useful information about the impacts of sunscreens in the environment. Laboratory experiment results may be specific to the designed experimental setup, and it may prove difficult to understand how such results would translate to in-situ habitats. Sea anemones in coastal environments present a model ecosystem at risk of contaminant pollution. Fluorescence and photosynthesis readings are viable field work measurements using an underwater PAM device and should be considered for future studies. In-situ underwater PAM measurements can provide realistic data of the levels of consequences of sunscreen in seawater by sampling the seawater and measuring the amounts of contaminants associated with changes in PAM measurements.

Since the control seawater samples contained high amounts of titanium and zinc, my study raises interesting questions about the amount of each presently in the ocean, its effects on marine organisms, and whether additional $TiO_2$ and ZnO from sunscreens could be expected to have any effect. It represents a basis to develop additional studies designed to examine the potential harmful effects of sunscreen contaminants on sea anemone functions.

**Conclusions**

My study found no significant evidence of negative impact of the sunscreen compounds oxybenzone, $TiO_2$, and ZnO on the photophysiology of sea anemones, and thus I conclude that the organisms in this experimental setup were not stressed. The endosymbiotic algae were able to perform photosynthesis consistently despite exposure to contaminants. The results of this experiment suggest that sea anemones did not regulate their GFP production or fluorescence

intensity in response to the treatments and demonstrates the need for further research regarding the role of GFPs in cnidarians. The primary aim of this study was to evaluate the differences between the chemical compounds and the "reef-safe" advertised mineral compounds found in sunscreens. However, conclusions cannot be made due to the variable compound concentrations in seawater measured by GC-MS and ICP-MS.

As more $TiO_2$ and ZnO sunscreens are produced and leak into the oceans, it is vital to understand the potential impacts that these compounds have on organisms. While this study did not provide evidence of negative effects, it does provide valuable information to guide future studies to further our understanding. This study demonstrates a key challenge in studying these types of contaminants, because the compounds being studied are not soluble in seawater and tend to aggregate together. There is a significant lack of research regarding sunscreen contaminants, specifically in sea anemones. Sea anemones play an important function in coastal ecosystems, and typically reside in areas high in human recreation. Many organisms are directly dependent on sea anemones and it is vital to further study the effects of anthropogenic contaminants on marine organisms and ecosystems. This experiment models a baseline for future studies in order to understand the threat of sunscreens to marine organisms.

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## Appendix

Table 4. Summary of MQY, EQY, Qm, Green fluorescence intensity and Red fluorescence intensity one-way analysis of variance (ANOVA) statistics to test for significant differences between treatments for individual days. Tukey's Multiple Comparison Post-Hoc test results are displayed when ANOVA: $p < 0.05$.

| | | ANOVA | | | | Tukey's Post-Hoc Test | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Day | Sum sq | Mean sq | F statistic | P value | Day | Treatments | P value |
| Maximum quantum yield | 0 | 0.058 | 0.005 | 1.679 | 0.116 | 10 | Oxybenzone 1µg/L - Oxybenzone 0.01µg/L | 0.02050 |
| | 3 | 0.056 | 0.005 | 1.434 | 0.199 | | Oxybenzone 1µg/L – $TiO_2$ 0.1µg/L | 0.00125 |
| | 7 | 0.041 | 0.004 | 1.665 | 0.12 | | Oxybenzone 1µg/L – $TiO_2$ 0.01µg/L | 0.01289 |
| | 10 | 0.055 | 0.005 | 3.298 | **0.0029** | | Oxybenzone 1µg/L - ZnO 0.1µg/L | 0.00580 |
| | 14 | 0.038 | 0.003 | 1.788 | 0.0913 | | Oxybenzone 1µg/L-ZnO 0.01µg/L | 0.00583 |
| | 17 | 0.049 | 0.004 | 1.585 | 0.143 | 25 | Oxybenzone 0.1µg/L-Control (Seawater) | 0.02701 |
| | 21 | 0.035 | 0.003 | 1.115 | 0.373 | | | |
| | 25 | 0.060 | 0.006 | 3.119 | **0.00435** | | | |
| Effective quantum yield | 0 | 0.034 | 0.003 | 1.232 | 0.298 | 21 | ZnO 0.01µg/L-$TiO_2$ 1µg/L | 0.00989 |
| | 3 | 0.015 | 0.001 | 0.822 | 0.609 | | | |
| | 7 | 0.024 | 0.002 | 2.153 | **0.0399** | | | |
| | 10 | 0.021 | 0.002 | 1.079 | 0.398 | | | |
| | 14 | 0.027 | 0.002 | 2.104 | **0.0445** | | | |
| | 17 | 0.023 | 0.002 | 1.547 | 0.155 | | | |
| | 21 | 0.049 | 0.004 | 2.523 | **0.017** | | | |
| | 25 | 0.017 | 0.001 | 0.812 | 0.618 | | | |
| Pressure over PSII ($Q_m$) | 0 | 0.392 | 0.043 | 6.05 | **0.00075** | 0 | Control (DMSO)-Control (Seawater) | 0.00037 |
| | 3 | 0.114 | 0.011 | 0.482 | 0.884 | | $TiO_2$ 0.01µg/L-Control (Seawater) | 0.04459 |
| | 7 | 0.258 | 0.025 | 2.62 | **0.0363** | | Oxybenzone 1µg/L-Control (DMSO) | 0.00389 |
| | 10 | 0.134 | 0.013 | 0.66 | 0.748 | | Oxybenzone 0.01µg/L-Control (DMSO) | 0.00234 |
| | 14 | 0.217 | 0.021 | 1.633 | 0.172 | | ZnO 1µg/L- Control (DMSO) | 0.00361 |
| | 17 | 0.153 | 0.017 | 1.157 | 0.38 | | ZnO 0.01µg/L - Control (DMSO) | 0.02642 |
| | 21 | 0.200 | 0.020 | 1.132 | 0.384 | | | |
| Green fluorescence intensity | 0 | 72592 | 7259 | 1.115 | 0.373 | | | |
| | 2 | 64846 | 6485 | 0.685 | 0.732 | | | |
| | 9 | 46128 | 4613 | 0.699 | 0.72 | | | |

| | | | | | |
|---|---|---|---|---|---|
| | 16 | 119138 | 11914 | 1.684 | 0.115 |
| | 25 | 66513 | 6651 | 0.931 | 0.515 |
| Red fluorescence intensity | 0 | 6382 | 638.2 | 1.287 | 0.268 |
| | 2 | 6836 | 683.6 | 1.786 | 0.0941 |
| | 9 | 3728 | 372.8 | 0.74 | 0.683 |
| | 16 | 7718 | 771.8 | 2.238 | **0.0328** |
| | 25 | 6534 | 653.4 | 1.68 | 0.114 |

Table 5. Summary of MQY, EQY, Qm, Green fluorescence intensity and Red fluorescence intensity one-way analysis of variance (ANOVA) statistics to test for significant differences between days for individual treatments. Tukey's Multiple Comparison Post-Hoc test results are displayed when ANOVA: $p < 0.05$.

| | | **ANOVA** | | | | | **Tukey's Post-Hoc Test** | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Treatment | Sum sq | Mean sq | F value | P value | Treatment | Days | P value |
| Maximum quantum yield | Oxybenzone 1 µg/L | 0.065 | 0.009 | 3.208 | **0.0113** | Oxybenzone 1 µg/L | 14-10 | 0.00877 |
| | Oxybenzone 0.1 µg/L | 0.053 | 0.007 | 2.776 | **0.0225** | | 17-10 | 0.03752 |
| | Oxybenzone 0.01 µg/L | 0.014 | 0.002 | 0.557 | 0.784 | Oxybenzone 0.1µg/L | 21-17 | 0.02052 |
| | TiO2 1 µg/L | 0.022 | 0.003 | 1.939 | 0.0957 | | | |
| | TiO2 0.1 µg/L | 0.018 | 0.002 | 1.157 | 0.354 | | | |
| | TiO2 0.01 µg/L | 0.027 | 0.003 | 1.63 | 0.163 | | | |
| | ZnO 1µg/L | 0.022 | 0.003 | 1.55 | 0.186 | | | |
| | ZnO 0.1 µg/L | 0.037 | 0.005 | 1.796 | 0.122 | | | |
| | ZnO 0.01 µg/L | 0.054 | 0.007 | 1.531 | 0.192 | | | |
| | Control (DMSO) | 0.028 | 0.004 | 1.847 | 0.112 | | | |
| | Control (Seawater) | 0.026 | 0.003 | 1.851 | 0.111 | | | |
| Effective quantum yield | Oxybenzone 1 µg/L | 0.007 | 0.001 | 0.34 | 0.929 | ZnO 0.1µg/L | 14-10 | 0.01540 |
| | Oxybenzone 0.1 µg/L | 0.016 | 0.002 | 1.458 | 0.217 | | 21-0 | 0.00131 |
| | Oxybenzone 0.01 µg/L | 0.008 | 0.001 | 0.74 | 0.64 | | 21-10 | 0.04589 |
| | TiO2 1 µg/L | 0.017 | 0.002 | 1.114 | 0.378 | | | |
| | TiO2 0.1 µg/L | 0.023 | 0.003 | 2.075 | 0.0756 | | | |
| | TiO2 0.01 µg/L | 0.014 | 0.002 | 2.017 | 0.0835 | | | |
| | ZnO 1µg/L | 0.007 | 0.001 | 0.586 | 0.762 | | | |
| | ZnO 0.1 µg/L | 0.021 | 0.0030 | 3.992 | **0.00305** | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ZnO 0.01 µg/L | 0.032 | 0.004 | 1.769 | 0.128 | | | |
| | Control (DMSO) | 0.023 | 0.003 | 1.778 | 0.126 | | | |
| | Control (Seawater) | 0.032 | 0.004 | 2.469 | **0.0382** | | | |
| Pressure over PSII (Qm) | Oxybenzone 1 µg/L | 0.157 | 0.022 | 1.674 | 0.186 | Control (Seawater) | 25-0 | 0.02002 |
| | Oxybenzone 0.1 µg/L | 0.125 | 0.020 | 1.453 | 0.264 | | | |
| | Oxybenzone 0.01 µg/L | 0.156 | 0.022 | 1.044 | 0.455 | | | |
| | TiO2 1 µg/L | 0.106 | 0.015 | 0.629 | 0.724 | | | |
| | TiO2 0.1 µg/L | 0.124 | 0.017 | 0.911 | 0.53 | | | |
| | TiO2 0.01 µg/L | 0.074 | 0.010 | 1.548 | 0.218 | | | |
| | ZnO 1µg/L | 0.231 | 0.033 | 1.806 | 0.145 | | | |
| | ZnO 0.1 µg/L | 0.197 | 0.032 | 2.522 | 0.0721 | | | |
| | ZnO 0.01 µg/L | 0.167 | 0.023 | 1.162 | 0.367 | | | |
| | Control (DMSO) | 0.106 | 0.015 | 0.744 | 0.639 | | | |
| | Control (Seawater) | 0.250 | 0.035 | 2.915 | **0.0362** | | | |
| Green fluorescence intensity | Oxybenzone 1 µg/L | 15299 | 3825 | 0.88 | 0.493 | | | |
| | Oxybenzone 0.1 µg/L | 21733 | 5433 | 0.833 | 0.52 | | | |
| | Oxybenzone 0.01 µg/L | 36993 | 9248 | 0.688 | 0.609 | | | |
| | TiO2 1 µg/L | 11488 | 2872 | 0.43 | 0.786 | | | |
| | TiO2 0.1 µg/L | 23761 | 5940 | 1.216 | 0.335 | | | |
| | TiO2 0.01 µg/L | 22139 | 5535 | 1.639 | 0.206 | | | |
| | ZnO 1µg/L | 21616 | 5404 | 0.463 | 0.762 | | | |
| | ZnO 0.1 µg/L | 3109 | 777 | 0.09 | 0.984 | | | |
| | ZnO 0.01 µg/L | 15995 | 3999 | 0.737 | 0.578 | | | |
| | Control (DMSO) | 30130 | 7533 | 0.636 | 0.643 | | | |
| | Control (Seawater) | 14972 | 3743 | 0.938 | 0.462 | | | |
| Red fluorescence intensity | Oxybenzone 1 µg/L | 2906 | 726.5 | 1.75 | 0.179 | ZnO 1 µg/L | 9-0 | 0.00901 |
| | Oxybenzone 0.1 µg/L | 2170 | 542.5 | 1.288 | 0.31 | | 16-9 | 0.01042 |
| | Oxybenzone 0.01 µg/L | 1843 | 460.8 | 1.604 | 0.216 | | 25-9 | 0.03275 |
| | TiO2 1 µg/L | 6532 | 1633 | 2.266 | 0.0981 | Control (Seawater) | 16-0 | 0.01210 |
| | TiO2 0.1 µg/L | 1546 | 386.5 | 0.952 | 0.455 | | 25-16 | 0.00426 |
| | TiO2 0.01 µg/L | 4374 | 1093.4 | 2.386 | 0.0855 | | | |
| | ZnO 1µg/L | 3311 | 827.9 | 4.978 | **0.00598** | | | |
| | ZnO 0.1 µg/L | 4299 | 1074.9 | 2.525 | 0.0731 | | | |
| | ZnO 0.01 µg/L | 130 | 32.5 | 0.058 | 0.993 | | | |

| | | | | |
|---|---|---|---|---|
| Control (DMSO) | 2198 | 549.5 | 1.242 | 0.325 |
| Control (Seawater) | 7866 | 1966.6 | 5.659 | **0.00325** |