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<SAMPLE DOCUMENT>

Objective:

To study how exposure of sunscreen affects coral by parameters such as photosynthesis efficiency, chlorophyll fluorescence, dissolved oxygen (DO) levels and overall coral health.

Motivation:

Underwater ecosystems like coral reefs attract large visitors via tourism and result in great economic benefits.[1] Furthermore, the value of the marine ecosystems combined with the organisms is unquantifiable. Its complex interactions with other marine flora and fauna are being understood. Their ecosystem is under threat due to overfishing, agricultural and industrial pollutants and anthropogenic factors.[2] Furthermore, over-tourism may cause additional stress via chemical pollutants such as dermal sunscreen released into sea water. Hence, it becomes necessary to understand what are the effects of sunscreen components on coral health, if any. This study comes at a critical time when global warming events are already causing widespread coral bleaching and pose great threat to such fragile marine ecosystems.

Outcomes:

The outcomes of this research will be following: We will answer if the chemical components of sunscreen formulation (hereby labelled as SF) cause any detrimental effect on coral health or not. We will also report what is the minimum concentration at which the negative effect(s) of SF are observed, if any.

Previous studies and relevance:

The effect of chemical pollutants to coral and associated marine life has been studied by several techniques looking at various aspects. We focus on the active chemical components of sunscreen formulations. Some of the chemical components such as TiO_2, ZnO, oxybenzone, etc. have been detected in coastal waters.[3–6] Crucially, some of the common sunscreen formulations available in US and European markets based on organic UV filters (such as benzophenone derivatives, camphor derivatives, salicylates) have been shown to induce lytic viral cycle causing eventual coral bleaching at concentrations of $10\mu g/L$.[7] This is relatively low concentration suggesting that corals are very sensitive to the local chemical environment. Hence, the presently proposed study is very relevant to assess the effect of the target sunscreen.

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Experimental Plan:

Controlled experiments with coral samples placed in tanks will be exposed to different concentrations of sunscreen listed in Table 1 to observe effect of exposure on coral health via biochemical parameters listed in Table 2, over a period of 5 weeks. Commonly used coral fragments of species such as - *Acropora* or *Pocillopora* will be used.

We will have five experimental setups (along with its 2 replicates) and one control set up (in total 11 tanks with corals, of which 10 are being exposed to sunscreen in varying concentrations).

Table 1: Plan of water tanks for the experiment

Tank label	Concentration of sunscreen ($\mu g/L$ of seawater)
Control	0
A1	5
A2	5
B1	10
B2	10
C1	25
C2	25
D1	50
D2	50
E1	100
E2	100

Table 2: Parameters in the study

Physical parameters	Biochemical parameters
Color change	Photosynthetic activity via chlorophyll fluorescence
Weight change	Tissue damage
Bleaching	

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Dissolved oxygen of sea water	

The physical and biological parameters under investigation to assess coral health are listed in Table 2. Their relevance and principle of technique is discussed briefly.

Corals (*Anthozoa/Cnidaria)* being colonial marine invertebrates build calcareous skeletal framework and have zooxanthellae (*Symbiodinium*) as a symbiont in tissues. Most importantly, the symbiont is photosynthetic and provides nutrition to the coral by performing the process during the day. About 30-40% mass of the coral is contributed via zooxantheallae.

Expulsion of symbiont eventually results in coral death, a phenomenon known as coral bleaching. Since, the symbiont is very crucial for coral health, it is widely used as a biomarker of its health via color and photosynthetic activity.

Degradation of coral health is typically marked by following processes:

- worsening photosynthetic activity resulting in decolouration
- complete colour change to white is a mark of coral bleaching, evidence of the elimination of symbiont.

Through following experimental techniques, we will assess the health of the corals.

1.) Fluorescence of Chlorophyll of the symbiont.
Maximum photochemical efficiency of PSII via the ratio of variable fluorescence (F_V) to maximum fluorescence (F_M) in dark adapted corals. This technique is widely used to assess the stress in photosynthetic systems. (In plants, the value in normal leaves is found to be 0.83 while it is less than 0.83 in stressed and/or diseased state)[8,9]. In the present case, the reference value would have to be assessed from initial readings of the coral (without sunscreen exposure and from the control group).

2.) Dissolved oxygen level.

Photosynthesis by symbiont produces oxygen which is released in the seawater. In normal corals an oscillation of dissolved O_2 is expected due to the variation of sunlight during day and night. The amplitude of this oscillation suggests the absolute amount of photosynthetic activity and hence coral health.

3.) Weight of the coral mass.
Change in the weight of the coral overtime gives us information about how healthy the corals are. For this, wet weight (without excess water) will be measured on a digital balance at the start of the study and will be followed every two weeks. A comparison with control will give us evidence of any retardation on the growth.

4.) Colour change via photographs.
Symbiont provides colour to corals via photosynthetic pigments. A colour change has been directly correlated to the health of corals via standard colour analysis of photographs.[7,10]

5.) Tissue damage status via microscopic section.
A cross-section of the coral can be taken to understand pattern of skeletal deposition to assess the effect on growth. Further, dark/coloured regions indicating symbiont in tissue sections give direct evidence of the status of zooxanthellae Such comparison with control group will give unequivocal evidence of any effect on the host-symbiont relationship due to chemical exposure.

Simulation of natural environmental condition:

The proposed experiment involves placing and acclimatizing them in a controlled environment prepared in a water tank for experiment. Maintaining a controlled environment for coral growth in critical for the proposed experiment. Water quality including amounts of trace chemicals, biological waste, pH has to precisely recorded and controlled. This requires a regular measurement of pH (**using a pH meter**), salinity (**using the seawater refractometer),** and light intensity (using the **light flux meter**) to ensure that all conditions are within the optimal range for coral survival. Standard flow setup with pump and water pressure spray for simulating water current will be used. A tight control of the physical conditions are needed to ensure valid results. For this automated measurement can be established via microcontrollers to record data automatically.

US-NOAA has provided detailed recommendations for establishing aquariums for experiments on coral, and the present planning is based on this document.[11]

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Required items/instruments:

pH meter
seawater refractometer
light flux meter
thermometer
microcontroller (Arduino)
PAR fluorometer (for photosynthetic activity)
Portable DO meter
Digital microscope (with camera)
Photographic camera
Digital balance
Sonicator

Tanks, piping, pumps
Wet lab items: glass-wares, pipette

Chemicals: calibration reference for pH meter, salinity refractometer, and for DO meter, DI water, acetone, NaOH standard solution.
Sea salt mix or natural sea water (200 L, assuming tank volume of 15L)
Disinfectants: ethanol or isopropyl alcohol, bleach
Coral feed
Notebook / PC for data logging

Data analysis plan:

This involves comparison of recorded data from 10 tanks with sunscreen exposure to that of the control. Compared parameters are listed in Table 2. For statistical analysis and data presentation open-source scientific tools (NumPy, SciPy and matplotlib)[12–14] will be used. This involves digitizing the recorded data as arrays on a PC.

Proposed Schedule:

1. **<u>Week 1-2 : Initial Setup of Water Tank:</u>**

o Coral samples are collected and fragmented to approximate size of 4-5 cm. This is appropriate for 15L of circulating water per tank.

o Tanks with volume 15L and pump for circulation are set up.

o Artificial Sea water is prepared using sea-salt mix and filtered, and sterilized. Preparation of artificial sea water is preferred due to the logistical cost of moving large amount of natural sea water.
Using product #S9883 from Merck, 600g of sea salt per 15 L of DI water is needed. pH adjusted using dilute solution of NaOH to 8.1-8.3.[11]

o Light source of intensity between 50-100 μmol/m^2/s (or equivalent) for 10-12 hours/day is set up with a timer (microelectronic controller). Lower light intensity is preferred to promote growth of symbiont alga.

o Temperature, and pH are constantly monitored (if possible, using a microcontroller). If not, available daily measurements will be performed.

o Corals are placed and we wait for their acclimatization (1-2 weeks)

o Prepare sunscreen with different concentrations for the introduction in the tanks. This involves testing the solubility of sunscreen in a transfer agent (ethanol or acetone). For control tank, the same amount of transfer agent will be added (without any sunscreen).

2. <u>Week 3 - Exposure to sunscreen:</u>

o Expose the corals to varying concentrations of sunscreen (in tanks A1 to E2).

o Daily measurements of chlorophyll fluorescence, dissolved oxygen and photograph, pH and temperature. Record data in a log book.

o Continuous monitoring of environmental conditions (temperature, salinity, pH, water flow, light).

3. <u>Week 4 to Week 5 - Data Collection and Analysis</u>

o Continue measuring chlorophyll fluorescence, dissolved oxygen and other parameters. Record data in a log book.

o Record growth data using digital balances and take photos for analysis. Record data in a log book.

o Microscopic investigation of the tissue section at the end of experiment to observe evidences of cellular damage or changes in coral structure. Record optical images.

o Prepare a report based on available data (for control and 10 tanks). Develop interpretation based on results.

Budget estimation:

Category	Item	Cost per item	No.	Total	Ref.
General equipment	Water tanks	6500	11	71500	https://www.kotobuki-kogei.co.jp/en/product/?cid=29

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	Pump	2500	11	27500	
	Microcontroller (with temperature, pH sensor)	4600	11	50600	https://axel.as-1.co.jp/asone/d/68-2108-35/?q=arduino%20micro
	Sensors	5000	11	55000	
	Lighting	4000	7	28000	https://shorturl.at/1kPA1
	Beakers	2000	2	4000	
	Gloves	5000	2	10000	
	Tubing	49500	3	148500	https://axel.as-1.co.jp/asone/d/80-0011-16/
	50mL sample tubes	5795	1	5795	https://axel.as-1.co.jp/asone/d/4-3632-02/
	Thermometer	84250	1	84250	https://www.jp.omega.com/pptst/HH911T-HH912T.html
	Glass container (250ml)	5100	4	20400	https://axel.as-1.co.jp/asone/g/NCGK072659/?cfrom=D0070100
	Glass container (500ml)	5500	4	22000	https://axel.as-1.co.jp/asone/g/NCGK072659/?cfrom=D0070100
	Glass container (1000ml)	7200	4	28800	https://axel.as-1.co.jp/asone/g/NCGK072659/?cfrom=D0070100
	Miscellaneous			75000	
	Shipping			50000	
Chemicals	Water (5L)	6400	33	211200	https://labchem-wako.fujifilm.com/jp/product/detail/W01W0104-1678.html

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	Sea salt mix (1kg)	18300	8	146400	https://www.sigmaaldrich.com/JP/ja/product/sigma/s9883
	Sodium chloride (for calibration of seawater refractometer sample prep)	1050	2	2100	https://labchem-wako.fujifilm.com/jp/product/detail/W01W0119-0166.html
	Sodium hydroxide (1N)	21500	1	21500	https://labchem-wako.fujifilm.com/jp/product/detail/W01ALF035629.html
	Acetone (3L)	5100	1	5100	https://labchem-wako.fujifilm.com/jp/product/detail/W01W0101-0035.html
	Ethanol(1L)	4800	2	9600	https://labchem-wako.fujifilm.com/jp/product/detail/W01W0105-0920.html
	Bleach (100g)	2650	2	5300	https://labchem-wako.fujifilm.com/jp/product/detail/W01W0103-1624.html
	Coral feed	3200	1	3200	https://algaeresearchsupply.com/collections/algae-research-supply-filter-feeder-formula-algae-culture/products/live-algae-filter-feeder-formula-1000ml
	pH meter calibration solution (500ml each for pH 4.0, 7.0 and 10)	12000	1	12000	https://www.jp.omega.com/pptst/PHA4_7_10.html
	miscellaneous			75756	

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	Notebook PC	248999	1	248999	https://axel.as-1.co.jp/asone/d/67-7578-30/
Human resource	5 weeks, 5 hours per day			137500	
Total (JPY)					**1560000**

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