BIOLOGY

ASSAYS

OVERVIEW

This chapter expounds on methods for assessing biological and chemical activity of extracts and samples which are mostly organic and plant-based. This includes bioassays and chemical assays that determine antibacterial activity, antimicrobial activity, antibiofilm activity, larvicidal activity, and inhibitory activity. The specific procedures used in the assays from the preparation of materials to the measurement of dependent variables are elaborated in this chapter. These test procedures vary according to the standardized tests employed by the researchers.

A. Antibacterial
(Grantoza, Mationg, Seguano, Oberio)

To prepare the different treatment concentrations, pure extracts were mixed with distilled water to obtain the intended concentrations using the following extract to water ratio: 1:3, 2:2, 3:1and 4:0. The following equation was used for the preparation of concentrations of treatments:

$$volume\ percent\ = \frac{volume\ of\ solute\ (mL)}{volume\ of\ solution\ (mL)} \times 100\%$$

The medium used for the antibacterial assay was Mueller-Hinton Agar (MHA), a culture media commonly used in antibacterial testing. Nutrient agar and nutrient broth were used for the revival process of the freeze-dried bacteria culture. The bacteria strains were inoculated into the nutrient agar by quadrant streaking to isolate pure colonies which were used for the assay. The media were placed inside the autoclave sterilizing it along with the petri plates, other glasswares and materials used for the experiment. Approximately 10 to 20 milliliters of the media were poured to each petri plate. The plates were cooled while the media solidified at room temperature and were then stored in the refrigerator at about 2- 8°C until use.

McFarland Turbidity Standard was used for adjusting the concentration of bacterial colonies suspended in the inoculum in preparation for inoculation on MHA plates. To make the 0.5 McFarland Turbidity Standard, 9.95 mL of sulfuric acid and 0.05ml of barium chloride were mixed in a test tube. The test tube was agitated well in order to mix the fine white precipitate of barium sulfate in the 0.5 McFarland turbidity standard tube (McFarland Standard 2014). If a dilution was necessary, a sterile pipette was used to add sufficient broth in order to obtain turbidity that would match the standard. A count of 1.50 x 108 colony forming units/mL was produced by the adjusted suspension.

Agar well diffusion method was used to test the antibacterial activity of the *D. alata* ethanolic extracts at different treatment concentrations (25%, 50%, 75%, 100%). Antibacterial assay plates were inoculated with respective bacteria before the agar wells were made. The extracts were then poured into the wells using a micropipette. The cultures were placed in the incubator at 37°C. Plates were initially checked for any zone of inhibition at the 18th hour of incubation, but data was only gathered after 24 hours of incubation. The diameter of the agar wells was 7 mm each. The zones of inhibitions on each plate were measured using a vernier caliper for more precise measurements in millimeters.

There were three readings in each plate with each four bacteria strains having six setups namely, the negative control (water), positive control (co-trimoxazole) and the treatment concentrations (25%, 50%, 75%, 100%). Each bacteria strain had 18 readings and the whole antibacterial assay process had a total of 72 readings all in all.

B. Anti-Biofilm
(Bat-og, Oreta, Villaflor, Jolito)

Antibiofilm activity of the mango peel extracts was determined through Crystal Violet Assay. Six petri plates were prepared and inoculated with the subculture. Approximately 30 mL of Tryptic Soy agar solution was poured into each plate. The petri plates were autoclaved at 121°C, 15 psi for 15 minutes.

From the stored pure culture, the *S.aureus* was transferred to a petri plate with an inoculating loop. Then, it was incubated at 37°C for 24 hours that allowed the bacteria to grow. After which, the culture was inoculated into two separate test tubes containing Tryptic Soy Broth.

Fifty µL of the culture was diluted with 5 mL Tryptone Soy Broth (1:100). 195 µL of the culture dilution were inoculated to individual wells of the 96-well plate underneath a laminar flow hood and left in the incubator to grow for 16 hours. After incubation, the plate was washed and air dried for 30 minutes to remove planktonic cells. 125-µL of the extracts were added after the bacteria formed a biofilm. It was incubated again for 16 hours at 37°C. Three replicates were done for each treatment, distilled water served as the positive control.

After incubation, the plate was washed by pipetting distilled water into each well twice. One hundred twenty-five µL of a 0.1% solution of crystal violet (CV) was added to each well. The plate was incubated again for 15 minutes at room temperature and then washed 4 times with distilled water and turned upside-down, left to dry overnight.

One hundred twenty-five (125) µL of 30% acetic acid was added to each well; then the plate was left to incubate for 15 minutes at room temperature. Absorbance was measured at 590 nm in a plate reader, using 30% acetic acid as a test plate ensuring the accuracy of the absorbance values.

The absorbance value of the blank was estimated to be zero with a range of -0.5 to +0.5. Their difference with the absorbance value of the experimental and positive control wells was equal to the value displayed by the plate reader. Absorbance values indicated the antibiofilm activity of each peel extract. After treatment, the remaining bacterial matter in the well was stained with CV. Relatively low absorbance values indicated that some of the biofilm had been dislodged by the peel extract.

C. Larvicidal

Larvicidal Activity of *Annona muricata* (Soursop) seed and Piper betle (Betel) leaf against *Aedes aegypti*
(Grande, Balmaceda, Taneña, Mediodia)

Larvicidal bioassays were conducted according to standard protocol from the World Health Organization 2005 guidelines for laboratory and field testing of mosquito larvicides in the Standards and Testing Division of the Industrial Technology Development Institute, Taguig, Manila. Preliminary testing of the extracts was carried out to establish the effective range of test concentrations by initially testing a wide range of concentrations which was later narrowed down until the lowest test concentration killed at least 10% of the population while the highest test concentration killed at most 90% of the population. Different concentrations of *A. muricata* extract (5 ppm, 10 ppm, 20 ppm, 30 ppm, 40 ppm) and *P. betle* extract (300 ppm, 600 ppm, 900 ppm, 1200 ppm, 1500 ppm) were prepared during the study. All experimental exposures were carried out using 200 mL beakers filled with 95 mL of deionized water. Twenty larvae which were a mix of third and early fourth instar were then added into each beaker and subjected to testing of the different test concentrations.

Three replicates were carried out simultaneously for each concentration. Mortality was recorded after 24 hours during which no food was offered to the larvae. The number of dead larvae was counted by transferring the larvae from the used beaker for testing onto a petri dish. The number of dead larvae was counted by transferring the larvae from the used beaker for testing onto a petri dish. A pasteur pipette was used to prod the larvae while observing for signs of movement. Larvae which were unresponsive to the prodding of the pasteur pipette were considered dead, whereas, responsive yet weak larvae were considered to be moribund. Mortality was recorded by counting the total number of dead and moribund larvae in a setup. Negative and positive controls were conducted alongside the established test setups with the negative control being 5 mL of 95% ethanol and the positive control being Abate.

Larvicidal activity of *Citrofortunella microcarpa* (calamansi) peel essential oil *Aedes aegypti*

(Carigaba, Leonida, Masculino, Mediodia, Garbo)

In the preliminary testing, the third and early fourth instar *Aedes aegypti* mosquito larvae were exposed to a wide range of test concentrations until there was a set of concentrations established that would give a larval mortality from 10% to 90%. The results were also compared to determine whether the dilution of the extract with the solvents involved had an effect on the mortality of the test organisms. For each concentration, at least four replicates were prepared. The mosquito larvae set-ups contained the appropriate volume of solution concentrations under the test. The same number of controls were set-up simultaneously.

For the final confirmatory test, the test organisms were exposed at concentrations ranging from 8 ppm to 11 ppm. After 24 hours, the mortality of the mosquito larvae for each set up was recorded. The mortality rate was determined by counting the number of deaths upon application of the formulated concentration. It was calculated using the following formula:

$$Mortality\ Rate\ = \frac{Number\ of\ dead\ larvae}{Number\ of\ larvae\ introduced} \times 100$$

The larvae were probed with a pasteur pipette and if there was no response from the larvae, which means that it did not move when it was probed, it was considered dead. In calculating the percentage mortality, moribund larvae, which are the larvae that are approaching death, were counted too and were added to the total number of dead larvae. Moribund larvae were qualified as those incapable of rising to the surface. They did not show any reaction when the water was disturbed.

D. Antimitotic

(Abellar, De Juan, Bela-ong)

After exposure of treatment to onion bulbs for 72 h away from direct sunlight, the number of cells undergoing prophase, metaphase, telophase, anaphase, and interphase was recorded, and the mitotic index (MI) was calculated. Chromosomal aberration was not evaluated due to limited equipment, and unavailability of the certain microscope required in order to perform the task.

After the completion of treatment, the roots (approximately 1-2 mm) were excised and collected, and immediately fixed in 3:1 (ethanol: acetic acid) for 24 hours (Tedesco and Laughinghouse 2012). Root tips were hydrolysed in 1N HCl for 15 min at 60°C, and stained with 2% orcein stain. After the

removal of root caps from well-stained root tips, a 1 mm of the mitotic zones was immersed in a drop of 45% acetic acid on a clean slide and squashed under a cover glass and examined microscopically (Ozmen and Sumer 2004). Red discoloured roots were observed under 100x magnification for different stages of cell division (Rintelen et al., 2017). Five hundred cells were analysed per root tip (Tedesco and Laughinghouse 2012), summing up to 4500 cells per treatment. The number of cells in each stage of cell division i.e. either prophase, metaphase, anaphase, or telophase, and including the non-dividing cells were counted and recorded. The average mitotic index of 3 root tips for each treatment was determined.

Mitotic index was calculated by using the formula:

$$Mitotic\ index = \frac{Total\ number\ of\ dividing\ cells}{Total\ number\ of\ cells\ examined}$$

E. Disk Diffusion

Antibacterial Activity of *Homalomena philippinensis* and *Merremia peltata L. Merr.* against *Staphylococcus aureus*
(Barrientos, Miraflores, Serisola, Mediodia)

Approximately 15 mL of Mueller-Hinton agar was poured into dry and sterile petri dishes. The medium was left to solidify for an hour. One loopful of bacteria was taken from the pure culture then streaked over the entire surface of the agar plate evenly. A pair of forceps was flame-sterilized. Using the forceps, a Whatman # 1 filter paper disc was picked up and immersed into the extracts for one hour. The bacteria was inoculated using an inoculating loop. After about 2 minutes, the moistened filter disc was laid gently on the seeded agar plate. The plates were inverted then incubated for approximately 18 hours.

Antibacterial Activity of Silver Nanoparticles
(Dogeno, Gamboa, Pefianco, Aban, Larroder)

Disk diffusion method was done to test the antimicrobial activity of the silver nanoparticles against *S. aureus*. The first agar plates were divided into four quadrants: each quadrant containing silver nanoparticles, silver nitrate, 15-g *M. oleifera sp.* seed extract, and distilled water. Filter disks were then placed in each quadrant. The petri dish was then incubated for 18 hours to let the bacteria culture grow. After the incubation period, the standard zone of inhibition (ZOI) was measured using a vernier caliper.

Antibacterial activity of copper-chitosan complexes
(Loquias, Placido, Mediodia)

Assessment of the antimicrobial activity of the synthesized copper-chitosan complexes was performed as outlined by the Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard 11th Edition by the Clinical and Laboratory Standards Institute (CLSI) at the Department of Science and Technology VI Regional Standards and Testing Laboratory. Mueller-Hinton (MH) Agar was utilized as a non-selective culture media. The experiment was carried out in triplicates.

The copper-chitosan complexes (0.1, 0.2, 0.5 wt% chitosan content) were suspended in distilled water and loaded onto blank sterilized Whatman No 1 filter paper disks. Ciprofloxacin (5 ug) served as the positive control in the form of commercially available ciprofloxacin-loaded antibacterial discs, while distilled water loaded onto blank sterilized Whatman No. 1 filter paper disks served as the negative control. Chitosan was also loaded onto blank sterilized Whatman No. 1 filter paper disks, totalling 6 treatments.

Prepared plates were incubated for 16-18 hours at 35±2°C in ambient air as recommended by the CLSI document M45-Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria.

The zone of inhibition of each treatment was measured using a Vernier caliper (read to the nearest mm). The susceptibility of *V. parahaemolyticus* to the copper-chitosan complexes was determined based on the breakpoint table provided by the CLSI test interpretation document M45-Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, wherein a zone of inhibition of > 21 mm indicates susceptibility to a compound tested with ciprofloxacin as the positive control while a zone of inhibition < 15 mm indicates resistance to the compound.

F. Minimal Inhibitory Concentration
(Barrientos, Miraflores, Serisola, Mediodia)

Thirteen screw capped 13 mm x 100 mm test tubes were sterilized and numbered accordingly. Using a 1.0 mL serological pipette, 1.0 mL of Mueller-Hinton broth was introduced into the 2nd to the 11th tube. For the 12th tube, 2.0 mL of Mueller-Hinton broth was introduced. Two mL of the prepared plant extract was pipetted into the first and second tubes. The second tube was vortexed for five seconds. Using a sterile 1.0 mL serological pipette, 1.0 mL of the contents of the second tube was aseptically withdrawn and transferred to the third tube, which was vortexed afterwards. The same process was continued until 1.0 mL was withdrawn from the ninth tube and subsequently added to the tenth tube. The contents of the tenth tube were vortexed. From the tenth tube, 1.0 mL was pipetted off the contents and it will be discarded.

One mL of the diluted bacterial inoculum was introduced into the tubes 1 to 11 and tube 13. In the 13th tube, 1.0 mL of the antibiotic standard was introduced. All tubes were tightly capped then the contents were vortexed. The tubes were incubated at 35°C for 16-18 hours. After the incubation period, the tubes were examined for bacterial growth by checking the turbidity in the tube. The tube with the lowest concentration of plant extract at which no growth or turbidity is observed was reported as the minimal inhibitory concentration (MIC) of the plant extract against *S. aureus*.

The MIC Assay was considered valid since the negative control tube has visible growth, the media control tube has no visible growth and the positive control tube has no visible growth.

G. α-Amylase Inhibitory Assay
(Alcalde, Tajo, Valencia)

Aloe vera extracts with different concentrations (0%, 20%, 40%, 60%, 80%, and 100% by volume) were first prepared in test tubes The diluting agent used is distilled water. The 0% sample had no aloe vera extract and only distilled water. Total volume of the different concentrations was 2 mL. 200 µL of 0.02 M sodium phosphate buffer, and 20µL of α-Amylase was added in the assay. In a concentration range of 10-100µL/mL, this solution was incubated at room temperature for 10 minutes. 20 µL of 1% starch solution was added on each test tube. 400 µL of 3,5 - dinitro salicylic acid (DNSA) reagent was added. The tubes were incubated in boiling water for five minutes and cooled at room temperature. The reaction mixture was diluted with 5 mL distilled water. The absorbance was measured at 540 nm using a UV-spectrophotometer. The control used is the blank sample and reference concentration is Acarbose, an α-amylase inhibitor.

H. Agar Well Diffusion

(Hembra, Henderin, Pareñas, Sinco)

Leaves of *Mangifera indica* (mango) contain phytochemicals which promote antibacterial activity. This study aimed to determine whether *M. indica* leaves extract can be an alternative antibacterial agent for triclosan.

The streak plate procedure was based on Sanders (2012) plating method. The medium was allowed to reach room temperature. Using an inoculum from the specimen, four-quadrant streak was performed to obtain well-isolated colonies. The specimen was contained on a swab, rolled several times over a small area near the edge of the plate, and streaked on the plate for isolation with a sterile loop starting where the swab was inoculated. The plates were then incubated at 35°C and examined after 24 hours.

A 0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175% v/v barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 mL of 1% v/v sulfuric acid (H_2SO_4) (Cockerill et al. 2012). Suspension of Staphylococcus spp. of 0.5 McFarland standard turbidity was done by visual comparison of the test tubes. To assist visual inoculum adjustment, bacterial suspensions were compared to McFarland standards against a Wickerham card (Hombach et al. 2015). The Agar Well Diffusion assay was performed in triplicates. Cultures of *Staphylococcus spp*. were inoculated separately on the solidified agar on each petri dish by streaking using a wire loop. About 100 mg/mL-1 of each test liquid soap was dispensed into separate wells using a 100 µg micropipette (Bbosa et al. 2007). The plates were then incubated at 37°C for 24 h. The sensitivity of the test organisms to the treatments was determined by measuring the diameter of the zone of inhibition surrounding the wells. After incubation, the diameters of the zones of inhibition were measured with a ruler read to the nearest mm (Bbosa et al. 2007).

COLLECTION

OVERVIEW

This chapter provides methods on the collection of various live and dead organisms as well as biological materials. This category covers factors regarding collection, storage, and transport such as proper timing, temperature, and handling. The conditions provided are necessary to ensure that the samples remain uncontaminated and suitable for use in research.

A. Mollusk Shells
(Faciolan, Leonora, Majaducon, Sinco)

Chromium in its hexavalent state, Cr^{6+}, is one of the prevalent heavy metals in aquatic ecosystems with its occurrence primarily attributed to industrial activities such as dye manufacturing and construction run-off. This paper presents the removal efficiency of organo-mineral composites from the shells of three mollusks abundant in the Philippines: *Crassostrea iredalei* (Slipper Cupped Oyster), *Perna viridis* (Green Shell), and *Telescopium telescopium* (Horned Snail).

Shells were obtained from a local wet market and were sent for speciation to Dr. Laureen Manalo, mollusk specialist at the University of the Philippines Visayas for authentication and confirmation of *Crassostrea iredalei* (*C. iredalei*), *Perna viridis* (*P. viridis*), and *Telescopium telescopium* (*T. telescopium*).

Shells were separated by species then boiled at 100°C for 10 minutes. Organic matter contained within the shells were removed prior to fragmentation by shattering. The shell fragments were then rinsed with distilled water. Subsequently, they were then oven-dried at 105°C for a total of 24 hours as prescribed by Zukri et al. (2018). Dried samples were then crushed into fine powder and sieved to particle sizes of ≤ 63 µm.

B. Blood

Sourced from Blood Bank
(Maquiling, Villanueva, Oberio)

The blood samples were acquired from the blood bank at the start of day 0. The blood bank ensured that no contaminants nor pathogens that can affect the results were present in the samples by analysis of Transfusion Transmitted Infections (TTIs). In order to ensure confidentiality, no name, age, sex, or any other personal data of the donor was given to the researchers.

Blood samples
(Alvarez, Oberio)

The blood samples were acquired from a local blood bank agency on Day 0. From each of the three donors, 450 mL of blood was extracted by a phlebotomist in a triple bag. CPDA-1 contained in the bag was used as an anticoagulant after blood extraction. The blood bank ensured that no contaminants or pathogens that can affect the results were present in the samples by analysis of Transfusion Transmitted Infections (TTIs). In order to ensure confidentiality, no name, age, sex, or any other personal data of the donor was given to the researchers.

C. Phytoplankton
(Cordova, Demandante, Occeña, Bela-ong)

The vertical tow as described by Milroy (2015) was used as the phytoplankton sampling method. To achieve deeper column sampling, collection of samples was conducted during high tide hours.

Phytoplankton sampling was conducted with the use of a conical plankton net with mesh size of 25 µm, a mouth diameter of six inches, and clamped rubber tube at the cod-end which collects the phytoplankton cells. The plankton net was acquired from University of the Philippines-Visayas (UPV), Miag-ao with a 50-cm mark as graduation. The net was lowered just above the bottom of the water and hauled vertically to the surface at a consistent speed of approximately one meter per second. To achieve a brisk pace while lowering the net, a weight of 2 kg was added to its cod-end. This essentially "flushes out" the net as it is lowered into the water. Once the net has been pulled back to the surface, the content of the cod-end was then transferred to properly-labeled, plastic, screw-cap sample bottles with graduations every 50 mL. The vertical tow was conducted until the phytoplankton sample amounted to over 200 mL in the sample bottle. The depth of each sampling site was determined using a Garmin Fish Finder and recorded.

The 5% Lugol's Iodine solution, purchased from Patagonian Enterprises, Jaro, Iloilo City, was used for the preservation of phytoplankton cells. Five drops of 5% Lugol's Iodine solution were added to each sample bottle.

D. Seaweeds

Handpicking

(Almarza, Gatila, Inosanto)

The three most abundant seaweed samples were collected at the coast of Taklong Island, Guimaras at around 15:00 to 16:00 in three different sampling sites. The seaweed species were handpicked via snorkeling at varying depths within the range of one (1) to two (2) meters. They were placed inside Ziploc® bags filled with seawater and were transported to the laboratory inside an icebox. They were then put inside a freezer with a temperature of -24°C for storage.

The seaweed samples were brought to the laboratory and washed with tap water to remove attached coral parts, stones, and epiphytes, and to thaw ice bits that formed around the samples from being stored in the freezer. The samples were then sun-dried for at least an hour to remove any excess moisture that may affect the measurement of dry weight of the samples.

E. Fish Samples

Clarias gariepinus* and *Chanos chanos

(Constantino, Guillergan, Yabut, Navarro)

Fish samples used for the study were chosen via convenience sampling. *Clarias gariepinus* samples were sourced from Zarraga since, according to Locara (2000), it is the main producer of catfish in Iloilo. Meanwhile, according to White (2013), Dumangas has one of the highest farmed milkfish production in recent years. Collection of ten samples of *C. gariepinus* from Beboy Pantatan in Zarraga, Iloilo was conducted every 4:00 PM, one day before the dissection. The number of fish samples collected per day was determined by the number of fish samples that can be completely dissected and examined in one working day. Fish samples were stored in aerated fry bags, along with water from the pond where the samples were harvested (Truter et al. 2017). Fry bags were placed in large buckets for transportation, with a total number of five (5) fish samples for each bucket. All 30 *Chanos chanos* samples were collected from a fish farm in Dumangas, Iloilo. The fry bags were pumped with oxygen and filled with water from the pond where the samples were harvested. Bags were then placed in large buckets (Villaluz 1984). *Chanos chanos* samples were transported to the Microtechnique Laboratory of SEAFDEC/AQD in Tigbauan, Iloilo for the dissection and examination of the gills and intestines. Different batches of fish samples were dissected on different dates.

Chanos chanos (milkfish)

(Cadorna, Chan, Salmon, Salazar)

Fresh-caught, farmed *Chanos chanos* (milkfish) with fork lengths of 20.2 to 26.8 cm, total lengths of 26.1 to 34 cm, and weights of 123 to 252 g, were collected from the Southeast Asian Fisheries Development Center (SEAFDEC) Brackishwater Station in Dumangas, Iloilo. After capture, the fish were immersed in icy water to shock them. Euthanasia was performed shortly after capture through spiking or iki jime, the physical destruction of the central nervous system. A dissecting needle was inserted into the cranial cavity of the skull found above the eyes and moved in back-and-forth and sideways motions to destroy the fish's brain and spinal cord (Davie and Kopf 2006). The fish were individually placed inside ziplock bags and immediately transported to a laboratory in Philippine Science High School - Western Visayas Campus in polystyrene boxes with 1:1 fish-to-ice volume ratio (Yeasmin et al. 2010). The fish were separated into two groups, one was immersed in 5% formalin for five (5) minutes (Yeasmin et al. 2010) while the other remained untreated. The fish samples from the two groups were stored in a chest freezer at a temperature of 0°C to 5°C and observed every 24 hours for seven days.

F. Bacterial Sample from Plastic Waste

(Canja, Hilis, Galan, Jolito)

Plastics are known for being durable materials while still maintaining a low cost of production. It is a very important material for commercial use all over the world. However, due to the lack of a reliable method of disposal, the risk of plastic pollution is steadily increasing throughout the years. This study aims to isolate and extract bacteria from the Iloilo City Engineered Sanitary Landfill, in Mandurriao, Iloilo City, and to assess their biodegradation potential on LDPE (Low-density polyethylene), HDPE (High-density polyethylene), and PET (Polyethylene terephthalate).

The bacteria were extracted from plastic waste using swab sampling and from soil samples which were taken from the dumpsite. The plastic wastes were randomly selected and a total of five swab samples were collected. The swabs sealed inside test tubes with tryptic soy broth were transported in a laboratory in PSHS-WVC for testing. For the soil, five samples were collected from a depth of 10 - 20 cm, then placed inside sterile containers and kept at a temperature of 4°C. (Bolo et al. 2015)

Random sampling was done by having an aerial view of the area (Coordinates: 10°42'34.7"N 122°31'25.8"E) and section it equally into 25 sites. Five randomly selected sites were chosen using a random number generator as shown in Figure 1.

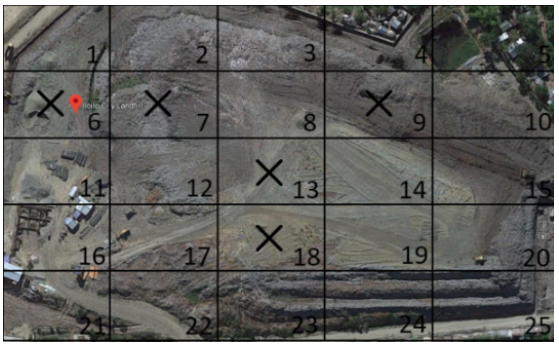

Figure 1. Sites were sampled by random selection.

Site 1 is located in area 7, site 2 in area 6, site 3 in area 13, site 4 in area 18, and site 5 in area 9.

G. *M. Indica* (Mango) Leaves

(Hembra, Henderin, Parenas, Sinco)

Leaves of Mangifera indica (mango) contain phytochemicals which promote antibacterial activity. This study aimed to determine whether M. indica leaves extract can be an alternative antibacterial agent for triclosan. The M. indica leaf samples were collected from Orchard Valley, Tigum Barrio Road, Pavia, Iloilo and were stored in a mesh bag. Prior to identification, the samples were then identified by the Department of Agriculture (DA) in Sta. Barbara, Iloilo.

DESIGN

OVERVIEW

When designing a device involving biological organisms, it is important to consider mimicking their natural habitats. In the case of Ampunan, Placer, and Robles, the natural habitats of spiders were considered in ensuring that each sector of the device was at least three times the width of a spider leg, the device temperature was regulated, and that the environment was dark. This is to ensure comfort and longevity of the living subjects. It is also important to break down the device into smaller, manageable parts, with specific and accurately measured dimensions . Finally, in the use of software such as CorelDRAW, it is advised to seek out the help of different professionals.

A. **UV-Visible**
(Ampunan, Placer, Robles, Manalo, Olvido)

The design for the spider web collector consists of three main parts: (1) cover, (2) storage units, and (3) base. In order to ensure accuracy, the design was rendered on CorelDRAW, and the cutting process was done using a laser cutter.

The cover is made up of two layers of clear acrylic sheet. For the top layer, 13 cm by 13 cm squares were cut out. These cutouts were used as coverslips and were bored with 9 holes to serve as ventilation. For the bottom layer, squares of 11 cm by 11 cm were cut out. These cutouts will not be included in the design. The bottom layer was glued with the top layer to act as the holder for the coverslips. A tape was attached to one side of the coverslip to serve as a tab for easier access.

The whole storage unit is composed of a grid of five by five square units. It consists of four outer walls, four inner long panels and 20 smaller panels. These walls were glued together to form storage units measuring 15 x 15 x 15 cm each (recommended area for *P. phalangioides* leg length).

For the base, the area was calculated with the average body length of the spider in consideration in order to avoid harming the spider during web collection. Holes were bored at the center of each storage unit. Wooden rods of three mm-diameter were inserted to these holes, which served as the web collectors. The walls around the base limit the movement of the storage units during web collection.

The spider web collector was assembled by placing the base at the bottom, the storage units in the middle, and the cover at the top. The interior of the storage units including a part of the base below the storage units were then painted black which provided a darker environment which is ideal for the spiders.

DISPOSAL

OVERVIEW

This chapter includes methods on the disposal of bacteria-related waste. It is important to first ascertain the specific laboratory safety protocols of the particular laboratory the researchers are working in. Waste must always be properly labeled and categorized according to levels of hazard and risk assessment. In particular, glassware must be segregated based on the type of substance they have handled. Once it is time to dispose of these, waste disposal measures must be followed to avoid the spreading of bacteria to different places, and to avoid unnecessary risk towards unrelated individuals.

A. **Culture**
(Barrientos, Miraflores, Serrisola, Mediodia)

Proper waste disposal methods were followed for all materials. All used agar and broth cultures were decontaminated immediately after the conduct of the experiment by mixing in hypochlorite for approximately an hour inside the biosafety cabinet. All of the spent agar media and broth were disposed of in the hazardous wastes bin. All glassware, including the culture tubes and dishes cleaned using antibacterial soap.

DISSECTION

OVERVIEW

This chapter includes methods for dissecting fish samples as preparation for further examination. This covers the extraction of the gill filaments and internal organs of the organism, as well as procedures to ensure optimum sample quantity upon examination.

A. Fish
(Constantino, Guillergan, Yabut, Navarro)

Fish samples were dissected by first cutting the left and right operculae open. The gill arches were then cut and removed from the cavity using dissecting scissors and tweezers. The gill filaments were then cut and removed from the gill arches, placed on a glass slide, and covered with a cover slip. An incision was then made from the anus of the fish up to its mouth, exposing the digestive tract (Fish Dissection 2019). The fish samples were then eviscerated. The small and large intestines were removed and placed on a petri dish filled with freshwater for *Clarias gariepinus*, and seawater for *Chanos chanos* to mimic the salinity of the fish pond where the fish species were collected.

ETHICAL CONCERNS

OVERVIEW

This chapter includes ethical considerations in performing specific procedures as well as the appropriate measures to maintain the integrity of the study. Research ethics provides the standards for conducting scientific research. It is important for researchers to adhere to these standards especially when handling living organisms out of respect for their dignity, rights, and welfare.

A. **Human Blood Samples**
(Maquiling, Villanueva, Oberio)

The blood samples were acquired from the blood bank at the start of day 0. The blood bank ensured that no contaminants or pathogens that can affect the results were present in the samples by analysis of Transfusion Transmitted Infections (TTIs). In order to ensure confidentiality, no name, age, sex, or any other personal data of the donor was given to the researchers.

EVALUATION

OVERVIEW

Evaluation is integral in analyzing and comparing the results of different experimental setups. It is a powerful tool when verifying the validity of test results in comparison to negative and positive controls. In evaluation, young researchers might consider finding related articles with the particular evaluation method that fits their research topic. Using this as a basis, methods may then be adjusted according to the specific needs of the study. One must also consider the software limitations at hand. It is best to identify a free software program which may be learned in a viable amount of time. Finally, in doing statistical analysis, researchers are advised to consult with experts to ensure their methods are accurate.

A. Fleshy Macroalgal Index
(Dalabajan, Hilay, Velasco, Navarro, Olvido)

The FMI was measured using the photoquad method. Photoquad method provides visual estimation but in a digital version. The remaining macroalgae attached to the rope were photographed. After taking a picture, the photo quadrant analysis software, photoQuad , quantified the FMI using the grid cell count option. A direct estimate of the species cover was automatically performed. The software offers a more versatile, quick and accurate result. The FMI of each unit will be measured before and after the exposure *T. gratilla* for comparison.

B. Biodiesel Properties
(Almarza, Gatila, Inosanto)

Sixteen biodiesel properties were calculated using the Fatty Acid Methyl Ester (FAME) composition of each species. Fuel properties derived from FAME profiles are the following: degree of unsaturation (DU), long chain sat- uration factor (LCSF), cold filter plugging point (CFPP), iodine value (IV), saponification value (SV), cetane numbers 1 and 2 (CN1 and CN2), saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFA), poly-unsaturated fatty acids (PUFA), kinematic viscosity, density, higher heating value (HHV), amount of C18:3, number of double bonds (Db), and oxidation stability.

C. Platelet Count and Morphology
(Maquiling, Villanueva, Oberio)

After extraction, platelet count was measured by running a sample through the hema-analyzer three times and taking the average. Mean platelet volume was measured similarly to the platelet count by running a sample of 1 mL through the hema-analyzer three times and taking the average. The pH was tested using a pH meter by sampling a 10 mL volume of the platelet concentrates and washing the bulb of the pH meter after every measurement. All successive measurements were then taken on Days 3 and 5 of storage.

Platelet morphology was analyzed by photographing microscope smears of the platelets in each setup and manually counting the ratio of activated platelets to the total number of platelets. For a qualitative comparison, microscopic analysis of the samples involving the shape and concentration of platelet change was also done to show their shape and configurations. Discoid and irregular shapes were noted among the platelets. Six photographs were taken from each slide and were then gridded and printed into paper for manual counting. A four-by-four area was then used to count the platelets. The number of irregularly-shaped platelets was then divided by the total number of platelets to obtain the percent change of platelet morphology.

D. Germination
(Legurpa Mi, Legurpa Ma, Oberio)

Seed priming is a technique used to improve the overall germination behavior of rice through the imbibition of solutions. This study employed three different priming methods (mannitol, glycerol, and sorbitol) on two local Philippine varieties of *Oryza sativa* (rice) and compared germination behaviors between primed and unprimed seeds.

Eight (8) setups were used for the two varieties (Red and Black) treated with three different priming agents and control of untreated seeds. Twenty-five seeds were sown in one 90-mm Petri dish which contained one layer of Whatman No. 2 filter paper moistened with 10 mL of water which was measured using a syringe. Each setup had three replicates which were made up of six Petri plates for a total of 150 seeds in each replicate and 144 Petri plates for the whole study.

Every day at 7 am in the morning and at 3 pm, seeds were watered using the syringe or as needed to maintain a 100% moisture level. Natural light was allowed to pass through the windows. The temperature was measured and recorded at 4 pm in the afternoon every day using a wall thermometer. Conditions were also observed and recorded such as the presence of insects, animals or any other factor that might affect the study. At 3 pm the number of seeds that have germinated was counted. A seed was deemed to have germinated once it reached a radicle length of 2 mm which was measured using a vernier caliper. These results were then recorded using Google Spreadsheets.

On the 7th day of the study, the seedling shoot and root length were measured. Two of the longest germinated seeds in each Petri plate were chosen to be measured. This was done using a Vernier caliper. These results were then recorded using Google Spreadsheets.

The germination percentage, germination rate index and shoot/root ratio were calculated using the formulas stated in Salah et al. 2015 and Awasthi et. al 2016.

Germination Percentage

$$GP = \frac{\text{Number of Total Germinated Seeds}}{\text{Total Number of Seeds Tested}} \times 100$$

Germination Index

$$GI = \sum(G_t/T_t)$$

Where the number of germinated seeds on Day t and Tt is time corresponding to Gt in days.

Shoot/Root Ratio

SL: RL

Where SL is the length of the shoot and RL is the length of the root.

EXTRACTION

OVERVIEW

This chapter provides different methods on how to prepare an extract from raw materials. Determining the type of method or solvent to be used based on the polarity of the compound to be extracted is essential in attaining a high extraction efficiency. Other factors that should be considered include particle size, temperature, duration, etc.

A. Ethanolic

Dioscorea alata (purple yam) peels

(Grantoza, Mationg, Seguano, Oberio)

The powderized samples were mixed with 95% ethanol and soaked for 48 hours (Khonkarn et al. 2010) in a 500-mL Erlenmeyer flask. Flasks were sealed with cling wrap and covered with black paper to prevent light from affecting the compounds present during the soaking process. The flasks were set aside at a safe place at room temperature. After soaking, the mixture was filtered using Whatman no. 1 filter paper into another erlenmeyer flask. Solid residues from the first extraction were further pressed for additional extracts and the products obtained from pressing were mixed with the earlier filtered extract. Filtered plant solution was set up for rotary evaporation (Eyela Autojack NAJ-100) at 40˚C with 100 rpm to remove solvent from the solution. Temperature used during rotary evaporation was decided using the Δ 20˚ Rule commonly used by scientists for effective extraction. Acquired crude extracts were contained in a sterilized erlenmeyer flask and was then sealed with a cling wrap. The flask was again covered with aluminum foil in order to prevent light from passing through it.

Clitoria ternatea (Asian pigeonwings)

(Triol, Dionela, Ecube, Mediodia)

The collected *Clitoria ternatea* flowers were washed with distilled water and oven-dried at 75˚C for 24 hours (Wang et al. 2014). The dried petals were ground into a fine powder using a NutriBullet blender. Amber bottles were filled with 125 g of powdered *C. ternatea* flowers, and the powdered petals were macerated in 95% ethanol with 1:10 mass to volume ratio for 24 hours. The mixture was filtered using No. 41 Whatman filter papers and was subsequently filtered using No. 1 Whatman filter papers (Suebkhampet and Sotthibandhu 2013). The filtered extract was then subjected to rotary evaporation (Ika Hb Digital) to obtain the crude extract, which was dissolved once again in 95% ethanol in a 1:1 mass-of-extract-to-volume-of-solvent dilution ratio.

Mangifera indica (mango) leaves

(Hembra, Henderin, Parenas, Sinco)

Leaves of *Mangifera indica* (mango) contain phytochemicals which promote antibacterial activity. This study aimed to determine whether M. indica leaves extract can be an alternative antibacterial agent for triclosan.

The *M. indica* leaf samples were washed with tap water twice (Krishnanda and Shabaraya 2016) then rinsed with distilled water to remove unwanted residues. Based on the methods of Zakaria et al. (2006) the leaves were then oven-dried, however, the drying period was extended to four days because the leaves were not crisp dry after 24 hours of oven drying. The leaves were then cut into small pieces using a pair of scissors and were powdered using a Hanabishi kitchen blender. The powdered samples were then macerated for 72 hours using 95% ethanol as the solvent and the resulting mixture was filtered using Whatman no. 1 filter paper (Diso et al. 2017), properly positioned inside a glass funnel. After which, the remaining solvent was then evaporated using the Biobase RE100-Pro rotary evaporator at 40˚C with 100 rpm (Zakaria et al. 2006).

B. Methanolic
(Venturina, Comuelo, Samaniego, Jolito)

Extracts from plants such as Bixa orellana, commonly known as Annatto, presents a potential to be an alternative to the commonly used synthetic stains. Thus, this study aimed to evaluate Bixa orellana methanolic extracts as a substitute for safranin in Gram Staining.

Bixa orellana extracts were used to stain *S. aureus*, *E.coli* and mixed bacteria smears. The powdered seeds (100g) were added to 1 L of methanol (CH_3OH) and stirred for 12 hours using a magnetic stirrer and stored without sunlight at room temperature for 12 hours. The extract was then filtered using Whatman Filter Paper No. 1 and was placed in an IKA RV10 Rotary Evaporator at 40°C until all methanol had evaporated. The extract was then stored in a refrigerator prior to staining.

As a confirmatory test, the crude extract (0.25g) was dissolved in 10 mL methanol (CH_3OH) and its pH was then measured using a pH meter. A 50 ppm solution of the reconstituted solution was prepared to find its absorbance which was measured with a UV-Vis spectrophotometer at 300-650 nm and compared to the graph of pure bixin.

C. Hot-water
(Yap, Bungay, Alfonso, Libo-on, Cordero)

Gracilariopsis heteroclada samples were sun-dried for 48 hours. After, it was powderized in a corn mill grinder and was sifted using a 250 um mesh. Thirty (30) grams of powder were boiled in 900 mL of distilled water for three hours at 100°C. The solution was filtered using a cloth and a sieve of 250 µm mesh size into the metal trays. The trays were frozen overnight at -80°C then lyophilized for 24 hours thereafter at 2 mmHg.

D. Crude Extract
(Elizalde, Herida, Jaudian, Mediodia)

Prior to extraction, the collected samples were sundried for 6 hours under sunlight and air dried in the laboratory at night for two days to greatly reduce the moisture of sponges. They were then minced into the finest possible particles. The dried sponges were stored in the refrigerator to prevent any contamination prior to extraction. The extraction process involved creating a mixture using 200 mL of methanol for every 25 g of dried *Callyspongia sp*. The mixture was agitated for two minutes every 30 minutes for two hours in a 500 mL Erlenmeyer flask. The mixture was brought to the Department of Science and Technology (DOST) Region 6 to collect the extract by evaporating the methanol using a rotary evaporator. The resulting extract mixture after evaporation was approximately 80 mL and was stored in an Erlenmeyer flask. The remaining evaporation was done in Philippine Science High School – Western Visayas Campus using a water bath at 40°C. The final crude extract collected weighed 0.53 grams and diluted in sterile distilled water to obtain an initial concentration of $4.8x10^{-2}$ g/mL.

E. Lipids
(Almarza, Gatila, Inosanto)

The sun-dried samples were minced using a pair of scissors as a means to pulverize them. Triplicates of one-gram minced seaweeds were measured for each species using a triple beam balance. The weighted seaweeds were placed inside test tubes and were added with one milliliter of chloroform and two milliliters of methanol resulting in a 1:2 ratio. The weighted seaweeds were placed inside test tubes and were added with one milliliter of chloroform and two milliliters of methanol resulting in a 1:2 ratio. The mixtures were vortexed for six minutes and an additional one milliliter of chloroform was added again and vortexed for another 30 seconds.

Finally, one milliliter of distilled water was added and vortexed for 30 seconds resulting in a final ratio of 2:2:1 of chloroform: methanol: distilled water mixture.

The mixture was filtered using a glass funnel and a filter paper setup in order to obtain their liquid components. The residues were then transferred into another set of test tubes where the separation of phases was going to take place. In order to do that, the samples were centrifuged at 1000 rpm for five minutes at room temperature. The upper phases (methanol layer) were siphoned out using a Pasteur pipette. The lower phases (chloroform layer) were then transferred to their respective preweighed test tubes.

The filtrates were treated to a hot water bath using a hot plate with the temperature set at around 185°C until the remaining chloroform contents of the solutions were removed completely, leaving the surface of the base of the test tubes clad with lipids only.

F. Chitosan

(Janiya, Lopez, Magtoles)

Five hundred (500) grams of crab shells were washed and dried under direct sunlight. Cleaned shells were crushed and put in a 1L beaker with 4% NaOH to boil at 100°C for one (1) hour. The samples were removed from the beaker and cooled down at room temperature. The samples were again crushed and pulverised to produce a powder using a mortar and pestle. The pulverised crab shells were sieved using a 0.2-mm mesh sieve to acquire finer particles of crab shell powder. The sample powder was then demineralized by adding 1% HCl to the sample powder in a ratio of 1:4 w/v powder to HCl for 24 hours. The HCl was drained from the mixture.

The sample powder was then treated by adding 2% NaOH solution in a 1:2 w/v ratio powder to NaOH solution. This treatment was done for one hour to remove the albumen. The treated powder was washed using distilled water. The sample was then soaked in boiling 50% NaOH for two (2) hours to deacetylate. The powder was filtered from the beaker and cooled down at room temperature for one (1) hour. The powder was washed carefully with 500 mL 50% NaOH. The powder was oven dried at a temperature of 120°C for 24 hours.

G. Hydroxyapatite

(Janiya, Lopez, Magtoles)

Five hundred (500) grams of crab shells were crushed to produce smaller pieces of shells by using a mortar and pestle. The crab shells were boiled in distilled water for two (2) hours to remove adherent meat. The crab shells were filtered from the beaker and air dried. The crab shells were again crushed and pulverised and were then sieved using 0.2 mm sieve to acquire fine particles. The acquired powder was heated in a furnace at 1000°C for five (5) hours for it to calcinate and form Calcium oxide powder. After heating, it was cooled down at room temperature. The powder was then mixed with distilled water in a 1:1 w/v ratio solution.

The phosphoric solution in 0.6M was added dropwise resulting in a 5:3 m_{powder}/v_{H3PO4} solution. The solution was kept at room temperature for 24 hours to precipitate. The precipitate was filtered using a Whatman No. 40 filter paper and washed carefully with distilled water. The precipitate was oven dried at 120°C for three (3) hours and sintered by heating it in a furnace at 900°C for four (4) hours.

H. Aloe Vera Plant
(Alcalde, Tajo, Valencia)

The leaves were carefully removed from the aloe plant. The roots were left in the pot to insure regrow of the plant. The leaves were separated and washed. The aloe gel was separated from the latex and rind by scraping using a stainless steel spoon. The fresh leaf gel was homogenized using an electric blender while the latex and the rind was powderized, also, using a blender.

The aloe gel was tested by the assay first, then the latex and rind were tested by the alpha amylase test two days after. The same aloe plant was the source of the aloe gel and aloe latex with rind. The latex and the rind was sun/shade dried for two days. The dried portions were manually broken to smaller pieces using a mortar and pestle and were further powderized using an electric blender. The aloe vera latex and rind were then placed in solvent-solvent extraction to be able to extract the constituent.

I. Microplastics from Sediment Samples
(Colacion, San Diego, Secondes, Oberio)

The obtained sediment samples were carefully placed onto separate aluminum foils. They were oven-dried at 60°C for 48 hours. Two sieves of 4 mm and 2 mm grid sizes were washed with distilled water and dried using a paper towel. They were stacked together with the 4 mm sieve on top of the 2 mm sieve. The dried sediments were passed through the sieves. The retained fraction on the 4 mm and 2 mm sieve were properly disposed of while those which passed through were temporarily stored for further processing.

Sieved samples of mass 500 g were mixed with 2000 mL of saturated NaCl solution. The mixture was stirred for five minutes. After stirring, the mixture was allowed to stand for an hour. A filtration setup utilizing Whatman No. 41 filter paper was constructed, as illustrated in Figure 2. The supernatant was carefully poured into the filtration setup to acquire the floating particles. The particles retained on the filter paper were allowed to dry inside a drying oven at 50°C for 48 hours.

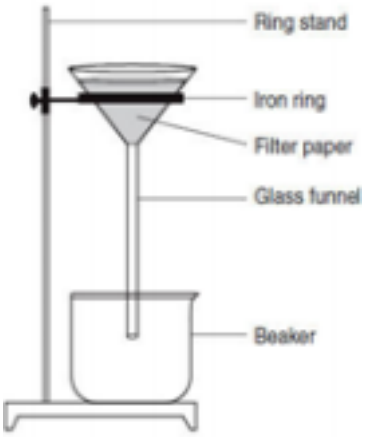

Figure 2. Set-up for density separation of microplastics

The oven-dried particles from the density separation step were transferred into a 50 mL glass beaker through gentle scraping with the use of a spatula. Twenty milliliters (20 mL) of 30% hydrogen peroxide was added to the beaker. The mixture was incubated at 60°C for 24 hours. The mixture was covered with a watch glass for the entire duration. The solution was filtered using Whatman No. 41 filter paper, and the acquired particles were oven-dried at 50°C for 48 hours before being stored inside glass Petri dishes.

IDENTIFICATION

OVERVIEW

This chapter covers methods involving morphological and molecular techniques in the identification of various organisms ranging from the microscopic to the macroscopic level. This category will specifically discuss the use of equipment and identification guides to distinguish species of parasites, seaweeds, and plants based on their form, structure, and appearance and the use of DNA sequence to identify bacterial species.

A. Bacteria
(Suarez, Apdon, Balinas, Baldonado, Hernando)

A TCBS agar plate containing the cultured bacteria with isolated colonies was packed tightly then secured to be delivered to the Philippine Genome Center in the National Institute of Molecular Biology and Biotechnology in the University of the Philippines Diliman for the 16S ribosomal RNA Extraction to Sequencing.

When the results arrived, the FASTA-formatted sequence was checked using the BioEdit software and was then inputted in the NCBI-GenBank. Basic Local Alignment Search Tool (BLAST) was used to find matching sequences in the NCBI databank. The highest matching sequence identifies the species of the bacteria.

B. Seaweed Species
(Almarza, Gatila, Inosanto)

Small representative portions of each individual sample were preserved in 70% ethanol for verification purposes. The species were identified by comparing the morphological characteristics of the samples to existing photographs of the species found on Taklong Island, Guimaras. The identified species were *Dictyota dichotoma*, *Sargassum cristaefolium*, and *Padina minor*.

C. Parasites
(Constantino, Guillergan, Yabut, Navarro)

The gill filaments were examined under the Olympus BX51 compound light microscope and the Howell Binocular Compound Microscope at 40x and 100x magnification. Photographs of parasites present in the gills were then taken at 100x magnification. The intestines were teased and examined under the Howell Binocular Stereomicroscope. Parasites found in the gills and intestines were identified up to the genus level based on morphology, using the book Health Management in Aquaculture by Cruz-Lacierda et al. (2001) and further verified by Mrs. Gregoria Pagador of the Fish Health Section of SEAFDEC/AQD. After identification, parasites were counted based on their types.

INCUBATION

OVERVIEW

This chapter covers methods involving incubation, particularly of chicken eggs. This category will specifically identify the equipment, temperature range, and relative humidity utilized and discuss the processes adapted such as egg candling and its corresponding criteria for exclusion of samples, and egg turning to ensure the integrity of the incubated eggs for research.

A. **Chicken Eggs**

(Aguirre, Sombiro, Valdestamon, Mediodia)

All chicken eggs purchased were put inside a manual industrial egg incubator with a temperature range of 38°C to 38.5°C and relative humidity of 57%. The eggs were candled on the third day of incubation to check their fertility, as measured by the visible growth of blood vessels in the egg when viewed under a candler. All infertile eggs were properly disposed of after candling. During the entire incubation period, the eggs were turned to a 45° angle from their vertical positions six times a day for seven days to ensure balanced temperature flow throughout the eggs and prevent the blood vessels from sticking to the shell.

INFECTION

OVERVIEW

In determining the efficacy of treatments against infectious disease, experimental units can be initially challenged with the pathogen and then administered with the treatment. As in this chapter, for example, the method involving in vivo infection of a viral disease in chickens is described.

A. Virus

(Sira, Valzado, Larroder, Cabarles)

Preparation of Virus-concentrated Solution

The LaSota vaccine, bought from a local livestock supply store, was diluted with a vaccine solvent specifically for freeze-dried avian vaccines provided by CPU - CARES. The ratio of the vaccine to the solvent was modified by the Department of Agriculture (DA) so that the final solution would be more concentrated with live viruses.

Infection of Samples

The final solution was injected to either the thigh or the breast part of the chicken samples. Cotton was also soaked with the solution and was taped at the top of the cages to contaminate the air with the virus. This was done under the supervision of Dr. Jaime Cabarles Jr. of CPU - CARES and Dr. Jonic Natividad of DA.

MICROBIOLOGY

OVERVIEW

This chapter is broad and it covers methods encompassed by the field of microbiology. The field includes many sub-disciplines (e.g. bacteriology and virology), all defined by a common object of interest which is microscopic organisms. The chapter includes culture methods, staining, assays, and use of equipment.

A. Microplate Use
(Cabalfin, Gerona, Labrador, Remaneses, Mediodia)

A 96-well microtiter plate was used to grow the biofilms of *S. aureus*. 200 L of the 80 Biofilm Eradication previously diluted liquid media was transferred to columns 2, 4, 6, 8 and 10 of the plate using a micropipette. The microtiter plate was sealed and placed in the incubator for 24 hours at 30°C. (Yadav, 2015). This was conducted inside the laminar flow hood.

B. Isolation & Culture

Algae Isolation
(Yeban, Baranda, Antenor, Sinco)

Samples from respective CM and F/2 media were dispensed to 15 mL test tubes for serial dilution of 10^{-1} to 10^{-10}. An inoculum of 1 mL was added to 9 mL of the respective media, with CM for brown algae and F/2 media for green algae. The 10th and 9th inoculated samples were kept in capped test tubes to avoid contamination, and exposed to a 40-W fluorescent lamp, continuous lighting at room temperature, 25°C (± 2°C) for a minimum of one week.

Agar-selective Bacteria
(Suarez, Apdon, Balinas, Baldonado, Hernando)

To culture the bacteria, a triangular, glass hockey was used. Initially, the hockey has to be sterilized with the use of heat. After which, 0.1 mL of the diluted coral mucus was placed on the agar plate, and then spread throughout the plate using the sterilized triangular, glass hockey. The agar plate was then closed and sealed, and then incubated at room temperature for three days.

After three days, the cultured bacteria were isolated using the Four Quadrant Streaking Method. A sterilized inoculating loop was used to streak the bacteria onto three new agar plates, after which they were then incubated for another three days at room temperature. This method was done once again after three days in order to ensure the purity of the samples. Morphological assessment was then conducted visually with the use of a vernier caliper, a compound microscope and an ultraviolet lamp.

After the assessment, an agar plate, containing the cultured bacteria with isolated colonies was packed tightly then secured to be delivered to the Philippine Genome Center in the National Institute of Molecular Biology and Biotechnology in the University of the Philippines Diliman for the 16S ribosomal RNA Extraction to Sequencing.

Staphylococcus aureus
(Cabalfin, Gerona, Labrador, Remaneses, Mediodia)

An inoculating loop was used to transfer an isolated colony from the agar plate to a test tube filled with 15 mL of Tryptic Soy Broth with 0.4 grams of glucose to create a subculture. The test tube containing the subculture was incubated for 18 to 24 hours at 30°C. A McFarland procedure was conducted to compare the optical densities of the cultures, using a bond paper with black and white stripes. A 96-well microtiter plate was used to grow the biofilms of S. aureus. 200 L of the previously diluted liquid media was transferred to columns 2, 4, 6, 8 and 10 of the plate using a micropipette. The microtiter plate was sealed and placed in the incubator for 24 hours at 30°C. (Yadav, 2015). This was conducted inside the laminar flow hood.

C. Co-culture
(Serra, Cabalfin, Lamzon, Mediodia, Catedral)

Separate cultures were prepared for the co-culturing of these organisms. The same method was used in order to acquire the needed population for each species. As soon as the dinoflagellate reaches cells/mL 1.00x104 and bacteria reaches 1.00x107 cells/mL. This was determined through counting the population of each sample dinoflagellate through hemocytometer (De la Peña and Franco 2013) and spread plate method for bacteria, and diluting the media to achieve the desired population density. Both samples were co-cultured to one another and were transferred into a 500 mL dextrose bottle with F - medium however the setup did not contain vitamin. Then will be placed in a well lit area equipped with aerators.

D. Simple Staining
(Triol, Dionela, Ecube, Mediodia)

The ethanolic extract was applied as a stain to 15 slides of *Staphylococcus aureus* and 15 slides of *Escherichia coli* bacterial smears using the simple staining method. Crystal violet, the positive control, was utilized as a stain to *S. aureus* and *E. coli*. Crystal violet and the ethanolic extract were used as the primary stain for the positive control and treatment, respectively. The primary stain was added to the bacterial smear and decolorizer was applied to remove the excess stain. Negative control without any stain was also used. The procedure for the simple staining was obtained from *Merck Microbiology Manual 12th Edition* (2010).

E. Gram Staining

***Staphylococcus aureus, Escherichia coli,* and mixture of the two bacteria**
(Triol, Dionela, Ecube, Mediodia)

The ethanolic extract was applied as a stain to 15 slides of *Staphylococcus aureus*, 15 slides of *Escherichia coli*, and 15 slides of mixed bacterial smears using the Gram staining method. Crystal violet, the positive control, was utilized as a stain to *S. aureus*, *E. Coli*, and mixed bacteria. Crystal violet and the ethanolic extract were used as the primary stain for the positive control and treatment, respectively. Gram's iodine solution was utilized as a mordant which was added after the primary stain. A decolorizer was also used to remove the excess stain and safranin was added as counterstain. Negative control without any stain was also used. The procedure for the Gram staining was obtained from *Merck Microbiology Manual 12th Edition* (2010).

Using Bixa orellana Extract as Stain
(Venturina, Comuelo, Samaniego, Jolito)

Extracts from plants such as Bixa orellana, commonly known as Annatto, presents a potential to be an alternative to the commonly used synthetic stains. Thus, this study aimed to evaluate Bixa orellana methanolic extracts as a substitute for safranin in Gram Staining. Bixa orellana extracts were used to stain *S. aureus*, *E.coli* and mixed bacteria smears. A drop of normal saline solution was placed on the slide. Using an inoculating needle, the cultured bacteria was smeared on the slide and allowed to dry. The slide was then passed quickly over the flame of an alcohol lamp three times. The slide with the heat fixed smear was then flooded with crystal violet for one minute and was then rinsed with distilled water. The smear was gently flooded with Gram's iodine for one minute and was then rinsed with distilled water. The smear was then decolorized using a 50 v/v% mixture of acetone and alcohol.

Endophytic bacterial isolates
(Halaba, Molinos, Superficial, Bela-ong)

Endophytic bacterial isolates were isolated from the roots of *Zea mays L. var. rugosa* (sweet corn) Sugar King variety at vegetative stage. A total of eight different bacterial strains were isolated, characterized; through gram staining and colony and cell morphology; and screened for a positive reaction for nitrogen fixation, ammonia production and zinc solubilization through the use of selective media. Jensen's media was used to screen for nitrogen fixing bacteria, peptone water with nessler's reagent for ammonia producing bacteria, and zinc incorporated media for zinc solubilizing bacteria. Gram staining was conducted to differentiate bacteria based on their different cell wall constituents. From each pure culture colony grown, a bacterial smear was prepared. Smears were prepared by heat fixing bacteria onto a sterile glass slide. The smears were then saturated with the following reagents: the primary stain crystal violet for 1 minute; Gram's iodine solution for 1 minute; 95% ethanol for 5 seconds; and the counterstain safranin for 1 minute rinsing the glass slide with distilled water after the addition of each reagent. Microscopy was conducted to determine the results for the gram staining. Purple stained bacteria were considered Gram-positive while pink stained bacteria were considered Gram-negative.

F. **Counting of Microalgal Cells Via Hemocytometer**
(Derramas, Gonzalez, Villaflor, Mediodía)

Ten milliliters (10 mL) of the algae culture was pipetted from the source container into a test tube. It was agitated in a vortex mixer for five seconds. A micropipette was used to extract ten microliters (10 µL) of the culture and was dispensed into slot A of the hemocytometer. Algae colonies in the five smaller squares of the hemocytometer's central square were counted. The algae colonies whose bodies overlap between squares were not counted. The concentration of algae per milliliter was computed based on the number of microalgal cells per microliter (µL).

G. **Antifeedant Assay**
(Bendicion, Dael, Genterola, Aban, Seredrica)

Anethum graveolens has been known to have insecticidal activity on several insect pests. Studies state that components of the essential oil of *A. graveolens*, which are mainly composed of carvones, and limonenes, can be used as antifeedant against insect pests. This study used the essential oil of *A. graveolens* in order to determine whether it has potential antifeedant bioactivity against *Cochlochila bullita* (lace bugs) on *Ocimum kilimandscharicum*.

Three mature leaves (fourth or fifth leaves from the bottom of a branch) of one *O. kilimandscharicum* were placed inside an 11 cm diameter petri dish, their stalks covered in sections of moist paper towel. This constituted one replicate. There were three replicates in total (Nair et al. 2012).

The basil leaves on which the lace bugs were placed were exposed to a concentration of essential oil from *A. graveolens* through dipping on the essential oil solution for ten seconds and then air-dried, whereas the negative control only included water (Shukla et al. 2012).

The dishes were arranged in a randomized complete block design and placed under conditions of 27 ± 1°C , the temperature range of 19°C-33°C is suitable for the development of lace bugs in laboratory conditions (Ju et al. 2011), and a photoperiod of 14:10 (L:D) hours (Nair et al 2012). Observations on the number of frass spots on the leaves per treatment were conducted every six (6) hour intervals for two (2) days (JeniLapinangga et al. 2018). The petri dishes were labeled as "Ag - (replicate number) (ppm concentration)" to denote the replicate number and concentration to be used. The control group were labeled as "(Control Group) - (replicate number)".

After every 6-hour interval, the leaves were assessed for damage by counting the number of frass spots. Frass spot numbers are highly correlated with leaf damage and serves as an index for the amount of *C. bullita* feeding on the basil (Nair et al. 2012). The number of frass spots per leaves per treatment was analyzed using One-Way ANOVA. The means were separated using Fisher's protected least significant difference (LSD) test.

MICROSCOPY

OVERVIEW

This chapter presents a detailed description of the methods for the proper handling and use of various types of microscopes. Additionally, it provides a rundown of the techniques involved in preparing different types of microscopic samples before processing, as well as the criteria for their identification and classification. Included among the wide range of specimens under this category are blood cells, phytoplanktons, algae, pollen, parasites, bacterial colonies, nanoparticles, and microplastics.

A. Haemocytometer

Counting Hemocytes

(Yap, Bungay, Alfonso, Libo-on, Cordero)

Hemolymph samples were withdrawn from one shrimp randomly selected from each tank 0, 1, 3, 4, 24, 72, and 120 hours after immersion has commenced. The hemolymph mixtures taken were of the following ratios: 100uL hemolymph: 200uL buffered formalin solution; 300uL hemolymph: 300uL anticoagulant solution; and 100uL hemolymph: 200uL anticoagulant solution for total hemocyte count, phenoloxidase activity, and respiratory burst activity, respectively.

For total hemocyte count, 10 uL of hemolymph-buffered formalin mixture was pipetted into the space formed between Neubauer hemocytometer and coverslip while avoiding bubble formation. This was viewed under a compound light microscope for counting. The calculation of the total hemocyte count was based on the formula:

$$Total\ Haemocyte\ Count\ = \frac{(N \times D \times 10^3)}{A \times 10} cells \cdot mL^{-1};$$

where N = total number of cells counted; D = dilution of haemolymph; A = total area counted (in mm^2, 10^3 = conversion factor from uL to mL, 10 = conversion factor from mm^2 to uL).

Respiratory burst activity was assessed using the extracted 300 uL of hemolymph mixture (200uL hemolymph: 100uL anticoagulant). The mixture was centrifuged at 1000g for 10 min at room temperature. The supernatant was removed and 100 uL of HBSS was added.

The resulting solution was incubated for 30 min at 37°C. After which the sample was washed three times with HBSS, stained with 100 uL of 0.3% NBT solution, and incubated for 30 min at 37°C. Then, the NBT was removed before adding 100 uL of absolute methanol. The methanol was removed, and the solution was washed three times with 70% methanol. The pellet was air-dried for 5 min before adding 120 uL of 2M KOH and 140 uL DMSO. The solution was transferred into microtiter plate wells and the optical density was read at 620 nm using a microplate reader. 120 uL of 2M KOH and 140 uL DMSO was used as the blank control reaction for subtraction from the absorbances of the samples. The formula used for the final absorbance was:

$$A_s = A_o - A_b;$$

where A_s = final absorbance of the sample; Ao = observed absorbance of the sample; and A_b = absorbance of the blank sample (solvent).

Counting Phytoplankton Cells

(Cordova, Occeña, Demandante, Bela-ong)

The procedure for counting phytoplankton cells using a hemocytometer was adopted from Delta Environmental (2015). Each phytoplankton sample from the screw cap bottles was transferred to a 250-mL graduated cylinder and then covered with cling wrap. The samples were left undisturbed at a shaded area at room temperature for 24 hours, allowing the phytoplankton cells to settle at the bottom of the graduated cylinder. The top layer of the phytoplankton samples was removed leaving 30mL of the samples.

The remaining sample was then transferred to another screw cap bottle and was homogenized by manual mixing. Two drops of the concentrated sample were transferred to the counting chambers of the hemocytometer.

A compound light microscope (LW Scientific) with a mechanically adjustable stage was used for counting phytoplankton cells. Counting was done for the two counting chambers of the hemocytometer.

Drawings and pictures of phytoplankton were used to count and identify the species. Reference literature is listed in Appendix B. Each phytoplankton was identified down to at least the genus level and, whenever possible, the species level. Those that remained unidentified were given accession numbers and their illustrations were appended.

B. Algae Identification

(Yeban, Baranda, Antenor, Sinco)

Pure algal isolates from the final serial dilution were examined under a compound microscope under the low power objective (10x magnification) and high-power objective (40x magnification). The morphology of the suspected microalgae species was considered using several manuals (Hyun & Serediak 2011; Hyun & Serediak 2006; Belcher & Swale 1976; Silva et. al. n.d.) for the identification of the microalgal isolates. The preliminary identification, based on the image, measurements, and parameters of the microalgal growth, was verified by an expert.

C. Parasite Identification

(Lopez, Aguirre, Dalisay)

When all of the samples have undergone processing, each sample was examined using a microscope. Using a pipette, about one (1) ml from each test tube was dropped on a glass slide and then covered using a glass slip. Under the low power objective, each slide was examined using the traditional S-direction scanning. All eggs found were counted and recorded.

To identify the genus of the parasite, the morphology of each suspected egg was compared to actual parasitic eggs. See Table 1:

Table 1. *Standard Morphology of Eggs*

Parasitic Egg	Morphology
Dipylidium spp.	Refer to Appendix B.1
Taenia spp.	Refer to Appendix B.2
Toxocara spp.	Refer to Appendix B.3
Trichuris spp.	Refer to Appendix B.4
Ancylostoma spp.	Refer to Appendix B.5

D. Cell/Colony Morphology

(Halaba, Molinos, Superficial, Bela-ong)

Endophytic bacterial isolates were isolated from the roots of *Zea mays L. var. rugosa* (sweet corn) Sugar King variety at a vegetative stage. A total of eight different bacterial strains were isolated, characterized; through gram staining and colony and cell morphology; and screened for a positive reaction for nitrogen fixation, ammonia production, and zinc solubilization through the use of selective media. Jensen's media was used to screen for nitrogen-fixing bacteria, peptone water with Nessler's reagent for ammonia-producing bacteria, and zinc incorporated media for zinc solubilizing bacteria.

Three sets of three test tubes: labeled 10-1,10-2, and 10-3 respectively, were filled with 9 mL of 0.9% saline solution. One gram of the sample was added to the test tubes labeled 10-1.

Before the sample settled, one mL of the suspension was transferred into the test tube labeled 10-2 using a micropipette. The procedure was repeated once again to achieve a concentration of 10-3.

Mixed culture colonies were cultured to determine the colony morphologies of individual isolates. One drop of the diluted samples from the test tubes labeled10-3 was pipetted onto one side of an agar plate. Using an inoculating loop, heat sterilized using an alcohol lamp, the sample was quadrant streaked on the agar plate. The agar plates were then incubated for 24 hours at 37° C. The plates were upside-down during the incubation period to avoid the interference of condensation in the growth of the microbes. After the incubation period, the colony morphology of the microbial plates was observed. Those with the same morphology were classified as the same colonies before the isolation of pure cultures to avoid multiple cultures of the same bacteria.

Colony morphology was done during the observation times before and after the isolation of pure culture bacterial colonies to ensure that the colonies isolated were correct. The shape, margin, elevation, size, color, and texture of the bacterial colony were identified. This determined the groupings, based on the similarities of their characteristics, for the isolation of pure culture colonies.

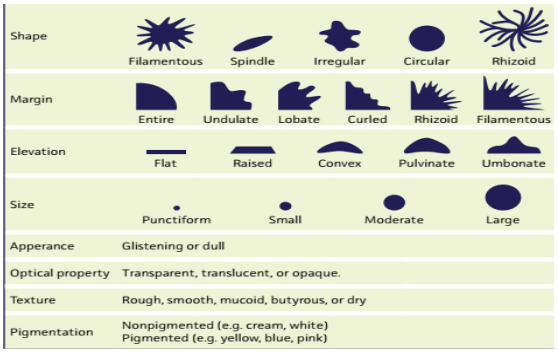

Figure 3. *Colony morphology guide (ATTC 2006)*

For the cell morphology, microscopy was conducted to determine the shape and arrangement of the bacterial cell.

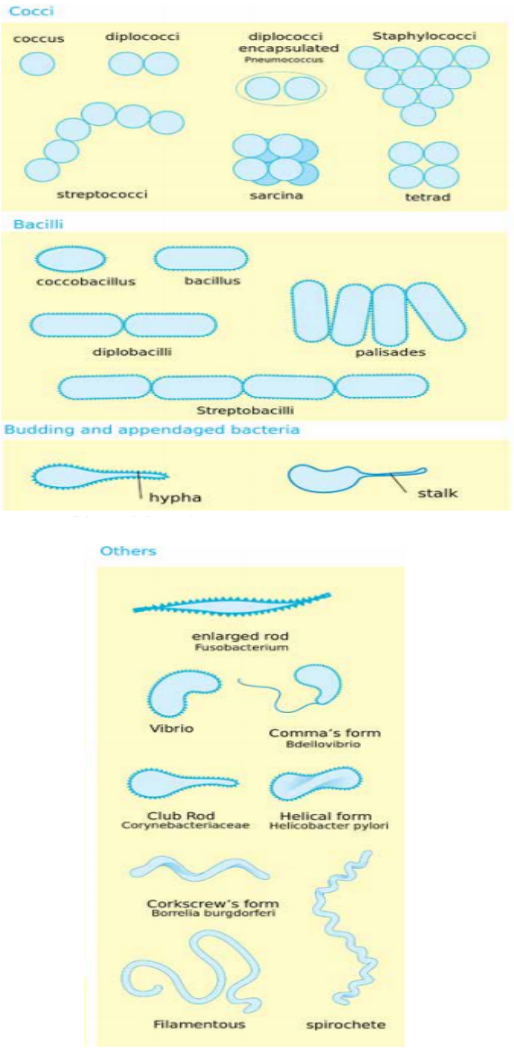

Figure 4. *Cell morphology guide (ATTC 2006)*

E. Atomic Force Microscopy (Socrates, Tang, Tionko, Bautista, Padernal)

Images of AgNPs deposited on a mica surface were taken to determine the size and observe the size distribution of the synthesized AgNPs at optimal conditions. For this purpose, a Shimadzu SPM-9700HT AFM was used in contact mode.

F. **Microplastics Visual Inspection**
(Colacion, San Diego, Secondes, Oberio)

The microplastics of size ≤ 2 mm acquired after the extraction process were inspected using a compound inverted microscope at 40x magnification. Microplastics were identified based on the criteria provided by Norén (2007). They were also classified into four types according to their morphological type – fibers, films, fragments, and pellets based on the criteria provided by Free et al. (2014) and Frias et al. (2018).

Table 2. *Criteria for the classification of microplastics based on definition (Free et al. 2014).*

Definitions and potential sources of microplastic types.

Microplastic type	Definition	Potential sources
Fragment	Hard, jagged plastic particle	Bottles; hard, sturdy plastics
Line/fiber	Thin or fibrous, straight plastic	Fishing line/nets; clothing or textiles
Pellet	Hard, rounded plastic particle	Virgin resin pellets; facial cleansers
Film	Thin plane of flimsy plastic	Plastics bags, wrappers, or sheeting

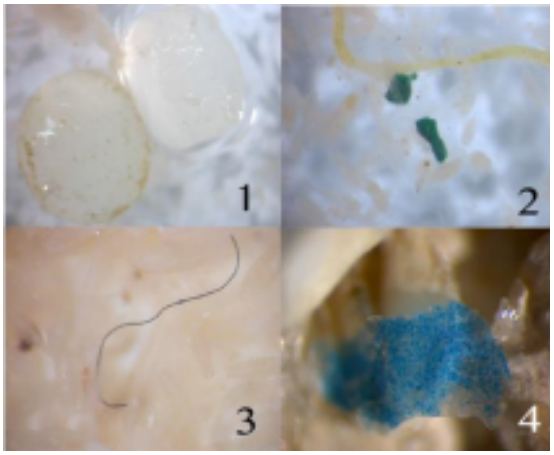

Figure 5. *Criteria for the classification of microplastics based on photos of different microplastic types: (1) pellets, (2) fragments, (3) fiber, and (4) film under a stereomicroscope (Frias et al. 2018).*

MOLECULAR BIOLOGY

OVERVIEW

This chapter involves methods from a descriptive study which includes the collection and preservation of samples, extraction of DNA, PCR amplification, gel electrophoresis, gene sequencing, and analysis of DNA samples using software and programs. The sample collection was done in Bagong Lipunan Market and Ivisan Market in Roxas City, Capiz, Philippines. DNA extraction, PCR amplification, and gel electrophoresis were conducted at Far Eastern University - Manila Molecular Laboratory.

A. DNA Extraction
(Areño, Cambel, Hilapad, Bela-ong, Castillo, Valdez)

DNA extraction of selected samples was performed using the Macherey-Nagel NucleoSpin Tissue kit (instructions included in the kit were followed).

B. Polymerase Chain Reaction
(Areño, Cambel, Hilapad, Bela-ong, Castillo, Valdez)

Extracted DNA was subjected to PCR amplification using the universal CO1 primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3').

Amplification was performed using the PCR mix consisting of 16.4 µL PCR grade water, 2.5 µL 10X PCR Buffer with 1.5 mM MgCl2, 2.5 µL 10mM DNTP, 0.5 µL Primer A, 0.5 µL Primer B, 0.10 µL Taq polymerase, 0.75 µL DMSO, 0.75 µL 3X BSA, and 1 µL DNA Template with a total reaction of 25 µL. PCRs were carried out by the following thermal regimen: 5 min at 95°C, then 35 cycles of 1 min at 94°C, 1 min at 43°C and 1:30 min at 72°C, followed by extension for 7 min at 72°C.

C. Agarose Gel Electrophoresis
(Areño, Cambel, Hilapad, Bela-ong, Castillo, Valdez)

Successfully amplified PCR products were subjected to gel electrophoresis to check the presence of DNA. The size and quality of PCR products were assessed in 1.5% agarose gel electrophoresis and stained with ethidium bromide.

D. DNA Sequencing (Areño, Cambel, Hilapad, Bela-ong, Castillo, Valdez)

Amplified PCR products were sent to the University of California, Berkeley for DNA sequencing.

E. Bioinformatics
(Areño, Cambel, Hilapad, Bela-ong, Castillo, Valdez)

All sequences were assembled in Geneious and aligned using MUSCLE in MEGA7. Each sequence was queried in BLAST for comparison of DNA sequences available in GenBank. Along with BLAST, BOLD was used to minimize the risk of using contaminated sequences. All identified species under BLAST search were checked on the IUCN red list of threatened species to identify endangered species.

For analysis of the base composition and visualization of the relationships among species included in the study, the software package MEGA7 was used. Phylogenetic analysis using the Neighbor-Joining (NJ) tree model was conducted. In addition, pairwise distances were also calculated along with the intraspecific and interspecific genetic divergences of the samples.

PLANT & ANIMAL CULTURE

OVERVIEW

This chapter includes the methods employed in culturing plant and animal specimens for the propagation of samples. Plant and animal cultures are grown in an artificial or natural environment and kept at favorable conditions for maintenance. In this process, it is important to consider growth factors (e.g. duration, temperature, pH), as well as the extraneous variables, that may affect the development and viability of the samples.

A. Cherry Tomato (*Solanum lycopersicum* var. *cerasiforme*)
(Pamati-an, Miraflores, Galino, Oberio)

Red Plum cherry tomato seeds, verified by the Department of Agriculture as true to the species that is named, as well as its hybrid and variety, were sown in an 8 in x 13 seedling tray using a soilless medium, Klasmann K TS1, which is mainly composed of white peat (FOREMOSTCO Inc. 2010). Two to three cherry tomato seeds were sown in each cell of the seedling tray, to a total of approximately 260 cherry tomato seeds sown in 104 cells. After 2 days, the seeds sprouted. The seedlings with the best growth attributes, indicated by the plant height, stem diameter, and the number of healthy true leaves were chosen for transplanting. Twenty-two (22) days after sowing the cherry tomato seeds, the seedlings produced true leaves. The seedlings were then transplanted to polyethylene pots with a diameter of 30 cm and height of 27.5 cm that were placed on pallets at the PSHS-WVC Academic Building rooftop.

B. *Allium* spp.
(Alogon, Horlador, Martinete, Mediodia)

Seeds were sterilized in 0.3% NaOCl solution for 5 minutes, washed thoroughly with sterilized distilled water three times, and transferred to Petri plates containing sterilized distilled water for germination.

After 8 days of germination in the dark at ambient temperature, plantlets were transferred to 9-cm-diameter petri plates with 5 mL 0.05M CaCl2 solution for further growth in the dark at ambient temperature, with each Petri plate having at most 100 plantlets.

C. Peanut Roots (*Arachis hypogaea*)
(Abellar, De Juan, Bela-ong)

Four peanut seeds were planted per pot, summing up to a total of 640 peanut seeds grown in 160 plastic pots. The pots were initially filled three-fourths of the way with rice hull compost soil, with the four peanuts seeds placed in a square orientation. An inch of soil was then added. These pots were situated in an area, wide enough for all pots to receive an equal amount of sunlight. These were grown in a controlled environment and given an equal amount of sunlight and water for 30 days before transferring half of the batch for UV exposure.

D. Onion Root Tips (*Allium cepa*)
(Abellar, De Juan, Bela-ong)

Fifteen commercial equal-sized *A. cepa* onion bulbs of 3 to 4 g were used. The bulbs were carefully unscaled, placed on top of beakers filled with tap water, and allowed to germinate at room temperature. After 48 hours, the onion bulbs were treated with 0.7% H_2O_2 for an hour. After the H_2O_2 treatment, onion bulbs with root tips which have grown up to 2-3 cm were washed for

an hour and were transferred to beakers containing the different treatments (mg/mL and 5 mg/mL crude ethanol extracts of both peanut plants exposed and unexposed to UV radiation, and tap water) for 72 h (Celik and Aslanturk, 2006). Assignation of bulbs to treatments was randomized.

E. Milkfish (*Chanos chanos*)
(Pasquin, Rodrigo, Villaluna, Aban)

Acquisition of *Chanos chanos* fingerlings was done with Southeast Asian Fisheries and Development Center (SEAFDEC) Region VI's affiliated institution, Dumangas Brackishwater Station (DBS). The fingerlings were placed in a 20-liter tank with dimensions of 43.18 x 22.86 x 27.94 (HxWxH) centimeters. They were then set to acclimatize for 48 hours under laboratory conditions. Water quality was maintained at a pH between 6 to 9, change in dissolved oxygen at 1 ppt to 3 ppt, and salinity at 0 ppt to 35 ppt. The *Chanos chanos* fingerlings were fed with commercial fish pellets daily and starved for 24 hours prior to and during the experiment. The water quality parameters (dissolved oxygen, pH, and temperature) were assessed using a PASCO PS-2230 water quality sensor, with an accuracy of ±0.6 mg/L right out of the box, provided by Philippine Science High School-Western Visayas Campus. Salinity was measured using an Original Equipment Manufacturer (OEM) brackish water salinity refractometer. The tests were done before and after the addition of extracts. Water quality was checked at 0, 1, 3, 6, 12, 24, 48, and 96 hours after the application of extracts.

F. Whiteleg Shrimp (*Litopenaeus vannamei*)
(Yap, Bungay, Alfonso, Libo-on, Cordero)

For the maintenance of the shrimp culture for the experiment, the seawater in the tanks was monitored with the following conditions: pH of 7.7 to 8.2, salinity of 33±1 ppt, temperature of 30±1°C, and constant aeration.

Commercial feeds were used and administered twice a day. Ten (10) shrimps were placed into separate 50 L tanks filled with 30 L of filtered seawater. Tanks used for each treatment (0, 100, and 200 mg/L hot-water extracts) were in triplicate.

G. Corn (*Zea mays L.*)
(Castañeda, Hallado, Lujan, Mediodia, Aguaras)

20-watt fluorescent bulbs were used and positioned 6 ft and 9 inches above the pots. Fluorescent light is an ideal source of artificial light for plants. A daily photoperiod of 14 hours was used as most facilities growing corn in a greenhouse use this duration of artificial light. Room temperature was kept at 25 C.

One corn seed was planted 2 inches deep into 2.5 kg of loamy soil in a 6 x 6 x 8 pot. The plants were watered every day with varying volumes depending on their growth stage requirements based on data. A 20% PEG-4000 solution was used to simulate drought conditions and was added at four-day intervals, while 0.23 g/ft^2 of potassium polyacrylate was added 15cm below the topsoil for water retention. Potassium Polyacrylate was not mixed with the soil to avoid breaking the polymers.

H. Corals (*Acropora spp.*)
(Dalabajan, Hilay, Velasco, Navarro, Olvido)

The branches were glued individually into cement cones with a diameter of 8 cm using an underwater epoxy. Marine epoxy was used because there are no recorded negative effects as coral adhesives and it is effective in attachment and transplant survival (Dizon et al. 2008). It is essential to plant the corals in order to simulate the natural conditions of the macroalgal-coral competition.

The treatments and control replicates (n=15 coral branches each) have separate metal racks. The metal racks were covered with a 1x1 cm grid metal screen to avoid other herbivores from entering. The racks were placed in designated areas around the island. Each of the coral branches within the

cement cones were interspersed 15-cm apart across the metal rack. As a means to minimize the extraneous variables and for easier measurement, there is a divider between each of the coral branches. The measurement of the whole metal rack is 115x46x50 cm (LxWxH). Three metal racks were used so that there would be 15 organisms for each experimental group for the statistical analysis.

I. Algae (*Chlorella sorokiniana*)
(Derramas, Gonzalez, Villaflor, Mediodia)

A culture of *Chlorella sorokiniana* was purchased from SEAFDEC, Tigbauan, Iloilo. The culture was sealed in a plastic bottle for transport to PSHS-WVC through an air-conditioned mode of transportation. It was then stored in a refrigerator at 3°C prior to usage to stabilize the algal cell concentration, which is accelerated by heat exposure. Twenty milliliters (20 mL) of *Chlorella sorokiniana* culture in exponential phase cultured in Conway medium was inoculated into each Erlenmeyer flask.

J. Lace Bugs (*Cochlochila bullita*)
(Bendicion, Dael, Genterola, Presno-Aban, Seredrica)

Anethum graveolens has been known to have insecticidal activity on several insect pests. Studies state that components of the essential oil of *Anethum graveolens*, which are mainly composed of carvones, and limonenes, can be used as antifeedants against insect pests. This study used the essential oil of *Anethum graveolens* in order to determine whether it has potential antifeedant bioactivity against *Cochlochila bullita* (lace bugs) on *Ocimum kilimandscharicum*.

Basil plants were procured from 3 Sunshine Garden, Tagbak, Jaro, Iloilo City. The basil plants were then transported to and raised in an improvised greenhouse created at Philippine Science High School - Western Visayas Campus (10°45'10.1"N 122°35'15.9"E). For four days, the basil plants were watered every morning and afternoon. *Cochlochila bullita* colonies were collected from basil plants located at Orchard Valley, Pavia, Iloilo (10°46'09.7"N 122°33'03.8"E). Adult lace bugs were collected by cutting the entire basil leaves on which the lace bugs were situated using scissors and placed inside mega box containers which were covered with perforated cling wrap. The lace bugs were then transported to the greenhouse and transferred onto the basil leaves. The basil plants were transferred inside the laboratory in the Student Learning Resource Center Building at Philippine Science High School - Western Visayas Campus. The lace bugs were acclimatized inside the laboratory for two to three days (Rojht et al. 2009; Sathe 2014). Male and female adult lace bugs were collected from the basil plants for the antifeedant assay. As described by Sajap and Peng (2010), adult lace bugs were classified according to sex using a hand microscope.

K. Mosquitoes (*Aedes aegypti*)
(Carigaba, Leonida, Masculino, Mediodia, Garbo)

The *Aedes aegypti* larvae used in the study were cultured in the Department of Science and Technology - Industrial Technology Development Institute (DOST-ITDI) Entomology Section Insectary and were reared according to their standard procedures following the guidelines provided by the World Health Organization (WHO). The larvae were reared at a laboratory condition of 25 ± 2°C and relative humidity of 70% ± 10%.

L. Bacteria from Plastic Waste
(Canja, Hilis, Galan, Jolito)

Plastics are known for being durable materials while still maintaining a low cost of production. It is a very important material for commercial use all over the world. However, due to the lack of a reliable method of disposal, the risk of plastic pollution is steadily increasing throughout the years.

This study aims to isolate and extract bacteria from the Iloilo City Engineered Sanitary Landfill, in Mandurriao, Iloilo City, and to assess their biodegradation potential on LDPE (Low-density polyethylene), HDPE (High-density polyethylene), and PET (Polyethylene terephthalate).

Bacteria were sampled directly from swabbing the plastic as well as from the soil sample obtained from the site. The bacterial sample in the swabs was plated on nutrient agar (NA) medium using the streak method of inoculation. Three NA mediums were utilized for the growth of the bacteria. The plates were incubated at 30°C for 24-48 hours. Colonies with different morphological appearances were then sub-cultured onto fresh NA.

Four grams of the soil sample were suspended in 96 mL of sterile distilled water and shaken vigorously for 2 minutes. It was heated at 60°C for 60 minutes in a water bath. The mixture was put to rest to allow the soil particles to settle. It was plated on a nutrient agar using the streak method. Incubation was done at 30°C for 24 - 48 hours. Identification was done using morphological observation (Al-Humam, 2016).

Samples were then plated in selective media, eosin methylene blue agar (EMB) and mannitol salt agar (MSA), to further categorize the bacteria. Out of the ten plates, two were selected for incubation based on the most distinct colonies and were again cultivated in these selective media. Colonies were selected based on their morphological characteristics: form, elevation, and margin.

Plates 1-2. *Bacterial strains EMBP2A and MSAP2A for incubation.*

Figures 6-15. *The bacterial culture inside Petri dishes.*

PREPARATION

OVERVIEW

This chapter includes methods for preparing different types of solutions and organic materials before they are subject to further processing. This broad category covers methods in different levels of molecular complexity. Some methods employ stoichiometry in preparing solutions and some involve mechanically processing raw plant products.

A. Substrates
(Faciolan, Leonora, Majaducon, Sinco)

For the preparation of substrate mixture, the total mass of each fruiting bag was 750 grams, comprising 98 percent of the alternative substrate with 1 percent molasses and 1 percent lime. Each treatment had five replicates to account for mushroom mortality.

There are 7 treatments: (i) the control, (ii) 100% rice stalk, (iii) 100% rice bran, (iv) 100% rice husk, (v) 50% rice stalk and 50% sawdust, (vi) 50% rice bran and 50% sawdust, and (vii) 50% rice husk and 50% sawdust. The control was based on the substrate mixture ratio used by WESVIARC.

B. Culture Media

Mueller Hinton Broth
(Barrientos, Miraflores, Serisola)

The Mueller-Hinton broth was prepared by suspending 10.5 grams of the medium in 500 mL of distilled water. The mixture was then mixed and boiled over a hot plate then autoclaved for 15 minutes with a setting of 121°C and 15 psi.

The Mueller-Hinton agar was prepared in a media bottle by suspending 20 grams of the medium in 500 mL of distilled water. The mixture was then boiled over a hot plate, autoclaved for 15 minutes with a setting of 121°C and 15 psi. The cooled agar was poured into sterile petri dishes on a level, horizontal surface to give uniform depth.

Tryptic Soy Broth and Nutrient Broth
(Elizalde, Herida, Jaudian)

In a 500 mL media bottle, 9.9 grams of Tryptic Soy Broth powder was suspended in 330 mL distilled water. The concentration may vary based on manufacturer specifications.

The suspension was stirred using a stirring rod until the media powder had fully dissolved. The prepared broth was then evenly distributed to their respective test tubes using a glass pipette. Each test tube was capped using cotton balls, covered with aluminum foil, and gathered for sterilization. Lastly, the prepared media was autoclaved at an optimal temperature of 121°C and pressure of 15 psi for 15 mins, cooled, and stored in the refrigerator prior to usage.

Tryptic Soy Broth and Nutrient Broth
(Cabalfin, Gerona, Labrador, Remaneses, Mediodia)

The glasswares (petri plates, test tubes, micropipette tips, stirring rods) were autoclaved (Delixi LS-B35L) for 15 minutes at the optimal pressure of 15 psi. Using a 500 mL media bottle, 20 grams of Tryptic Soy Agar was mixed with 500 mL distilled water. The mixture was cooked in the hot plate (LMS HTS-1003) and continually stirred until the mixture cleared up. Using a separate 500 mL media bottle 2.4 grams of Nutrient Broth was suspended in 500 mL of distilled water. The solution was mixed using a sterile stirring rod until the solid was dissolved. Peptone water was prepared with 0.5 grams of Bacteriological Peptone, and 4.8 grams of Sodium Chloride. It was then suspended in 500 mL of distilled water in a 500 mL media bottle. The media was autoclaved for 15 minutes at 15 psi and was cooled down afterward. In four sterile test tubes, 15 mL of Nutrient Broth mixture was pipetted. Peptone water of 10 mL was pipetted into two test tubes. Nutrient agar (20-30 mL) was poured into each agar plate. After the agar has solidified it was stored in the refrigerator at 20C.

TCBS Agar

(Suarez, Apdon, Balinas, Baldonado, Hernando)

Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS) Agar was prepared with the use of distilled water amounting to 400.00 mL to suspend 35.64 grams of the dehydrated agar in a media bottle. The mixture was then stirred on a hot plate until it was boiling and the dehydrated agar completely dissolved in the solvent. Once completely mixed, the agar solution did not undergo autoclaving as instructed but was cooled to 50°C and poured into sterilized Petri dishes. The agar plates were then left to cool overnight in the refrigerator to be preserved until they are used.

C. Stock Solution

Lemongrass

(Sira, Valzado, Larroder, Cabarles)

One kilogram of *Cymbopogon citratus* (lemongrass) leaves was washed with distilled water. The *C. citratus* leaves were then loaded into the juicer with 1 L of distilled water gradually being added at the same time. The produced aqueous solution was collected in a 1 L plastic container and then sealed and stored away from the sunlight until use in the preparation of serial dilutions.

Calamansi Essential Oil

(Carigaba, Leonida, Masculino, Mediodia, Garbo)

Two stock solutions of 10,000 ppm each (0.1 mL extract in 10 mL acetone and 0.1 mL in 10 mL 95% ethanol) were prepared and diluted in dechlorinated water as per the concentration used in the assay. Ethanol and acetone were used as solvents because the essential oil is not miscible in water, if applied directly.

Anethum graveolens essential oil

(Bendicion, Dael, Genterola, Presno-Aban, Seredrica)

Anethum graveolens has been known to have insecticidal activity on several insect pests. Studies state that components of the essential oil of *Anethum graveolens*, which are mainly composed of carvones, and limonenes, can be used as antifeedants against insect pests. This study used the essential oil of *Anethum graveolens* in order to determine whether it has potential antifeedant bioactivity against *Cochlochila bullita* (lace bugs) on *Ocimum kilimandscharicum*.

The essential oil of *Anethum graveolens* was acquired from Plant Therapy Essentials Oils Corporate, 510 2nd Ave S, Twin Falls, ID 83301, the United States of America through an online shopping site, Lazada PH, as a medium. The essential oil concentrations were diluted according to a previous study (Khosravi and Sendi 2013) in which 0.01, 0.02, 0.04, and 0.08 mL of essential oil were mixed in 0.4 milliliters of dimethyl sulfoxide (DMSO) inside a 100mL volumetric flask. Distilled water was added into the volumetric flask until the final volume of 100 mL was reached. This provided a 100 mL solution of 100, 200, 400, and 800 ppm concentrations of essential oil. A negative control group of distilled water was also prepared.

Chromium (VI) Stock Solution

(Faciolan, Leonora, Majaducon, Sinco)

Chromium in its hexavalent state, Cr^{6+}, is one of the prevalent heavy metals in aquatic ecosystems with its occurrence primarily attributed to industrial activities such as dye manufacturing and construction run-off. This paper presents the removal efficiency of organo-mineral composites from the shells of three mollusks abundant in the Philippines: *Crassostrea iredalei* (Slipper Cupped Oyster), *Perna viridis* (Green Shell), and *Telescopium telescopium* (Horned Snail).

A stock solution of 1000ppm Cr^{6+} solution was prepared by dissolving potassium dichromate ($K_2Cr_2O_7$) in distilled water (Baijnath et al. 2014; Onchoke and Sasu 2016). The dichromate ($Cr_2O_7^{2-}$) ion present in $K_2Cr_2O_7$ contains chromium in the hexavalent oxidation state (Cr^{6+}). The dichromate is being used instead of its trivalent counterpart, chromate (CrO_4^{2-}), in compliance with Method 7196A of the American Environmental Protection Agency, and the Standard Method for the Examination of Water and Wastewater followed by DOST-VI. The stock solution was subsequently diluted to the 100ppm standard solution from which 1, 5, 10, and 50ppm solutions were prepared.

D. Treatment Solution
(Derramas, Gonzalez, Villaflor, Mediodia)

Using a graduated cylinder, four liters of distilled water were measured and poured into a clean plastic container. After this, 57.3 mg of potassium dihydrogen phosphate (KH_2PO_4) was measured and mixed into the water via agitation to create the control (base) solution. One liter (1 L) of the KH_2PO_4 solution was then measured for each of the four treatments using a graduated cylinder and transferred into respectively labeled beakers. From the separated solution, 180 mL was measured using a graduated cylinder and was transferred into each of the five Erlenmeyer flasks designated to hold the replicates of the control setup with the treatment containing 0 mg of nitrogen. The remaining 350 mL of the solution was stored inside a beaker. This process was then repeated for the three other treatments wherein 5 mg, 10 mg, and 15 mg of nitrogen as potassium nitrate (KNO_3) was added respectively to the one liter of base solution for each treatment.

E. Cadmium and Lead Metal Solution
(Bandiola, Galotera, Sampiano, Mediodia)

Synthetic solutions with a concentration of 10 mg/L and 30 mg/L for lead and cadmium ions, respectively, were prepared from the following salts: $3CdSO_4 \cdot 8H_2O$ (cadmium sulfate octahydrate, Scharlau) and $Pb(NO_3)_2$ (lead nitrate, Farco) (Vera et al. 2019). Using the Shimadzu AUX220 (Min 0.1 g; Max 220 g) analytical balance, 60.44 mg of $3CdSO_4 \cdot 8H_2O$ and 47.95 mg of $Pb(NO_3)_2$ were weighed. The salts were then poured into three one-liter volumetric flasks. Deionized water was poured to mark into each of the volumetric flasks. The flasks were agitated until there were no visible solid salt particles in the solution. The three-liter heavy metal solution was transferred and stored in a four-liter HDPE bottle. A pH of 6.0 was obtained using 0.1 M of hydrochloric acid. A strip of pH paper with an accuracy of 1 pH was used to determine the pH of the solution prior to adsorption.

The salts (NaCl, KCl, and $CaCl_2$) were weighed using the Shimadzu Analytical Balance. For the different concentrations, the respective amount of salt needed was measured and dissolved in 250 mL of distilled water in separate beakers. The solution was then tested with the probe to test if the solution has achieved the desired salinity level. When the desired level was not obtained, the solution was manually diluted with distilled water or increased in salt concentration.

The salts were weighed using Shimadzu Analytical Balance (AUX220), located at the Biology Stockroom of PSHS-WVC. For the 4 dS/m treatment, 0.64 grams of each salt was weighed. For the 6 dS/m treatment, 0.512 grams of each salt was weighed. For the 8 dS/m treatment, 0.1024 grams of each salt was weighed. These values were scaled down according to the Electrical Conductivity formula. A spare plastic container acted as a tray for the weighing of the salt samples. A plastic spoon was used to transfer salt from the original container to the acting tray. The containers for the salt were closed immediately upon opening to prevent moisture from accumulating inside the container, which may have led to the contamination of the salt samples.

EC = TDS / 640 (at 25°C); wherein
EC = Electrical Conductivity
TDS = Total Dissolved Salts in mg/L

The salts were dissolved in 250 mL of distilled water in separate one-liter beakers using separate stirring rods. This adjustment in proportions was performed in order to avoid wastage of salt samples and distilled water. The solutions were then tested with the conductivity probe to check if the desired level of salinity was obtained. When the solution was not equal to the desired level, the solution was manually diluted with distilled water or increased in concentration.

Virus-Concentrated Solution
(Sira, Valzado, Larroder, Cabarles)

Freeze-dried live LaSota strain Newcastle disease virus vaccine was diluted using a vaccine solvent. The volume ratio of vaccine to solvent was modified by authorized personnel from the Department of Agriculture to assure a more virus-concentrated final solution.

F. **Flavonoid Treatments**
(Sombiro, Valdestamon, Aguirre Mediodia)

For the treatment with anthocyanin only and quercetin only, 0.0151g each of anthocyanin and quercetin was weighed on an analytical balance. The two flavonoid powders were then separately transferred to 50-mL beakers and mixed with 50 mL of dimethyl sulfoxide (DMSO). The solutions were stirred until the powders dissolved. Each solution was transferred to a one-liter beaker where it was diluted with distilled water until the 1-liter line was reached. The mixture was stirred.

For anthocyanin and quercetin, 0.0076 g each of anthocyanin and quercetin were combined and the same process was followed. All treatments were then transferred to reagent bottles and stored in a refrigerator for 24 hours at a normal refrigeration temperature,1.6 C.

G. **Calcium Chloride Solution**
(Florentino, Santos, Templonuevo)

Three varying calcium chloride concentrations were made at 1, 2, and 3 percent w/v concentrations. Calcium chloride weighing 2.5, 5.0, and 7.5 grams were separately mixed in distilled water in three 250mL volumetric flasks.

H. **Sodium Alginate Solution**
(Florentino, Santos, Templonuevo)

Three percent of sodium alginate solution was made by putting 1.5 grams of sodium alginate in a 50 mL conical tube. It was then initially added with a small amount of distilled water and was mixed using the vortex mixer and was diluted to mark. Continuous mixing was done after every addition of distilled water until it was fully mixed. The mixture was then left to settle overnight to allow it to dissolve completely.

I. **Corn Stalks**
(Rentoy, Angot, Mabaquiao, Larroder)

The corn stalks were removed from its dirt and leaves, and the outer sheaths were peeled to reduce impurities and to produce a smoother surface. The stalks were chopped, blended, and used immediately after. Using a top-loading balance set to 0.1 milligrams accuracy, 200 g of the blended corn stalks were weighed in preparation for the next procedure. The outer sheath remained intact in the first repetition, but since it resulted in a rough bioplastic sheet, in the end, the outer sheath was peeled off in the succeeding repetitions.

J. **Viscose**
(Rentoy, Angot, Mabaquiao, Larroder)

A sodium hydroxide solution was used in order to convert cellulose pulp into alkali cellulose. The solution was made by preparing a 500 mL solution of 18% hydroxide. Afterward, ninety grams of NaOH was added into a 500 mL volumetric flask filled to form the solution. The pulp was then poured into the beaker containing the solution then covered with aluminum foil and left to stand for 60 minutes. The solution was then transferred to a Buchner funnel which afterward the alkali cellulose was then hand pressed to remove as much solution as possible.

The alkali cellulose underwent xanthation which was done by adding 7.70g of carbon disulfide to the alkali cellulose in a one-liter media bottle and left to soak at 30°C for two hours. The bottle was rotated periodically to ensure uniform xanthation. The yellow pulp had turned a deep orange color afterward 240.28mL of cold water (5°C) and 48.56 g of 18% sodium hydroxide were added. The result was a viscous, thick, orange-colored liquid which was then stirred for two hours using an electric mixer. The “viscose” was then transferred into a clean media bottle for storage which was loosely sealed and stored at 5°C in a refrigerator for 24 hours.

K. Bacterial Smears

(Triol, Dionela, Ecube, Mediodia)

Staphylococcus aureus, Escherichia coli, and a mixture of the two bacterial smears were prepared by placing a drop of normal saline solution on a glass slide and mixing it with a small colony of bacteria obtained from an agar slant tube using an inoculating loop which was obtained from the Biology Science Research Assistant (SRA) of Philippine Science High School - Western Visayas Campus (PSHS-WVC). The bacteria were then heat-fixed by having the glass slide pass over a flame. Seventy-five slides of *Staphylococcus aureus* bacterial smear, 75 slides of *Escherichia coli* bacterial smear, and 45 slides of mixed bacterial smear were prepared in a biosafety cabinet.

L. Pyrolysis

(Diaz, Golo, Villaluna, Aban)

Research regarding biochar remediation efficiency of excess nutrients such as phosphates is limited due to its low adsorption capacity. The study aimed to determine the potential of pineapple peel-derived biochar in adsorbing phosphates. Pineapple peel biochar was produced via pyrolysis at 300°C, 400°C, and 500°C, and then characterized using a Fourier Transform Infrared Radiation (FTIR) spectrometer.

The procedure for ash and biochar separation was acquired from the study of Wang et al. (2016). The resulting products from pyrolysis were immersed in 0.1 M HCl solution for 12 hours, washed with distilled water repeatedly, then oven-dried at 60°C for 12 hours. After cooling down to room temperature in a desiccator, the solids passed through a 100-mesh sieve to obtain the final biochar sample. The resulting biochar produced from each temperature were referred to as PP300, PP400, and PP500.

Pineapples were obtained from Leganes Public Market, Iloilo. The peels were thoroughly separated from the flesh by careful scraping using a knife. The peels were air-dried for 24 hours, then oven-dried at 70°C for 12 hours. The oven-dried peels were ground using a blender and passed through a 1 mm sieve. Five grams of pineapple peels were weighed using an analytical balance and were placed into ten constant-weighed ceramic crucibles for each pyrolysis temperature.

All crucibles used for pyrolysis were previously oven-dried at 110°C for 1 hour, placed inside a desiccator for 15 minutes, then weighed. Afterward, the crucibles were oven-dried again for 30 minutes at the same temperature, and then placed again inside the desiccator for 15 minutes, and finally weighed again. After weighing, the oven drying and the 15-minute cooling process were repeated until successive weighing was agreed to within ±0.0005 g.

The dried pineapple peels underwent slow pyrolysis and were incubated at the peak temperature (300°C, 400°C, 500°C) for 2 hours using a muffle furnace at the DOST- Region VI laboratory. Ten crucibles with a total of 50 grams of the sample were pyrolyzed for each temperature. After pyrolysis, the crucibles were then cooled to room temperature in a desiccator, then weighed using an analytical balance.

The procedure for ash and biochar separation was acquired from the study of Wang et al. (2016). The resulting products from pyrolysis were immersed in 0.1 M HCl solution for 12 hours, washed with distilled water repeatedly, then oven-dried at 60°C for 12 hours. After cooling down to room temperature in a desiccator, the solids passed through a 100-mesh sieve to obtain the final biochar sample. The resulting biochar produced from each temperature were referred to as PP300, PP400, and PP500.

M. Rice Husks and Mango Peels Absorbents

(Bandiola, Galotera, Sampiano, Mediodia)

One kilogram husks of *Oryza sativa* (rice) from Iloilo Central Market was initially washed with tap water in a colander and then with distilled water thrice by batches. After washing, the rice husks were evenly placed on a glass baking tray and oven-dried using the Binder ED53 laboratory oven for eight hours at 70°C to remove any moisture. It was then ground using the Oster household blender. Ten kilograms of ripe Philippine mangoes, *Mangifera indica,* ranging from class four to five on the ripeness chart (Nambi et al. 2015), was obtained from Iloilo Central Market.

The excess flesh on the mango peels was removed and the peels were sliced into small squares using a kitchen knife. The mango peels were initially washed with tap water in a colander by batch and then with distilled water thrice. The mango peels were then evenly placed on a glass baking tray with a skin-side down orientation to prevent curling of the mango peels when drying. They were then oven-dried for 72 hours at 70°C (Iqbal et al. 2009b). After drying, the dried mango peel adsorbents were placed inside a plastic container and were crushed manually. Both the adsorbents were sieved using the Hubbard Scientific Sieve Mesh Size 10 at a particle size larger than 2 mm. After sieving, the adsorbents were stored in separate air-tight containers at constant room temperature until the implementation of the column adsorption.

N. Seed Priming Agents

(Legurpa Mi, Legurpa Ma, Oberio)

Seed priming is a technique used to improve the overall germination behavior of rice through the imbibition of solutions. This study employed three different priming methods (mannitol, glycerol, and sorbitol) on two local Philippine varieties of *Oryza sativa* (rice) and compared germination behaviors between primed and unprimed seeds.

This study is an experimental study that investigated and compared the effect of three different priming agents (sorbitol, mannitol, and glycerol) with specific concentrations on two different local varieties of rice (red and black). Several germination parameters were used to compare the different setups in order to test the seed priming effect on seed germination.

The methods were provided by Mr. Stephen Timple from International Rice Research Institute (IRRI) taken from De Guzman 2018. This study was conducted at the second-floor laboratory of Philippine Science High School - Western Visayas Campus' SLRC Building. Three different Priming Agents were used in the study and one unprimed control for each variety. These concentrations below were taken from previous studies on seed priming (Theerakulpisut et al. 2017) (Debnath et al. 2017).

The table below shows the priming agent, what concentration was used, and their treatment numbers.

Table 3. *Priming agents.*

Priming Agent	Concentration	Treatment Number
mannitol	2% (Theerakulpisut et al. 2017)	1
glycerol	5% (Debnath et al. 2017)	2
sorbitol	0.25M (Theerakulpisut et al. 2017)	3

Twenty-five grams of mannitol was measured using an analytical balance (Shimadzu aux-220) and then dissolved in 500mL of water in a 2L beaker and stirred using a stirring rod in order to achieve the desired 2% concentration. A 100mL graduated cylinder was used to measure 25mL of glycerol and 475mL of water to create a 5% glycerol solution, in a 2L beaker, mixed using a stirring rod. The same process was repeated to achieve a 0.25M sorbitol solution with 15mL of sorbitol and 485mL of water.

During the priming process, the seed weight to solution volume ratio of 1:5 was used. 100g of seeds from each variety were soaked in 5% solution of glycerol for 24 hours, the same was done for mannitol and sorbitol. This was done in 2L beakers. Soaked seeds were then recovered from the solution, spread on a metal tray with paper towels and allowed to dry for 24 hours.

O. *Oryza sativa* (Rice) Seed Priming

(Legurpa Mi, Legurpa Ma, Oberio)

Seed priming is a technique used to improve the overall germination behavior of rice through the imbibition of solutions. This study employed three different priming methods (mannitol, glycerol, and sorbitol) on two local Philippine varieties of *Oryza sativa* (rice) and compared germination behaviors between primed and unprimed seeds.

250g of seeds of both Black and Red rice were obtained from a local farmer. The priming agents; mannitol, glycerol and sorbitol, were all obtained from Patagonian Enterprises. The beakers, stirring rods, Petri dishes, flasks and graduated cylinders were all from PSHS WVC's Chemistry Supply.

Seeds were tested for their moisture content at Western Visayas Agricultural Research Center. A grain moisture tester (Riceter f511) was used to measure the moisture content. A small number of seeds were placed into the test chamber and then the handle was turned to crush the grain and the moisture content was instantly provided.

Salt Treatment Setups
(Geroche, Sombiro, Villegas, Olvido, Patricio)

Salinity is one of the leading causes of crop yield loss worldwide. Harmful cations and anions are present in the soil as seawater intrusion is the main cause of soil salinization in the Philippines. With this, the study focused on the effect of three types of salts: NaCl, KCl, and $CaCl_2$ on the germination stage of rice.

The NSIC Rc 442 rice variety seeds that were used for the study had a moisture content (MC) of 13.4%, which fell within the desired MC range of 8% to 14%. The seeds and their certification were obtained from WESVIARC.

Pre-germinated seeds were separated between pure and impure seeds. Fifty seeds of rice were randomLy placed in 9cm-diameter Petri dishes. Twenty milliliters of each solution were added to the Petri dishes. Distilled water was used for the control set-up.

P. ***Bixa orellana***
(Venturina, Comuelo, Samaniego)

Extracts from plants such as Bixa orellana, commonly known as Annatto, present the potential to be an alternative to the commonly used synthetic stains. Thus, this study aimed to evaluate Bixa orellana methanolic extracts as a substitute for safranin in Gram Staining. Bixa orellana extracts were used to stain *S. aureus*, *E.coli*, and mixed bacteria smears.

The seeds were separated by hand then washed with distilled water and sterilized in a hot air oven at 60°C for 24 hours (Braide et al. 2010). The seeds were then powdered using a grinder.

SAFETY PROTOCOLS

OVERVIEW

When handling biological material, researchers must be cautious of the possible presence of parasite species of varying infectious stages. To avoid ingestion, proper personal protective equipment (PPE) must be used throughout all stages. These typically include laboratory gowns, surgical gloves, and surgical masks, but may vary depending on the type of material being handled. Finally, young researchers must always remember to properly disinfect articles of clothing, and thoroughly wash their hands after each handling of these samples.

A. Fecal Samples
(Lopez, Aguirre, Dalisay)

Segregation of waste materials was followed thoroughly. All materials that were used were labeled. Ideal proper laboratory attire was worn. Laboratory gown, pair of gloves, and masks were used during the entire duration of the study. All glassware was handled with care. All glassware were rinsed three times into the waste container before they were cleaned. Extreme caution was followed when handling the chemicals. Glasswares were stored in a safe place to avoid scratches and breakage. After every experiment, the glasswares were cleaned and dried as soon as possible prior to storing. All the glassware used were placed on a pot full of water and were left for boiling after using them. The glassware were then rinsed with tap water twice, they were first rinsed with liquid detergent and then rinsed for the second time with distilled water. Glasswares were then placed upside down in a tray for drying. Uncontaminated materials and contaminated materials were labeled. Then when dry, the materials were ready for storage or for disposal. Glasswares contaminated with biological material were emptied and decontaminated by boiling. They were rinsed thoroughly with tap water, drained, and air-dried.

SCREENING

OVERVIEW

Screening is one of the most reliable methods for gathering data from phenomena occurring beyond the microscopic scale. A variety of reagents, procedures, and samples can be utilized to cater to different scientific investigations. This chapter provides a brief description of the different methods of screening for chemical and biochemical studies.

A. Antibacterial
(Elizalde, Herida, Jaudian)

A 10-fold serial dilution was performed in order to dilute the extract in the prepared broths into 8 different concentrations using a micropipette. The bacteria samples were then inoculated into the treated test tubes using a micropipette with 100 µm each. One test-tube for every trial was reserved for normal growth without any extract present as a negative control. The test tubes were then incubated for 18-24 hours under an optimal temperature of 30°C. The turbidity of each test tube was measured using a UV-2100 spectrophotometer with a wavelength of 600 µm.

B. Phytochemical
(Andonaque, Dorado, Ledesma)

The antioxidant activity of Lansium domesticum seed extracts was determined by phytochemical screening, adopting and modifying methods from Tiwali, et. al. (2011). After drying, crushing, and sifting, Lansium domesticum seeds were added to amber reagent bottles containing hexane, methanol, and ethyl acetate, respectively, with a solvent to dry weight ratio of 5:1. The presence of phenols was tested by adding 3-4 drops of 5% ferric chloride solution to 1 mL of the stock solution, with a change in color to brownish-black indicating a positive result. Flavonoids were tested by adding 10% sodium hydroxide solution dropwise into 1 mL of the stock solution in a test tube. At the appearance of an intense yellow color, 5N hydrochloric acid was then added dropwise until the solution turned colorless, indicating the presence of flavonoids. For saponin testing, one mL of the stock solution was placed in a graduated cylinder, diluted with 20 mL of distilled water, and manually agitated for 15 minutes. The formation of a foam layer roughly 1 cm thick at the top indicated the presence of saponins. Alkaloid testing was done by adding 5-15 drops of Wagner's reagent into 1 mL of stock solution in a test tube. The formation of a brown precipitate indicated the presence of alkaloids.

C. Anthocyanin (Triol, Dionela, Ecube, Mediodia)

The ethanolic extract of *Clitoria ternatea* was tested for the presence of anthocyanin following the procedure from the book entitled "A Guidebook To Plant Screening: Phytochemical and Biological" edited by Guevara (2005). Three milliliters of the ethanolic extract were added with five drops of 1% (v/v) hydrochloric acid (HCl) and was subjected to boiling. A change from the original color of the extract to an orange-red to blue-red color upon addition of HCl was used as an indicator for the presence of anthocyanin in the extract.

D. Nitrogen Fixation (Halaba, Molinos, Superficial, Bela-ong)

Endophytic bacterial isolates were isolated from the roots of Zea mays L. var. rugosa (sweet corn) Sugar King variety at the vegetative stage. A total of eight different bacterial strains were isolated, characterized; through gram staining and colony and cell morphology; and screened for a positive reaction for nitrogen fixation, ammonia production, and zinc solubilization through the use of selective media. Jensen's media was used to screen for nitrogen-fixing bacteria, peptone water with Nessler's reagent for ammonia-producing bacteria, and zinc-incorporated media for zinc solubilizing bacteria.

Bromothymol blue was used as an indicator for the screening of nitrogen-fixing bacteria. The reagent was provided by the Chemistry SRA of PSHS-WVC.

Following the procedures for the making of Jensen's media by Richard et al., 20 grams of sucrose, one gram of dipotassium phosphate, 0.500g of magnesium sulfate, 0.500g of sodium chloride, 0.100g of ferrous sulfate, two grams

of calcium carbonate and 15g of agar were suspended with 1 L of distilled water in a culture bottle. The mixture was then boiled using a hot plate and continuously stirred using a sterile glass stirring rod. After boiling, the solution was sterilized in an autoclave at 121° C for 15 minutes. The solution was then allowed to cool down for 3-4 minutes before being distributed into sterile agar plates. The plates were filled until the bottoms were fully covered and allowed to cool and solidify inside the biosafety cabinet. The plates were then labeled and stored at 2-8° C.

Nitrogen-free malate agar (Jensen's media) was used in the screening for nitrogen fixation with bromothymol blue (BTB) acting as an indicator (Gothwal et al. 2007). The isolates were then incubated at 37°C for 24 hours. Isolates that exhibit nitrogen fixation will provide nitrogen that the media lacks and will change its color from creamy white to blue.

E. Ammonia Production
(Halaba, Molinos, Superficial, Bela-ong)

Endophytic bacterial isolates were isolated from the roots of Zea mays L. var. rugosa (sweet corn) Sugar King variety at the vegetative stage. A total of eight different bacterial strains were isolated, characterized; through gram staining and colony and cell morphology; and screened for a positive reaction for nitrogen fixation, ammonia production, and zinc solubilization through the use of selective media. Jensen's media was used to screen for nitrogen-fixing bacteria, peptone water with Nessler's reagent for ammonia-producing bacteria, and zinc-incorporated media for zinc solubilizing bacteria.

The guidelines reported by HiMedia were followed in the preparation of peptone water. In a culture bottle, 10g of peptone and 5g of sodium chloride were suspended with 1 L of distilled water. The mixture was then boiled using a hot plate and continuously stirred using a sterile glass stirring rod. After boiling, the solution was autoclaved for sterilization at 121° C for 15 minutes. Then, 16 test tubes were each filled with 10 mL of peptone water solution while the remaining were left inside the culture bottle. Both were then labeled and stored at 2-8° C.

The guidelines reported by HiMedia were followed in the preparation of peptone water. Inside a culture bottle, 10g of mercuric chloride, 7g of potassium iodide, 16g of sodium hydroxide were suspended and mixed with 100 mL of distilled water. The mixture was then stirred continuously using a sterile glass stirring rod. The solution was then allowed to cool down before being stored in the refrigerator at 2-8° C.

Isolates were inoculated into 10 mL peptone water in separate test tubes then incubated for 2-3 days at 28±2 degrees Celsius. After the addition of 0.5 mL of Nessler's reagent, isolates that exhibit ammonia production will cause the water to discolor due to the sudden addition of ammonia to the solution.

F. Zinc Solubilization
(Halaba, Molinos, Superficial, Bela-ong)

Endophytic bacterial isolates were isolated from the roots of Zea mays L. var. rugosa (sweet corn) Sugar King variety at the vegetative stage. A total of eight different bacterial strains were isolated, characterized; through gram staining and colony and cell morphology; and screened for a positive reaction for nitrogen fixation, ammonia production, and zinc solubilization through the use of selective media. Jensen's media was used to screen for nitrogen-fixing bacteria, peptone water with Nessler's reagent for ammonia-producing bacteria, and zinc incorporated media for zinc solubilizing bacteria.

Following the procedures for the making of zinc chloride medium by Kamran et al., 0.5g of zinc chloride was suspended with 500 mL of TSA. The mixture was then boiled using a hot plate and continuously stirred using a sterile glass stirring rod. After boiling, the solution was autoclaved for sterilization at 121°C for 15 minutes. The solution was then allowed to cool down for 3-4 minutes before being distributed into sterile agar plates. The plates were filled until the bottoms were fully covered and allowed to cool and solidify inside the biosafety cabinet. The plates were then labeled and stored at 2-8° C.

Zinc chloride (ZnCl2) medium plates were used in the screening for zinc solubilization. The isolates were aseptically inoculated as spot on the respective medium plates and covered with aluminum foil. They were then incubated in the dark at 28° C for 1 day. Isolates exhibiting zinc solubilization formed clear zones around the bacterial colonies due to the bacteria breaking down the zinc present in the media into simpler forms.

SELECTION

OVERVIEW

This chapter discusses the selection criteria for different aspects of research studies. It includes the selection of study sites and of animal subjects.

A. Study Site

Site for Dog Feces Collection

(Lopez, Aguirre, Dalisay)

Iloilo is one of the four (4) provinces in Panay Island located in Region VI Western Visayas of the Philippines to which its capital is Iloilo City. Currently, there are seven (7) districts in Iloilo City which are Arevalo, Iloilo City Proper, Jaro, La Paz, La Puz, Mandurriao, and Molo. Based on the 2010 Census on Population and Housing (CPH), the current population of Iloilo City is 424,169. According to a study conducted by Robinson et al. 1996, the human to dog ratio in the Philippines is one to three (1:3). This means that for every dog, there are three (3) humans accompanying it. Based on this data, there should be about 142,000 dogs in Iloilo City. Among these districts only one (1) district was selected. The number of barangays was considered due to the limited time frame given for the study. Added with safety reasons, the district of Arevalo was selected as the district to be the study site. It is located 10.6859 N, 122.5118 E having 13 barangays which are Bonifacio, Calaparan, Dulonan, Mohon, Quezon, San Jose, Santa Cruz, Santa Filomina, Santo Domingo, Santo Nio Norte, Santo Nio Sur, Sooc, and Yulo Drive.

Site for Collection of Microplastics

(Colacion, San Diego, Secondes, Oberio)

Anilao, Iloilo was selected as the coastal community to investigate due to its large impact on the province's seafood industry, as well as its accessibility to the researchers. Three (3) coastal areas in the municipality, specifically Barangays Dangula-an (10°58'58.2" N 122°46'44.2" E), Pantalan (10°57'5" N 122°45'52" E), and San Carlos (10°58'48" N 122°46'42" E) were selected as sampling sites based on the fish-ing and mariculture activities in the areas. The sediment sample collection was conducted during low tide in the coastal areas. A transect of length 100 m was placed along the high tide line of each coastal area. Three quadrats of dimension 0.25 m x 0.25 m were then randomly placed along the 100 m stretch. A metal trowel was used to collect sand to a depth of approximately 2 cm from the surface. The obtained samples were placed inside a glass container with a metallic lid, stored inside an ice chest, and transferred to the PSHS-WVC Research Laboratory. A total of three (3) samples per site were collected.

B. Domestic Dogs

(Lopez, Aguirre, Dalisay)

Selection criteria for the dogs included the presence of leashed dogs in households and consent from the owners to collect samples. The fecal samples needed to be approximately one (1) to three (3) days old. Information such as the age, breed, diet, and medical treatment such as vaccination and deworming of the dogs were also taken. After one household was selected we selected the next household approximately 10 households away from the first one to avoid the auto infections of parasites in dogs.

STORAGE

OVERVIEW

Storage is the action of preserving samples for future use. This section deals with the storage and preservation of human platelets. Though some samples take a while to degrade, always ensure that the length of storage does not compromise the quality of the sample. It is always recommended to work on the sample upon harvest/preparation unless otherwise required for the study.

A. Platelet Storage and Preservation
(Alvarez, Oberio)

CPDA-1 has been added during blood extraction (contained in the blood bag) during blood extraction at around 63 mL per blood unit to preserve the platelets and prevent coagulation. The samples were stored at a volume of 20 mL at 20-24oC with constant gentle agitation in their respective satellite bags. Three replicates were prepared, and each replicate consisted of the three setups: l-carnitine (positive control), saline (negative control), and ALCAR.

The chosen concentration for the preservatives used in the study was 15 mM based on a pilot study of Deyhim et al. (2014) in determining the best concentration and volume of l-carnitine in preserving PCs.

L-carnitine solution was prepared by dissolving the l-carnitine powder in sterile, normal saline at a concentration of 15 mM.

A volume of 1 mL l-carnitine solution was added to the positive control setup (Deyhim et al. 2014). ALCAR was prepared and added to the variable setup the same way as l-carnitine. The preservatives were added to the PCs one day after blood extraction.

One mL of l-carnitine and ALCAR each were introduced into the platelet bag via aseptic infusion one day after the extraction. Insulin syringes were used after filtering the preservative solution through a 0.22 µm filter. Sterile normal saline was used to dissolve the l-carnitine and ALCAR powders. As a control, an equal volume of 1 mL of saline was also added to the third setup. The site of puncture was sealed and a stripper was used to mix the preservative with the blood bag contents. A biosafety cabinet level II located in a nearby hospital from the site of storage was used. Transportation of samples was done using an approved Styrofoam box and was returned after the application of preservatives.

TREATMENT

OVERVIEW

In experimental studies, specific treatments are administered to experimental units to determine their effects, usually to establish whether the treatment is effective or is at par with current standard treatments. This category covers methods in different forms of treatment administered to various experimental units. Some methods employ manipulation of main treatment components, while some manipulate only the concentration of the same treatment. Other methods focus on the manipulation of storage conditions.

A. Virus-infected Samples
(Sira, Valzado, Larroder, Cabarles)

Infected samples were drenched using 1 mL syringes with aqueous extracts from the *C. citratus* plant every afternoon. Groups from vaccinated samples vaccinated against Newcastle's Disease (ND) were labeled as Group VA (vaccinated, group A), Group VB (vaccinated, group B), Group VC (vaccinated, group C), and Group VD (vaccinated, group D). Groups from the unvaccinated samples were labeled as Group UA (unvaccinated, group A), Group UB (unvaccinated, group B), Group UC (unvaccinated, group C), and Group UD (unvaccinated, group D). Samples from groups VA and UA were administered with aqueous extract dosed at 1 mL extract, groups VB and UB with 1 mL extract/5 mL, groups VC and UC with 1 mL extract/10 mL, and groups VD and UD with 1 mL extract/20 mL. Survival rate and live weight gain were measured 14 days after the start of treatment. Survival rate was computed by treatment group using the equation (total number of chickens - number of deaths)total number of chickens) x 100. With regards to the side effects of the phytochemicals on chickens, the study of Raza et al. (2015) showed that plants containing alkaloids, flavonoids, saponins, and tannins that were tested against the ND virus in vitro and in vivo exhibited positive results and did not show any adverse reactions on chickens.

B. Light Cycle for Algal Cultures
(Derramas, Gonzalez, Villaflor, Mediodia)

Twenty milliliters (20 mL) of Chlorella sorokiniana culture in exponential phase cultured in Conway medium was inoculated into each Erlenmeyer flask. The flasks were linearly arranged in a ventilated, isolated room. The cultures were subjected to light exposure with a 20-watt fluorescent lights distance of 40.64 cm (16 in) away from the flasks on a light/dark regimen of 14/10 h simulation of the day and night light exposure cycle (Patel et al. 2012). After submerging the connected tubing into the filled flasks, the water aerator, set on low, was switched on to induce agitation of the medium by continuously bubbling the air in the flasks. These conditions were maintained for the entire duration of the observation period.

The setup was monitored for nine days. The lights were switched on and off at 6:26 AM and 8:26 PM, respectively. This simulated a 14/10-hour day and night cycle.

C. Seed Priming
(Legurpa Mi, Legurpa Ma, Oberio)

Seed priming is a technique used to improve the overall germination behavior of rice through the imbibition of solutions. This study employed three different priming methods (mannitol, glycerol, and sorbitol) on two local Philippine varieties of Oryza sativa (rice) and compared germination behaviors between primed and unprimed seeds.

The priming agents mannitol, glycerol, and sorbitol were prepared. During the priming process, the seed weight to solution volume ratio of 1:5 was used. 100g of seeds from each variety were soaked in a 5% solution of glycerol for 24 hours, the same was done for mannitol and sorbitol. This was done in 2L beakers. Soaked seeds were then recovered from the solution, spread on a metal tray with paper towels, and allowed to dry for 24 hours. After that seeds were brought to WESVIARC to have their moisture contents measured following the same methods as the first test.

D. Germination

(Geroche, Sombiro, Villegas, Olvido, Patricio)

Salinity is one of the leading causes of crop yield loss worldwide. Harmful cations and anions are present in the soil as seawater intrusion is the main cause of soil salinization in the Philippines. With this, the study focused on the effect of three types of salts: sodium chloride (NaCl), potassium chloride (KCl), and calcium chloride (CaCl2) on the germination stage of rice.

Each researcher was assigned to a table, in which they were responsible for counting the number of germinated seeds daily, performing maintenance, and properly managing the set-up when it was needed.

The germination rate was recorded for every petri dish daily for ten consecutive days. Counting began at 4:30 PM daily.

Ambient temperature and relative humidity were measured using the LabdiscEnviro daily for10 consecutive days. Ambient factors were gathered at 8:00 AM, 12:00 PM, and 4:00 PM daily.

The leaf color of the seeds was measured by matching the leaves of the best seeds on a Leaf Color Chart produced by the Philippine Rice Research Institute (PhilRice) acquired from the Department of Agriculture- LGU Oton.

Ten of the best seedlings per petri dish were chosen to represent the petri dish. Shoot and root length were measured using a plastic Vernier caliper with a precision of 1mm. The germinated seed's shoot was laid flat beside the Vernier caliper, and the measurement was taken. The same process was repeated for the roots. In the case of the roots, the longest root was chosen as the main root for measurement.

The 10 best seedlings used for the length of the roots and shoots were also utilized for the weight of the roots and shoots. The roots and shoots were separated from the seedlings using tweezers and laid on tissue paper. The roots and shoots were then patted dry and stored for measurement. The roots and shoots were weighed using Shimadzu Analytical Balance (AUX220), and a plastic petri dish cover was used as a tray for the samples. The roots and shoots were weighed within two hours upon being separated from the seedling.

The germination rate (or periodic germination percentage) provides a measure of the time course of seed germination. It was measured daily, and its unit of measurement is in percent (%). Its formula is as given below:

$$Germination\ Rate = \frac{GR_0 - GR_1}{50} \times 100$$

Equation 1. Formula for calculating Germination Rate

Where GR_0 = Total number of germinated seeds today and GR_1 = Total number of germinated seeds yesterday

The germination percentage was recorded after the 10th day. Germination percentage expresses the proportion of the total number of seeds that are alive. A seed is considered germinated if its main radicle has reached a length of 2 mm (Vibhuti et al. 2015). Good seeds have more than an 80% germination rate. The rate of germination is an indicator of vigor. The formula for germination percentage is as given below (Vibhuti et al. 2015).

$$Germination\ Percentage = \frac{No.\ of\ total\ germinated\ sees}{Total\ no.\ of\ tested\ seeds} \times$$

Equation 2. Formula for Germination Percentage

A one-way Analysis of Variance (ANOVA) at a 95% confidence interval (α = 0.05) was used to evaluate whether a significant difference exists between the germination percentage, lengths, and weights of both shoots and roots, in relation to the type of salt and the level of concentration utilized. This test was performed using Statistical Packages for the Social Sciences (SPSS) version 23.