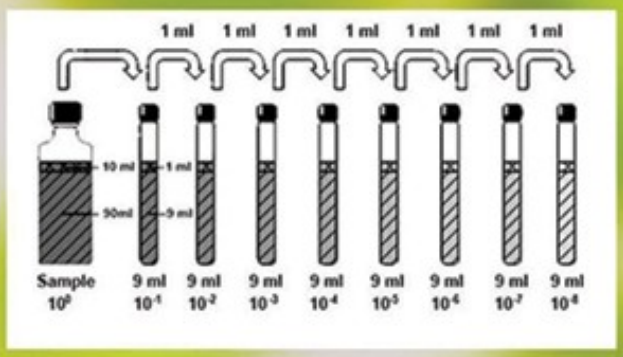
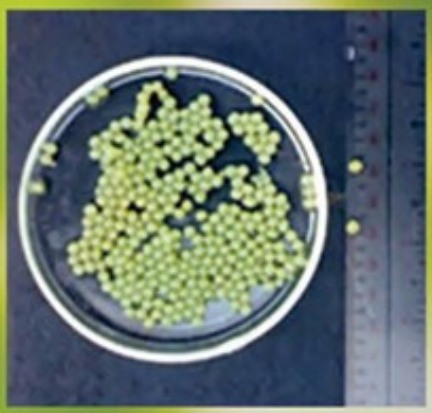
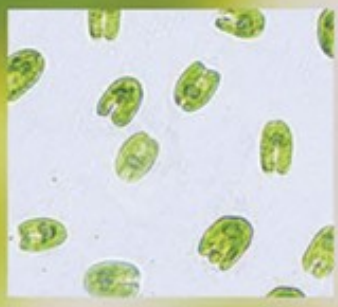
Methods in Microalgal Studies

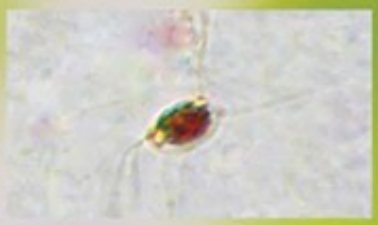
EDITORS

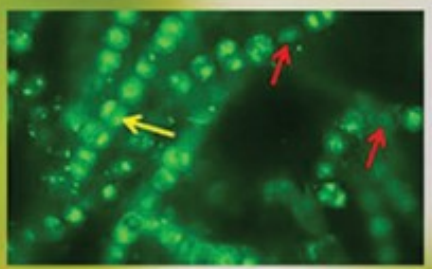
MRM Goss WL Rivera NK Torreta

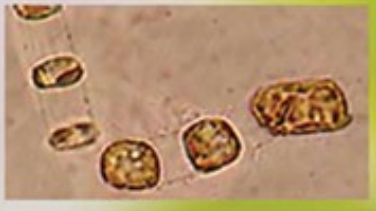












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Methods in Microalgal Studies

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PREFACE

A number of circumstances led to the realization of this book. First, the experience of the editors particularly Professor Emeritus Milagrosa Martinez-Goss, with colleagues and stakeholders during conventions and in the recently concluded CHED funded program entitled "Capability Building Program on Microalgae Research" pointed to the need for an authoritative book that would serve as a shared reference embodying their collective capabilities and interests in the different methods in the study of microalgae. Second, there was a need to come up with an affordable, locally written reference material that would highlight locally available materials and equipment needed in conducting and performing experiments in microalgal research. The fact that many of those working in phycology are not majors in the field, but got there only by force of circumstance, meant that they are not familiar with its methods, terminologies and identity of organisms, and this made a common reference imperative. It was clear that an accessible, affordable, reliable and comprehensive volume to which workers could refer to was urgently needed. Third, a volume like this would not only be immensely useful in providing guidance to the many involved in the field, but would also go a long way in standardizing practices and methods in laboratories and schools.

The completion of this book has taken several years to be fully realized. The groundwork was laid down when it was recognized that there was a need to come up with a book envisioned to embody the state of the art in the microalgal research that would serve as a reliable compendium of microalgal techniques – a standard reference that would be the legacy of the authors for the future, and an archetype or model for what is to come. This led to the next stage, which was to get in touch with the leading authorities in the different areas of phycology touching on microalgae. Their coming together and the formulation of the contents of this book, as authors and reviewers, over a period of several years, with many drafts and revisions along the way, is what has led to this work.

This tome has an Introduction and 16 chapters and the latter are divided into six major topics, such as, 1) Sterilization, Disinfection, Isolation and Purification, 2) Culture, Maintenance, and Live Preservation of Microalgae, 3) Preservation, Fixation, and Storage of Algal Specimens, 4) Estimation of Algal Biomass, 5) Microscopy, and 6) Approaches in Algal Taxonomy. These were written by 26 scientists who are/were from the teaching and research community in the Philippines and abroad. All the chapters in this book underwent two separate reviews, one headed by Dr. Martinez-Goss and the other one was done by the Board of Reviewers of Philippine Science Letters. There were a total of 28 expertise who willingly and carefully reviewed parts of this book.

Grateful recognition is due to all of them for their contributions, in the various chapters, glossaries and indices, and assistance in making this work a reality. In particular we are indebted to the invaluable contribution in the completion of this book from researching to editing and formatting to Mr. Edwin N. Camaya. We also wish to thank Ms. Roselyn P. Padernal for her kind help in correcting the final draft. The financial assistance of the UPLB FI- DA-Bar Project no. 2012-080 “Optimization of Growth and Functional Food Ingredients in the Edible Cyanobacterium *Nostoc commune* Vaucher for Commercial Production” under the leadership of Dr. Milagrosa R. Martinez-Goss is hereby acknowledged. Likewise, we would like to thank Maria Adeline Diongco for making the cover design.

MR Martinez-Goss
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NK Torreta

UNIVERSITY OF THE PHILIPPINES LOS BAÑOS

Office of the Chancellor

MESSAGE

I commend the team of Dr. Milagrosa R. Martinez-Goss, Dr. Windell L. Rivera, and Prof. Nerissa K. Torreta for undertaking the monumental task of producing the book titled, "Methods In Microalgal Studies," published by the prestigious ISI scientific journal, Philippine Science Letters, and the University of the Philippines Los Baños.

This compilation, with a total of 16 chapters, encompassing 6 major topics on microalgal research, written by 26 scientists, and reviewed by 28 Filipino experts, provides an important addition to the growing body of scientific works adapted to Philippine conditions.

This publication addresses the rising need to specify and standardize the fundamental methodologies in conducting basic and applied researches amidst the growing interest in microalgae research in the country, not just in academe but also in industry, such as in pharmaceuticals, food, feed, and fuels. It will guide the increasing number of practitioners and ease data comparison across various studies.

UPLB and the scientific community acknowledge the CHED-funded program entitled "Capability Building Program on Microalgae Research" as the catalyst for this book and the UPLB-FI-DA-BAR Project entitled "Optimization of Growth and Functional Food Ingredients on the Edible Cyanobacterium, Nostoc commune Vaucher, for Commercial Production," for the financial support it provided.

In line with our vision of a future-proof UPLB, the University supports this noteworthy undertaking towards standardizing current and upcoming research in the study of microalgae.

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INTRODUCTION

Windell L. Rivera [1*], Milagrosa R. Martinez-Goss[2], and Nerissa K. Torreta[3]

Early records of freshwater microalgae in the Philippines were noted by Georg von Martens when he discovered two new species of *Cladophora*, i.e., *C. diluta* and *C. luzonensis* from the collection of the Prussian East Asia Expedition in Rizal and Laguna, headed by FJE Meyen when they visited the Philippines in 1831 (Cordero 1990). However, the earliest mention of the algae, specifically marine macroalgae, in the country was made by Blanco in his book, Flora de Filipinas (1837-1845). Almost within this period, in 1838-1842, an American expedition called the "Wilkes Exploring Expedition" visited the Pacific Ocean and its surrounding islands, including the Philippines. There was no phycologist in the group, thus, the specimens collected were examined by JW Bailey and WH Harvey. Aside from the marine macroalgae that they studied and identified, they also worked on microalgae, like the diatoms. These specimens are now kept in the Farlow Herbarium of the Cryptogamic Herbarium of Harvard University (Edgar 1979). About eight decades after, the first comprehensive taxonomic book of Philippine marine diatoms was published by Albert Mann in 1925 (Mann 1925). These were the specimens collected by the Albatross Voyages from 1888-1904, in the Pacific Ocean and the surrounding islands, and the mounted slides are kept in the General Diatom Collection of the United States National Museum in Washington, D.C., USA (Mann 1907; Mann 1925).

The earliest work on Philippine cyanobacteria (blue-green algae) were done by Dickie (1876 a, b) from the botanical collection of HN Moseley, the naturalist of the British HMS Challenger Expedition (1872-1876) (Martinez 1984). Dickie identified two species of *Lyngbya* and a species of *Oscillatoria* that were collected in the vicinity of Zamboanga City and Camiguin Is. in Mindanao, Philippines. Sixty-four years later, the first of a series of papers on Philippine Myxophyceae (Cyanobacteria) was published by our late National Scientist, Dr. Gregorio T. Velasquez (Velasquez 1940). His greatest work on this group of microalgae was published in the Phillippine Journal of Science in 1962 where 162 species and three forms belonging to 33 genera and eight families were described (Velasquez 1962). Dr. Velasquez provided the impetus to work on this microalgal group among his colleagues and students. Hence, over a period of 100 years (1870s-1970s), a great number of publications on cyanobacteria were achieved in 1970s written mostly by Filipino than foreign scientists (Martinez 1984).

More recently, the microalgae and the phytoplankter have been the subject of books and publications which includes JB Pantastico's (1977) Freshwater Algae of Laguna de Bay and vicinity; Tamayo-Zafaralla's the Microalgae of Taal Lake and the Seven Lakes of San Pablo City, including the algae in Crocodile Lake in Los Baños, Laguna (Tamayo-Zafaralla 1998, 2014); and Martinez and co-workers' published taxonomy and ecology of algae in fishponds and fishpens of Laguna de Bay (Martinez 1978, 1984; Martinez and Eakle 1977). Dinoflagellates have been the focus of many researches and publications especially since the first recorded red tide in the Philippines in 1983 (Yap-Dejeto et al. 2018). The book on "Harmful Dinoflagellates of the Philippines" by Azanza and co-workers (2017) is a timely synthesis on this subject matter.

The growth in awareness and knowledge about microalgae in the Philippines has been extensively augmented by both veteran scientists and young researchers who became cognizant of the need for an authoritative volume covering the different aspects of the study of microalgae, from

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sterilization and disinfection to isolation, laboratory culture, mass culture, maintenance and propagation, immobilization, enumeration, microscopic observation and taxonomy. The increasing number of workers engaged in this area of phycology, rapid scientific advances, and heightened interest due to the importance of microalgae in the economic and environmental realms have made such a volume much more crucial.

This book lays the groundwork for those working on microalgae. The first chapter is on sterilization and disinfection, the logical starting point for those dealing with microorganisms. The chapter that follows is on isolation and purification of microalgae, and the next two concern the culture of freshwater and marine microalgae. Chapter 5 is on maintenance and preservation of microalgal cultures. Chapter 6 on immobilization of microalgal cells for various purposes. Chapter 7 on production of microalgal paste for use as feed for mollusks, crustaceans and other aquatic organisms. Chapter 8 on the preparation of diatoms for light and electron microscopy. Chapter 9 on the preservation of microalgal specimens in fluids. Chapter 10 on the preparation of herbarium specimens of microalgae. Chapter 11 deals with the enumeration of microalgae using the haemocytometer while Chapter 12 is on the analysis of chlolorophyll, a key component of microalgae. The two chapters that follow are on microscopy: microscopes and basic microscopic techniques, and fluorescence microscopy. Finally, chapters 16 and 17 are on taxonomy: classical taxonomy, and molecular techniques for algal taxonomy.

It is hoped that this work will respond to the needs of teachers, students, researchers and stakeholders; provide instruction and guidance; help standardize practice in schools, laboratories and institutions; stimulate further interest in microalgae and phycology in general, and point the way to future directions in the field.

This book is a work in progress for it still lacks some aspects like methods on the study of dinoflagellates, cryptophytes, charophytes, and many more. Hopefully, future editions will address these topics.

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Chapter 1. Sterilization and Disinfection in the Culture of Microalgae

Nerissa K. Torreta[1]

I. Introduction

Microalgae are a diverse group of photosynthetic microorganisms that may either be prokaryotic (cyanobacteria and prochlorophytes) or eukaryotic (chlorophytes, euglenoids, diatoms, dinoflagellates, rhodophytes, and phaeophytes). They are morphologically and phylogenetically diverse. Considered as versatile cellular factories, they contain pigments, lipids, proteins, polysaccharides, bioactive compounds, and other products that are economically and ecologically important (Koller et al. 2014). Hence, the culture and maintenance of these microalgae is essential.

An important aspect in the culture of microalgae is sterilization. From the preparation of culture media to inoculation, even in the maintenance and storage of microalgal cultures, sterilization needs to be done**. Sterilization** is a process that destroys or removes all viable microorganisms, including spores and viruses. It is a process in establishing aseptic conditions and is important in maintaining isolated strains of microalgae (Fernandez, et al., 2008; Madigan et al., 2015). Kawachi and Noel (2005) stressed that the use of sterile technique, in combination with sterile equipment and supplies, minimizes contamination, resulting in more reliable cultures free of unwanted organisms.

Often confused with sterilization is **disinfection.** Collins et al. (2004) defined **disinfection** as a process that implies the destruction of vegetative organisms that might cause disease or spoilage. It usually employs chemicals and does not necessarily kill spores. Disinfection, then, is a process that kills or reduces the number of pathogenic microorganisms in an environment or a surface (Kawachi and Noel, 2005). For instance, the use of hypochlorite (like Clorox or Zonrox) including the use of Quats (quarternary ammonium compounds) or 70% ethyl alcohol in cleaning table surfaces is disinfection which is an integral part of the sterile technique.

II. Pre-sterilization: Cleaning Procedures for Glassware

It is important to thoroughly clean new glass and plastic vessels before first use because there may be chemicals or other contaminants resulting from their manufacture which may be harmful to the microalgae. Also, dirty culture vessels and used glass pipettes must be cleaned before they are reused. Table 1.1 below summarizes the cleaning procedures that can be employed for these purposes.

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Table 1.1. Summary of cleaning procedures prior to sterilization (modified from Kawachi and Noel, 2005).

Container	Cleaning Procedure
New Glassware	• Immerse in dilute hydrochloric bath (1 M HCl) for 1 week Rinse several times in running tap water • Rinse with distilled or deionized water before drying • Alternatively, wash in neutral detergent commercialized for laboratory use (like Cleanmate) followed by thorough rinsing and drying. • It is also recommended to check the safety of cap materials before use because some may release toxic materials during autoclaving
Dirty Culture Vessels	• Dirty culture vessels, particularly, those with contamination, should be autoclaved to kill all cells • After autoclaving, liquid or agar is cooled and discarded and briefly rinsed in running water • Clean vessels by immersing in neutral detergent bath overnight followed by scrubbing with brush and sponge • Rinse in running water until no more suds are seen (rinsing > 10 times is recommended) • Final rinse is done with distilled water or deionized water • Cleaned vessels are dried in a dust-free area
Reusable Glass Pipettes	• Immediately rinse with tap water so that cells do not dry onto the glass surface • If immediate rinsing is not possible, immerse in detergent bath for 1 or 2 days • An ultrasonic bath and siphon-type pipette washer are used to completely remove the detergent • Pipettes are rinsed with distilled water and dried in 150ºC oven. Cotton plugs are inserted into the top of the pipette and the end is briefly flamed to remove excess cotton • Pipettes are placed in canister to be autoclaved or sterilized by dry heat method

III. Sterilization Methods

Sterilization ensures total inactivation of microbial life. It is done to prevent contamination by unwanted organisms and may also serve to eliminate unwanted chemicals. Sterilization can be done by means of several methods. The method of choice depends on the purpose and material used, either empty glassware/plastic ware or medium-containing vessels, but also on the facilities available in a laboratory.

Two general methods of sterilization may be used: physical or chemical sterilization (Kawachi and Noel 2005; Collins et al. 2004). Physical sterilization involves using either heat (moist or dry), filtration or radiation. Chemical sterilization makes use of different chemicals.

A. Physical

Physical sterilization refers to sterilization (the killing or removal of all microorganisms, including bacterial spores) by physical means, such as the use of heat (moist heat or dry heat). For instruments such as inoculating wires, the opening of test tubes, transfer loops or glass pipettes, sterilization can be done by holding them in a Bunsen flame (or even alcohol lamp) until they are red hot. Micro-incinerators, electrically operated and intended to replace Bunsen burners, are becoming very popular for this purpose (Fig.1.1).

1. Heat

Heat sterilization is sterilization with the application of heat. This process can either be by the moist heat or the dry heat method.

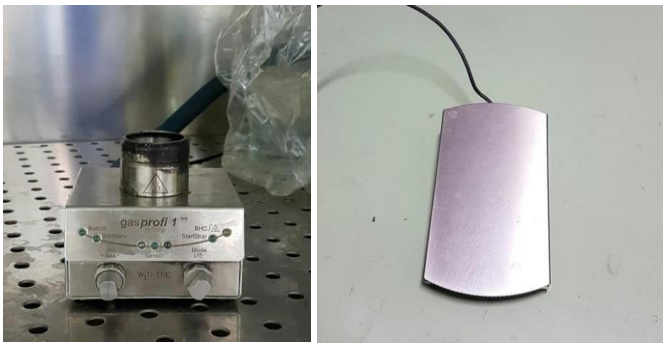

Figure 1.1. Electrically operated micro-incinerators, like this Fireboy, are equipped with foot pedals (right figure).

Moist Heat Sterilization

Materials for the culture of microalgae like glassware, petri dishes, pipettes and even the culture media, are usually sterilized through this method. Before sterilization, culture vessels and other glassware are wrapped in paper and held in place with rubber bands. Culture media should occupy only a third or half of the culture vessel space. These are stoppered with cotton plugs, covered with foil or paper, and held together by rubber bands. Once done, all the materials should be carefully arranged inside the autoclave for effective sterilization and to avoid breakage.

Reliable sterilization with moist heat requires temperatures above that of boiling water. The autoclave, which contains steam under pressure, is the most dependable sterilization method (Fig. 1.2). Steam must directly come in contact with the material to be sterilized. **Moist heat sterilization** kills microorganisms by **coagulating** their proteins. Latent heat is liberated when steam condenses on a cooler surface. Hydrolysis and breakdown of bacterial proteins occur. Heating at temperature of 100°C or more kills vegetative forms of bacterial pathogens. Most pathogens can be killed within 10 minutes or less. Endospores and some viruses are not destroyed as quickly. The temperature of steam reaches 121°C at twice the atmospheric pressure. All organisms and endospores are killed within 15 minutes. In general, moist heat is much more effective than dry heat.

Dry Heat Sterilization

Dry heat sterilization technique requires longer exposure time (1.5 to 3 hours) and higher temperatures than moist heat sterilization. Dry heat ovens are used to sterilize items that might be damaged by moist heat or that are impenetrable to moist heat (e.g., powders, petroleum products, sharp instruments). Metal instruments, glassware and the like are usually dry sterilized. Materials that are organic in nature undergo protein denaturation, oxidative damage and toxic effects of increased levels of electrolytes may also occur. For culturing algae this type of sterilization is not usually recommended.

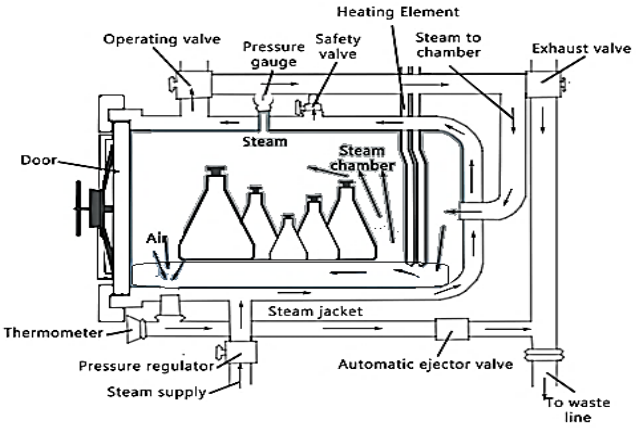

Figure 1.2. An autoclave is a closed chamber with high temperature and pressure (adapted from Tortora et al., 2004).

Sterilizing by dry heat is accomplished by conduction. The heat is absorbed by the outside surface of the item, then passes towards the center of the item, layer by layer. The entire item will eventually reach the temperature required for sterilization to take place. Dry heat does most of the damage by oxidizing molecules. The essential cell constituents are destroyed and the organism dies. The temperature is maintained for almost an hour to kill the most difficult of the resistant spores.

There are two types of dry-heat sterilizers. One is the **static-air type sterilizer**. This **oven-type sterilizer** has heating coils at the bottom of the unit that cause the hot air to rise inside the chamber via gravity convection. This type is slow in heating, requires time to reach sterilizing temperature, and is not uniform in temperature control throughout the chamber. The other type, the **forced-air or mechanical convection sterilizer** is equipped with a motor-driven blower that circulates heated air throughout the chamber at a high velocity, permitting a more rapid transfer of energy from the air to the instrument.

A **dry heat cabinet** is easy to install and has low operating costs. It penetrates materials, is non-toxic and does not harm the environment. Likewise, it is **non-corrosive** for metal and sharp instruments. However, dry heat sterilization is a time-consuming method because of the slow rate of heat penetration and microbial killing. Also, high temperatures are not suitable for most materials.

2. Filtration

Filtration is the removal of microbes by the passage of a liquid or gas through a screen-like material with small pores. This method is used to sterilize heat-sensitive materials like vaccines, enzymes, antibiotics, and some culture media. Filtration is done under aseptic conditions when these chemicals are introduced into and mixed with the culture media that has already been sterilized.

Examples of these include **membrane filters,** which have uniform pore size like 0.22 and 0.45 µm pores. These filters are used to filter most bacteria but do not retain spirochetes, mycoplasmas and viruses. On the other hand, 0.01 µm pores retain all viruses and some large proteins and are used in industry and research. **High Efficiency Particulate Air Filters (HEPA)** are used in operating rooms to remove bacteria from air.

3. Radiation

Sterilization by **ionizing radiation** is the use of short-wavelength, high-intensity radiation to destroy microorganisms. Gamma rays, X-rays, electron beams, or higher energy rays have short wavelengths (less than 1 nanometer). This radiation reacts with DNA, causing mutations which result in damaged cells, and produces peroxides. These are commercially used for the sterilization of disposable items **(cold sterilization).**

Non-ionizing radiation uses longer wavelengths and lower energy like ultraviolet (UV) light and infrared light. As a result, non-ionizing radiation does not have the ability to penetrate substances, and can only be used for sterilizing surfaces. It damages DNA by producing thymine dimers, which cause mutations. It also affects the three-dimensional (3D) structure of proteins and nucleic acids and is suitable for transparent fluids and surfaces.

Microwave radiation, a form of non-ionizing radiation, has wavelengths ranging from 1 millimeter to 1 meter. Heat is absorbed by water molecules. This may kill vegetative cells in media. Bacterial endospores, which do not contain water, are not damaged.

B. Chemical

Physical sterilization provides an efficient way to remove all contamination. However, it is not always appropriate because it may damage certain materials. In this case chemical sterilization is done. Chemical methods for sterilization make use of harmful liquids and toxic gases without affecting the material. Sterilization is effective using gases because they penetrate quickly into the

material like steam. Some risks may be encountered like the chances of explosion and this method may be expensive.

Gases commonly used for chemical sterilization are a combination of ethylene oxide and carbon dioxide where carbon dioxide is added to minimize the chances of an explosion. This method is widely used for the sterilization of medical devices, but it is not a routinely available technique for algal cultures. Ethylene oxide kills all microbes and endospores, but requires exposure of 4 to 18 hours. Moreover, ethylene oxide is a potent carcinogen. Ozone gas is another option which oxidizes most organic matter. Hydrogen peroxide, nitrogen dioxide, glutaraldehyde and formaldehyde solutions are other examples of chemicals used for sterilization. Kawachi and Noel (2005) summarized sterilization types including their applicability and limits (Table 1.2.).

Table 1.2. Summary of sterilization types, including applicability and limits (adapted from Kawachi and Noel, 2005).

Category	Sterilization Method	Effective Method	Application	Limitation
Physical	Flame	Direct heat with fire (Bunsen burner)	Surface sterilization (test tube openings, transfer loops, glass pipettes)	Non-heat-resistant materials (e.g. most plastics)
Physical	Autoclaving	2 atm steam pressure. 121°C; time varies (10, 20 min for small liquid vol; 1 h for large vol)	For general use: liquids and agar, glass and metal vessels, equipment	Non-heat-resistant materials; pH change; metal contamination
Physical	Dry heat	250°c, to 3 to 5h; current protocol at 150°C for 3 to 4 H repeated 3 times in 3 d	Dry goods: glass and metal vessels and equipment	Non-heat-resistant materials; liquids
Physical	Filtration	$\leq$ 0.2 um pore size filter	Liquid with heat-labile	Small volumes, high-viscosity liquids, viruses not eliminated
Physical	Microwave	10 min at 700 W; 5 min with intervals at 600 W. For dry goods: 20 min at 600 W with water, 45 min without water	Liquids: small volume of media; dry goods: glassware, vessels	Small liquid volumes; dry goods with water require elimination of water
Physical	Ultraviolet radiation	260 nm, 5-10 min	Surface of materials, working area	Ultraviolet-sensitive plastics
Chemical	Bleach (sodium hypochlorite)	1-5mL for 1 L water, several hours	Large volume of water for aquaculture	Cysts may survive; neutralization required (e.g. sodium thiosulfate, 250 g L^{-1} stock solution; 1 mL for 4 mL of bleach)
Chemical	Ethanol	50-70% solution	Popular, general disinfection	Some resistant microorganisms
Chemical	Ethylene oxide	Airtight room or pressure cabin	Plastic and rubber products, non-heat-resistant products	Explosive: chemical residue is problematic or toxic

Chemical	Corrosive sublimate, $HgCl_2$	0.1%; add same amount of NaCl and dissolve with distilled water	Antiseptic and disinfectant	Poison; not for materials in contact with live cells
Chemical	Phenol (carbolic acid)	3% solution	Antiseptic and disinfectant	Poison; not for materials in contact with live cells
Chemical	Saponated cresol solution	3-5% solution	Antiseptic and disinfectant	Poison; not for materials in contact with live cells
Chemical	Formaldehyde (formalin)	2-5% solution	Antiseptic and disinfectant	Poison; not for materials in contact with live cells

IV. Disinfection

Physical and chemical methods of sterilization are effective methods. However, sometimes it is not practical to autoclave or subject many items to high heat, especially if the items can be damaged through repeated exposure to heat. Treatment of inert surfaces and heat labile materials can be accomplished through the use of disinfectants. Below are different types of disinfectants (Rutala et al. 2019)

Types of Disinfectants

1. Phenolics

Phenolics are phenol (carbolic acid) derivatives. These biocides act through membrane damage and are effective against enveloped viruses, rickettsiae, fungi and vegetative bacteria. They also retain more activity in the presence of organic materials than other disinfectants. Some examples are cresols which is derived from coal tar and biphenols, which are effective against gram-positive staphylococci and streptococci. An advantage of using phenols and phenolics is that they are stable, persist for a long period after being applied and remain active in the presence of organic compounds. Available commercial products are Lysol and Pine-Sol. It is rarely used today because it is a skin irritant and has a strong odor.

2. Halogens

Halogens like iodine, chlorine and fluorine are good disinfectants. They are effective alone or in compounds. Iodine, like iodine tincture (in alcohol solution), was one of the first antiseptics used. Chlorine compounds are good disinfectants on clean surfaces. However, they are quickly inactivated by organic matter thus reducing their biocidal activity. Hypochlorites, the most widely used of the chlorine disinfectants, are available in liquid (e.g., sodium hypochlorite), household bleach and solid (e.g., calcium hypochlorite, sodium dichloroisocyanurate) forms. About 5.25% chlorine is found in household bleach. To be effective, this disinfectant should be made fresh and used within the same day it is prepared.

3. Alcohols

Alcohols are effective against lipid-containing viruses, bacteria and fungi, but not endospores or naked viruses. They act by denaturing proteins and disrupting cell membranes. Examples include ethanol (drinking alcohol) and isopropanol or rubbing alcohol. The optimum bactericidal concentration is in the range of 60% to 90% by volume and their activity drops sharply when diluted below 50% concentration. Isopropanol is a better disinfectant than ethanol. It is also cheaper and less volatile. Absolute alcohol is also not very effective. They are used to clean instruments, wipe down interior of cabinets and bottles. Alcohols are considered non-corrosive.

4. Heavy Metals

Heavy metals include copper, selenium, mercury, silver, and zinc. Soluble salts of mercury, silver lactate, mercuric chloride and mercurous chloride are efficient bactericidal agents. Silver nitrate

and mercuric chloride are commonly used in tiny amounts such as 1:1000 aqueous solutions. Action is through attack on protein structure and disruption of enzyme functions.

5. Quaternary Ammonium Compounds (Quats)
Quaternary ammonium compounds or quats are cationic (positively charged) detergents. These are effective against gram positive bacteria and less effective against gram- negative bacteria. They are generally ineffective against viruses and spores. The mode of action of quats is through inactivation of energy-producing enzymes, denaturation of essential cell proteins, and disruption of the cell membrane. Many of these compounds are better used in water baths, incubators, and other applications where halide or phenolic residues are not desired.

6. Aldehydes
Aldehydes include some of the most effective antimicrobials like formaldehyde and glutaraldehyde. These aldehydes inactivate proteins by forming covalent crosslinks with several functional groups. Formaldehyde is an excellent disinfectant in 2% aqueous solution. Commonly used as formalin, a 37% aqueous solution, it was used extensively to preserve biological specimens and inactivate viruses and bacteria in vaccines. However, it irritates mucous membranes because of its strong odor. Glutaraldehyde is less irritating and more effective than formaldehyde. It is commonly used to disinfect instruments.

Laminar Flow Hoods
Laminar flow hoods provide an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures. Specifically, these are physical containment devices that act as primary barriers either to protect the material within the hood from different sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood during routine procedures (Coecke et al. 2005). Three kinds of laminar flow hoods, designated as Class I, II and III, have been developed to meet varying research and clinical needs (Coecke et al. 2005, Slavin and Schell 2012).

Class I laminar flow hoods provide significant levels of protection to laboratory personnel and to the environment when used with good microbiological techniques. However, these do not provide cultures with protection from contamination. These are similar in design and air flow characteristics to chemical fume hoods.

Class II laminar flow hoods are designed for work involving BSL-1, 2, and 3 materials. They also provide an aseptic environment necessary for cell culture experiments. A Class II biosafety cabinet should be used for handling potentially hazardous materials (e.g., primate- derived cultures, virally infected cultures, radioisotopes, and carcinogenic or toxic reagents).

Class III biosafety cabinets are gas-tight and provide the highest attainable level of protection to personnel and the environment. A Class III biosafety cabinet is required for work involving known human pathogens and other BSL-4 materials.

Laminar Flow Hood/ Cell Culture Hood Layout
A laminar flow hood or **cell culture hood** should be large enough to be used by one person at a time and should be comfortable to use. It should be easily cleaned inside and outside and provided with adequate lighting. The work space in the cell culture hood should always be clean and uncluttered. Likewise, every material should easily be reached and within sight. Disinfect each item placed in the cell culture hood by spraying it with 70% ethanol and wiping it clean.

The arrangement of items within the cell culture hood usually adheres to the following right-handed convention, which can be modified to include additional items used in specific applications (Fig. 1.3). It is important to have a wide, clear work space in the center with the cell culture vessels. The pipettor is in the front right, where it can be reached easily. Reagents and media are in the

rear right to allow easy pipetting. The tube rack is placed in the rear middle holding additional reagents, while a small container to hold liquid waste should be positioned in the rear left.

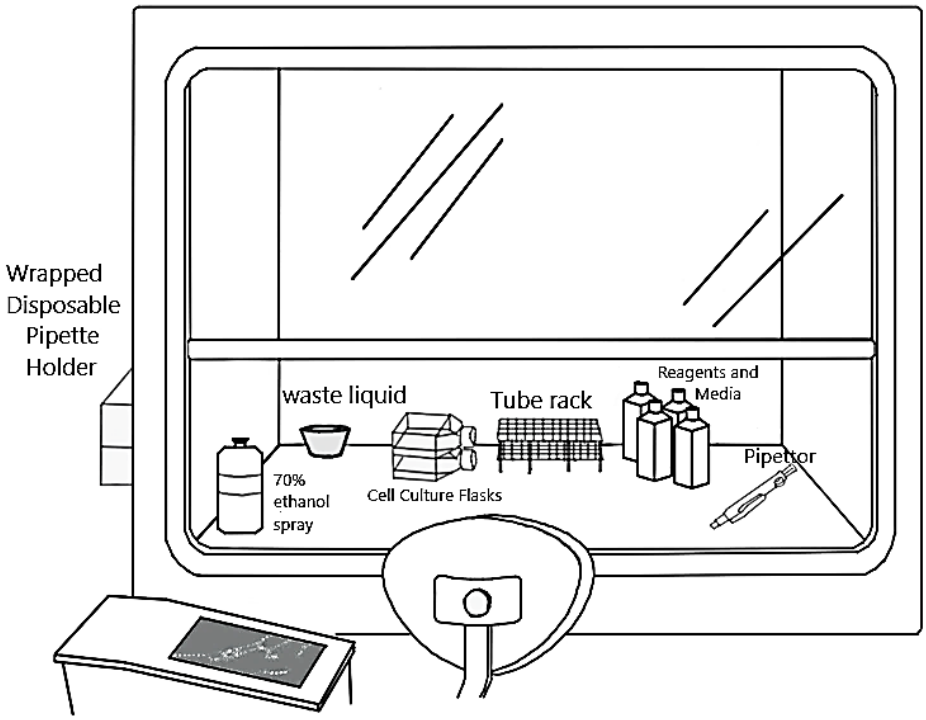

Figure 1.3. Basic layout of a cell culture hood for right-handed workers. Left-handed workers may switch the positions of the items laid out on the work surface. (adapted from Introducing a New Class of Imaging Systems. 2020. https://www.thermofisher.com/ph/en/home/references/gibco-cell-culture-basics/cell-culture-equipment/laminar-flow-hood.html)

V. Summary

Effective sterilization and disinfection must be done to remove or destroy all forms of microbial life, including viruses, bacteria and fungi (as vegetative forms or spores) that may prevent the growth and development of algal cultures. Cleaning merely reduces the number of contaminants present while disinfection removes most pathogenic organisms. Sterilization is the killing or removal of all organisms. Among the different types of sterilization methods presented, moist heat sterilization is the most useful for algal cultures. Alcohols, aldehydes, halogens and quats are often used in disinfection. It is therefore very important to select the right methods and perform them well to produce algal cultures that can be used for research and teaching activities.

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Chapter 2. Isolation and Purification of Microalgae

Katherine L Pintor[1] and Pierangeli G. Vital[2]*

I. Introduction

Microalgae are highly diverse group of photosynthetic microorganisms that are ubiquitous in nature and predominantly found in freshwater, brackish and marine ecosystems. Communities of microalgae can also be found and isolated in extreme environments such as salt waters, geothermal outflow, deserts, and mining effluent pools (Lopez-Rodas et al. 2008; Duong et al. 2012). These primary producers have a massive biodiversity ranging from 200,000 to several million species dispersed in different genera; however, a large chunk of the population has never been identified and studied (Norton et al. 1996). Microalgae can either be unicellular or multicellular, and can exist individually, in chains, or in complex groups. Their sizes typically range from a few to hundreds of micrometers (μm), depending on the species type. They can also tolerate and survive a wide range of pH, temperature, salinity, and light intensity levels. Naturally they reside at multiple depths in the water column and can occur as either pelagic or benthic, and free-living or attached to a specific substrate (e.g. sediments, rocks, and aquatic plants) (Hillebrand et al. 1999; Fogg 2001; Pulz and Gross 2004; Barra et al. 2014; Barka and Blecker, 2016).

In recent years, microalgae have been widely used and explored as novel sources of biologically important bioactive metabolites and highly valuable substances that can be used for the production of feed, food, biofuels and pharmaceutical products. As a result, growing attention has been directed towards the commercial production of microalgae, but only certain species of microalgae are suitable for biotechnological applications (e.g. *Chlorella vulgaris, Arthrospira platensis,* etc.) (Khatoon and Pal 2015; Mobin and Alam 2017). To separate the desired microalga from heterogenous samples, unialgal cultivation must be carried out. A number of traditional isolation techniques have been established and used since the 1950s, including the use of a micropipette or glass capillary under an inverted microscope, streak plate method, serial dilution and selective enrichment of the targeted microalgae by the addition of nutritional requirements

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and specific growth conditions (e.g. silicon source for diatoms) (Duong et al. 2012; Lee et al. 2014). In this regard, various methods of isolation and purification of microalgae are elaborately described in this chapter, hoping to equip students and researchers with essential and appropriate techniques that can be used in microalgae-related studies.

II. Sample Collection

Depending on the target species, whole water samples can be collected at various depths from the water column. But the assemblage of microorganisms present in water samples can be extremely sensitive to environmental factors including pressure, light, and temperature changes. Therefore, samples must be handled carefully to prevent too much agitation that may shear and break algal cells. In order to ensure cell viability of water samples, keep them in sterile bottles covered with cotton plugs and store inside an ice box or any container with stable temperature not exceeding to 32-35°C to preserve cell integrity. It is also essential to shake the bottles occasionally to wash the cells off the bottom and prevent from packing down. One can also record the physico-chemical characteristics (e.g. dissolved oxygen, conductivity, pH, etc.) of water in the study site since these parameters are typically used as indicators of water quality, which could then explain the phytoplankton diversity in the samples.

The materials needed in sample collection are glass bottles with cap or cotton plug (Niskin, Gatorade® or catsup bottles), plankton net with a pore diameter of 10-120 micrometers (pore size depends on the target microalga; ordinary mesh cloth net or screen can also be used as an alternative), ice-filled container/polystyrene box and multi-parametric device to measure the physico-chemical properties of water including pH level, salinity, dissolved oxygen (DO), temperature, total nitrite, total nitrate, total phosphorus, biological oxygen demand (BOD), chemical oxygen demand (COD), and conductivity.

From the target site, around 300 to 500 mL of whole water sample will be collected using a sterile bottle attached to a plankton net. In the absence of a plankton net, make use of any filter or screen available. Since natural waters usually contain numerous zooplankton and other unwanted organisms grazing on microalgae, pre-filtering of water samples immediately after obtaining from the site is recommended in order to remove both biological and physical contaminants. After filtration, quickly transfer the samples inside an ice box while being transported back to the laboratory to avoid desiccation. Upon reaching the laboratory, store and maintain water samples on an ice-filled container or inside the refrigerator (do not place inside the freezer) until further use. However, some microalgae die quickly within hours after sample collection, thus it is also recommended to proceed with the isolation if the target species are quite abundant already in the water sample.

III. Enrichment of Culture

Enrichment of algal culture refers to the addition of selected nutrients (e.g. nitrate, ammonia, silica and phosphorus) in the culture medium to boost the growth of the target microalgae. Although this initial step towards **"single-cell isolation**" can promote algal growth, it can also be a limiting factor. Hence, the specific nutrients and its concentrations must be identified in order to avoid bacterial overgrowth, which can make the culture anoxic to microalgae (Andersen 2005).

The materials commonly used for the enrichment of cultures are liquid culture medium (BG-11, Bold's Basal Medium, Algae Culture Broth), micro- and macro-nutrients, trace metals, inoculating loops and beakers.

Different culture medium can be utilized to cultivate microalgae. BG-11 with added nitrogen source is most suitable for the enrichment of non-nitrogen fixing cyanobacteria, while the removal of nitrogen source from the culture medium can be used for the isolation of nitrogen-fixing cyanobacteria. On the other hand, Bold's Basal Medium is commonly used to enhance the growth

of green algae or chlorophytes; while Algae Culture Broth is a general medium used for cultivation of all types of microalgae from soil or water samples. Once sterile media is prepared, there is an option to add supplementary micro- and macro-nutrients (e.g. phosphate, silica, ammonium, and nitrate) and/or organic substances (e.g. Vitamin B, yeast extract, amino acids, urea, etc.) if the target species are the osmotrophic type of microalgae. The addition of antibiotics would also aid in removing microbial contaminants. However, culture medium with antibiotics could adversely affect microalgae during the enrichment process. To steer clear of the deleterious impact of antibiotics, the correct dosage must be known before usage. The commonly used antibiotics are kanamycin, chloramphenicol and gentamycin for bacteria, and nystatin for fungi. Listed in Table 2.1 are the recommended concentrations of antibiotics for algal cultivation by Ermakova-Gerdes and Vermaas (1999). Trace metals (e.g. Cr, Mn, Fe, Co, Ni, and Cu) are also essential for microalgal growth and can be incorporated directly in the culture media. However, these can also be a limiting factor in microalgal growth, so chelating agents (e.g. EDTA) should be added in the solution. To make a trace metal stock solution, combine 2.1 g zinc chloride, 2.0 g cobalt chloride, 0.9 g ammonium molybdate tetrahydrate, and 2.0 g copper sulfate, and bring to a final volume of 100-mL with distilled water. This solution normally appears to be cloudy, to make it clear, add few drops of hydrochloric acid.

Table 2.1: Frequently used antibiotics for elimination of bacteria in microalgal cultures (adapted from Ermakova-Gerdes and Vermaas 1999).

Antibiotic:	Recommended working concentration:
Ampicillin	50 – 300 µg/ml
Chloramphenicol	10-100 µg/ml
Gentamycin	10 – 100 µg/ml
Kanamycin	10 – 150 µg/ml
Tetracycline	10 – 100 µg/ml

On other hand, some species of microalgae such as diatoms and chrysophytes require silicon for growth. Normally the concentration of silica in freshwater bodies ranges from 5 to 25 mg/L and 5 to 7 mg/L in saline waters. When cultivating diatoms, prepare a stock solution of silicon by adding 30 g of sodium metasilicate and bring to a final volume of 1-L with distilled water. The working concentration is 1 mL per L of culture. Nickel can also be added for those microalgae that utilize urea as their nitrogen source and since nickel is needed to form urease. Once the culture medium is ready, add 20 to 100 mL of microalgae culture (the amount of inoculum depends on the total volume of culture broth), and incubate at 20-28°C with continuous aeration under a specific light intensity and lighting regime (e.g. 8000 lux with 12-h light and 12-h dark photoperiod). Examine the culture every day for the growth of target species through microscopy. Once healthy target cells are abundant, individual cells can now be isolated.

IV. Standard Isolation Methods

A. Streak Plate Method

The **streak plate method** is a basic but one of the most essential techniques in the field of microbiology, primarily for the isolation of discrete colonies on the agar plates. According to Andersen (2005), this particular technique is the best preferred isolation method of microalgae from either soil or water samples. It is also very simple and straightforward; and it can also be used to establish axenic algal cultures devoid of additional treatments. However, there are some species of microalgae that do not exhibit good growth on solid agar. For instance, the flagellates *Peridinium* sp., *Heterosigma* sp., and *Aureococcus* sp. are not capable of sustaining growth on solid agar because they prefer liquid medium or soft/semi-solid agar (0.3 to 0.6%), while most microalgae favor solid agar with a concentration ranging from 0.8% to 2.0%.

To obtain well-isolated colonies, the **quadrant streak plate technique** is frequently used. This particular method shares the same principle with serial dilution whereas the original water sample loaded onto the metal loop is diluted by successive streaking across the agar. As a result, there is a considerable reduction in the number of microorganisms from the initial streak up to the last one. The third and the fourth streaks usually contain lesser microorganisms, and thus produce isolated colonies on the agar surface after incubation at 20 to 28°C for 7 to 20 days.

The materials needed are inoculating loops, agar plates (e.g. Algae Culture Agar), and Bunsen burner or alcohol lamp.

At least 30 minutes before starting the procedure, place the bottles with water samples inside the laminar flow cabinet to let the algal cells settle to the bottom. Once the cells have accumulated at the base, proceed with flame sterilization of wire inoculating loop using an alcohol lamp or a Bunsen burner while holding the loop at an angle of about 45º. Wait until it is red hot, and then allow it to cool down for 1 min. Scratch the bottom of the sampling bottle using the inoculating loop to get a substantial amount of microalgal cells. Slightly open the agar plate and immediately streak the inoculating loop over a quarter of the plate's surface using a back-and-forth motion producing close parallel streaks. Make sure to just gently streak the loop onto the agar surface, so as not to scratch or gouge the agar. By using an alcohol lamp, re-flame the loop until red hot and allow it to cool down for 30 sec to 1 min. Go back on the edge of the first streak, drag down the loop and repeat streaking onto the next quadrant without overlapping the previous streak. This time, perform streaking in a zigzag pattern, and the parallel streaks must not be close to each other. Repeat the latter for the third and fourth quadrants (Fig. 2.1). Incubate for 7 to 20 days under 12h:12h light and dark photoperiod at 20-28°C (conditions may vary for different species). After the incubation period, check for isolated colonies in the last quadrant (Figure 2.2). By using a disposable loop or heat-sterilized metal inoculating loop, gently pick a single colony from the agar plate and observe under the microscope for purity. If only one species of microalgae is observed, inoculate the microalgae in sterile glass bottle containing 100-mL of algae culture broth. Allow it to grow for 14 to 20-d under the same culture conditions mentioned above. This step is intended for purification and enrichment purposes. However, if analysis by microscopic observation reveals that consortium of microalgae is still present in the isolated colony, then repeat the quadrant streak plate method until unialgal culture is achieved.

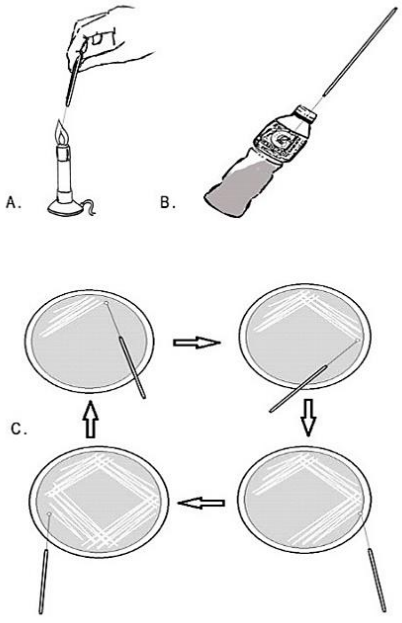

Figure 2.1: The Quadrant Streak Plate Method. (A) Heat-sterilizing of inoculating loop, followed by (B) dipping of metal loop inside the bottle to obtain microalgal cells, and (C) streak plating on the agar's surface.

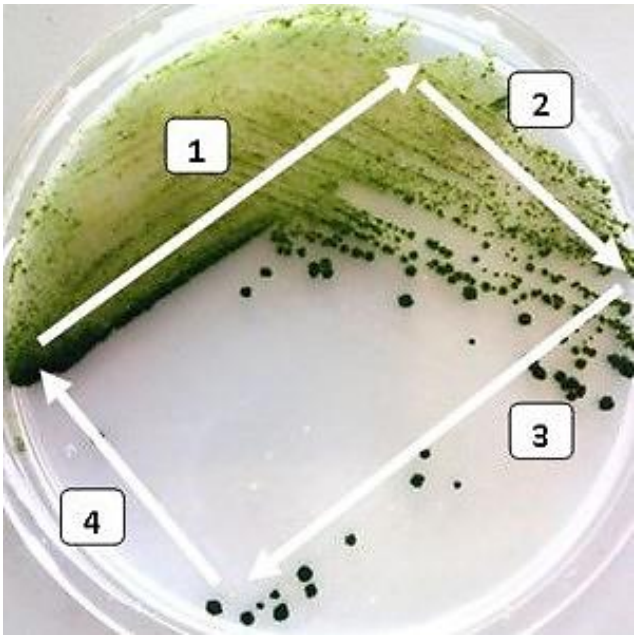

Figure 2.2: Example of quadrant streak plate method after 20-d incubation of *Chlorella vulgaris* grown on BG-11 agar plate (photo by Katherine L. Pintor)**.**

B. Micropipette Technique (for single-cell isolation)

The micropipette technique is another conventional method used for isolation, but it requires a great deal of practice and hands-on experience. This method is usually employed for single-cell isolation using a Pasteur pipette or a glass capillary tube, which will be remolded into a "micropipette" or "capillary pipette" with a very fine tip (Fig. 2.3). This is made by holding the tip with sterile forceps while being heated under low flame. If the Pasteur pipette is exposed to high heat, it will not stretch out into the desired shape and length, and there is a huge tendency that it will snap off instantly. Take note that the forceps must be placed approximately 1 cm from the end of the tip. The diameter of the finely pulled tip can be varied as well by changing the speed of pulling: (a) the slowly-pulled tip would create a larger bore hole, while (b) a rapidly-pulled tip would lead to a narrow end. Make sure that the diameter of the micropipette tip matches the size of the desired algal cell to guarantee single-cell isolation. It is advised that the tip's diameter should be more or less twice the size of the algal cell to ensure fine micromanipulation and to avoid "fluid shearing forces" that can inflict damage to the cells (Andersen 2005).

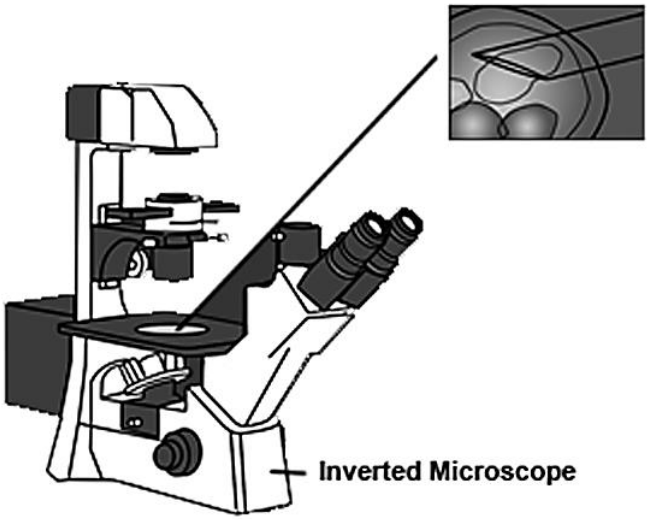

Figure 2.3: Single-cell isolation with the use of micropipette and inverted microscope.

In conducting this technique, the materials needed are Pasteur pipettes, alcohol lamp, lighter, inverted microscope, glass slides, forceps, inoculating loops, beakers, glass bottles, and a basic liquid culture medium for microalgae.

By using sterile forceps, hold the tip of the Pasteur pipette and flame the adjacent area until it slightly softens (prevent it from completely melting). Pull it away from the flame and immediately extend the tip in a horizontal direction with the use of the forceps. Do not heat the Pasteur pipette very long to prevent it from breaking. Stretch the heated glass tip to a length of 4 to 6 cm. Make sure that the diameter of the tube's hole should be larger than the desired microalgal cell. This will avoid shearing of cells as it gets into the micropipette (Fig. 2.4).The resulting micropipette tip can either be straight or curved. To obtain a straight tip, simply break off a very small portion of the end of the tip after extending it to a desired length, while a curved tip is produced by bending the end of the micropipette tip right after it is finely pulled. The curved tip is helpful when the cells are placed in a deep container, but the straight tip is easier to manipulate especially under the microscope. Another advantage of a straight tip is that it requires less effort in releasing the algal cell inside the tip. Once a micropipette tip is created, flame-sterilize an inoculating loop and scratch the bottom of the glass bottle containing the microalgal cells. Mix gently with a drop of sterile water placed on top of a glass slide. Observe under the microscope and look for individual cells of the desired microalgal species. Locate the extended pipette tip under the microscope, position it above the target microalgae and then gently tap the cell or group of cells using the tip. Allow capillary action to draw the cell up and into the micropipette tip. Transfer it onto another glass slide containing a drop of sterile water or algae liquid culture medium by softly blowing the other end of the pipette. Secure a clean cotton inside the opposite end of the tip in order to filter the air being used to flush out the microalgae. Check if the target cells are obtained by observing under the inverted microscope. Repeat the procedure until at least 10 to 20 cells of the desired microalgae are isolated. Transfer the cells from the glass slide into a sterile glass bottle containing 50-mL of algae culture broth. Incubate for 7 to 20 days at 20 to 28°C with a photoperiod of 12 h light and 12 h darkness (conditions may vary). Continuous aeration can be applied, or as an alternative, manually shake the bottles 6-8x per day to avoid clumping of cells that may lead to algal mats, and to ensure equal nutrient distribution in the culture broth. Every 3 to 5 days, check the culture through microscopy to see if there are other microorganisms present. If the culture is contaminated by another species of microalgae, repeat all the steps mentioned above until unialgal culture is achieved.

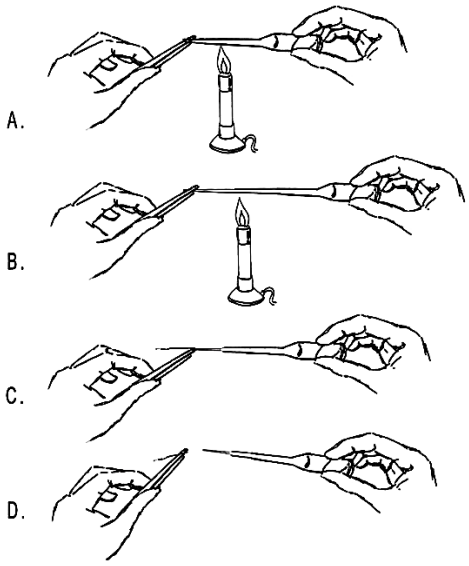

Figure 2.4: Single-Cell Isolation by Micropipette Technique. (A) Hold the narrow tip of the Pasteur pipette and expose the middle part to flame coming from the Bunsen burner or alcohol lamp. (B) Allow the glass to soften but prevent it from completely melting. (C) Extend the end of the tip of the Pasteur pipette to produce a long thin tube. (D) Break the end of the tip by using forceps.

C. Serial Dilution Techniques

The serial dilution technique (Fig. 2.5) is most often used when attempting to culture random species of microalgae. It can also effectively isolate microalgae that are present in abundant numbers in water samples. The main goal of this method is to reduce a dense culture of cells as the dilution increases.

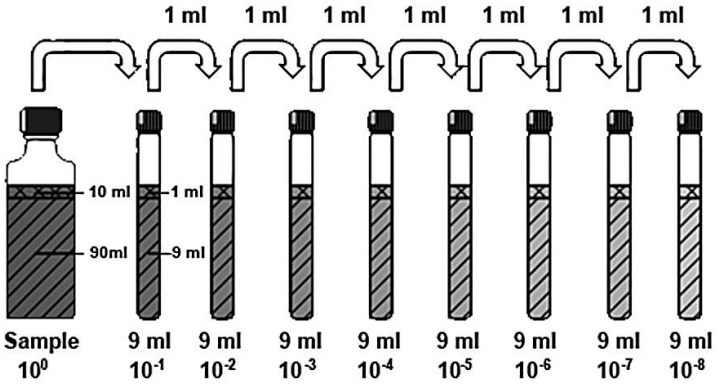

Figure 2.5: A series of sequential dilutions to reduce the number of algal cells

The materials needed for this technique are sterile screw-capped test tubes, test-tube rack, algae culture broth, 10-mL and 1-mL pipettors, alcohol lamp and vortex mixer.

Prepare 10 pcs of sterile test tubes with 9 mL of algae culture broth. Label tubes with 10^{-1} to 10^{-10} indicating dilutions. Add 1-mL of enriched microalgal sample into the first tube (10^{-1}) and gently invert the test tube twice. Obtain 1-mL of liquid from this dilution by using a pipettor, and transfer into the next tube (10^{-2}). Mix gently by inverting 3 to 5x. Repeat the third step for the remaining tubes until 10^{-3} to 10^{-10} dilutions are achieved. Place the test tubes with algal inoculum inside a shaking incubator or manually shake the tubes 6-8x per day if it will be placed inside a growth chamber. Incubate at 20 to 28°C under 12-h light and 12-h darkness for 7 to 20 days. Light intensity, photoperiod and incubation time will depend on the species of microalgae being isolated. Examine every 3 days by aseptically withdrawing a small amount of water sample from each dilution tube and observe under the microscope. This procedure is necessary to check how many species of microalgae are present in the samples. A single species of microalgae is expected to thrive in higher dilutions. However, if it still contains 2-3 species of microalgae, another round of microdilution is recommended in order to achieve a culture with only one species of microalgae.

V. Purification Method: Axenization using antibiotics and selective culture media

Removal of biological contaminants using antibiotics

To achieve an axenic culture of a microalga (a unialgal culture free from other microorganisms such as bacteria, protozoa and fungi), antibiotics (alone or in combination) must be added to the culture with actively growing cells. This will help diminish the growth of unwanted organisms, while still allowing the microalga to continue growing. The correct concentration of antibiotics and exposure time should be determined first to ensure that it is not lethal to the target microalga.

The total elimination of unwanted microorganisms in a xenic culture is extremely difficult to achieve since they exhibit mutualistic or symbiotic relationship with microalgae. Bacterial and fungal resistance to a wide array of antibiotics is another challenge during axenization. In some studies, treating algal cultures with antibiotic(s) has to be done repeatedly since the latter are prone to degradation due to the presence of oxygen, temperature and light. As a result, the potency of each antibiotic decreases. Mixtures of antibiotics with different modes of action to target different organisms are commonly used as well (Gonzalez-Pleiter et al. 2013; Molina-Cardenas et al. 2016).

The materials needed for axenization of algal cultures are antibiotics (Tables 2.2-2.9), pipettor, sterile Erlenmeyer flask, enriched algal culture, deionized water, 0.22 µm syringe filter, 100% Ethanol and Amber bottles. In preparing the antibiotic(s), dissolve the lyophilized powder with distilled water or ethanol (check solubility). Filter-sterilize using a 0.22 µm syringe filter, and aliquot in sterile microtubes to prevent degradation.

To reduce bacterial load, pre-wash the algal cells with distilled water and centrifuge at 3,500 rpm for 15 minutes. Remove the supernatant and repeat washing the cell pellets. Standardize the initial number of cells by using a hemocytometer or adjusting the optical density (e.g. OD_{750}=1.0) since this will be used to calculate the survival rate (%) of the microalga after the antibiotic treatment. A dilution series of each antibiotic cocktail (e.g. 0.5%, 1%, 5%, 7%, 10%) listed in Tables 2.1 to 2.9 can be prepared to see which concentration will not be lethal to the microalgae. On the other hand, application of a single antibiotic can also be used to remove biological contamination (Table 2.10). For repeated doses, apply antibiotics every 24-h in a span of three days. Then incubate the culture for 24 hours up to 4 weeks at 20-28°C under light or dark incubation (conditions may vary). Every 24 hours, obtain a sample from each dilution and count the number of algal cells or measure the OD to check algal growth. Compare the initial and final algal cell densities to determine if the antibiotics have detrimental effects to the microalgae. It is also important to examine the viability of microalgal cells through microscopy by checking their shape, color/pigmentation and cell wall integrity. In addition, any apparent discoloration (e.g. from green or brown for diatoms to white/yellow) of the cultures indicates that the working concentration of the antibiotics being used is lethal to the microalgae. The color change reflect alteration in pigment composition due to a perturbation in the normal physiological state of the microalgae. Moreover, to determine the efficiency of the treatment in eradicating bacteria and fungi, pipette out 1-mL of algal culture and spread plate onto PDA (potato dextrose agar) and NA (nutrient agar) plates. Incubate in the dark (or wrap the agar plates with aluminum foil) at 20-28°C for 48-72 hrs, and check for bacterial/fungal growth. Choose the antibiotic dilution with the highest microalgal survival rate and lowest bactericidal load. Once an axenic culture is established, regularly check DAPI and SYBR Green-stained samples under the epifluorescence microscope to ensure the absence of biological contaminants especially bacteria.

Stock solutions of antibiotics

Table 2.2: Cocktail #1 for golden-brown microalgae or haptophytes (Molina-Cardenas et al. 2016).

Antibiotic	**Concentration (*µg/ml*)**
Ampicillin	250
Kanamycin	100
Neomycin	500
Streptomycin	50

*Repeat treatment every 24-h for 3 days minimum; dark incubation

Table 2.3: Cocktail #2 for green microalgae and diatoms (Eigemann et. al. 2013).

Antibiotic	**Concentration (*µg/ml*)**
Ampicillin	250
Gentamycin	50
Kanamycin	100
Neomycin	500
Tetracycline	100
Streptomycin	50
Chloramphenicol	50

*Dark incubation for 3 days minimum

Table 2.4: Cocktail #3 for marine microalgae (Campbell 2013).

Antibiotic	Concentration (*µg*/*ml)*
Cefotaxime	500
Carbenicillin	500
Kanamycin	200
Augmentin	200

*Dark incubation for 1 to 2 weeks

Table 2.5: Cocktail #4 for marine freshwater and marine microalgae (Thompson 2009).

Antibiotic	Concentration (*µg*/*ml)*
Penicillin	100
Streptomycin	50
Chloramphenicol	10

*Dark incubation for 3 to 4 weeks

Table 2.6. Cocktail #5 for cyanobacteria (Bolch and Black-burn 1996).

Antibiotic	Concentration (*µg*/*ml)*
Cycloheximide	100
Nystatin	100

*Light incubation for 3 to 4 weeks

Table 2.7. Cocktail #6 for filamentous cyanobacteria (Ferris and Hirsch 1991).

Antibiotic	Concentration (*µg*/*ml)*
Ampicillin	100
Imipenem	100
Penicillin	100
Cefoxitin	100
Cycloheximide	100

*Add of 2.5% sucrose, 0.5% yeast extract, 0.5% bacto-peptone; dark incubation for 24-h

Table 2.8. Cocktail #7 for filamentous cyanobacteria (Choi et al. 2008).

Antibiotic	Concentration (*µg*/*ml)*
Neomycin	100
Cycloheximide	20

*Addition of 0.1% glucose; light incubation for 24-h

Table 2.9. Cocktail #8 for nitrogen-fixing cyanobacteria (Hong et al. 2010).

Antibiotic	Concentration (*µg*/*ml)*
Imipenem	100
Cycloheximide	20

*Dark incubation for 24-h

Table 2.10. List of single antibiotic treatments that can be used for cyanobacteria (Rippka 1988; Bolch and Black-burn 1996; Sarchizian and Ardelean 2010; Hong et al. 2020; Vasquez-Martinez et al. 2004).

Antibiotic	Concentration (*µg/ml)*
Ampicillin *Addition of 0.02-0.1% casamino acids and 0.5% glucose; dark incubation for 24-48 h	100-1000
Imipenem *Addition of 2.5% sucrose, 0.5% yeast extract and 0.5% peptone; dark incubation for 18-24 h	100
Kanamycin *dark incubation for 24-h	100-150
Streptomycin *dark incubation for 24-h	100
Cycloheximide *dark incubation for 24-h	20-100
Chloramphenicol *dark incubation for 24-h	100
Carbenicillin *dark incubation for 24-h	100

VI. Summary

Compared to other organisms, microalgae are considered an untapped resource of diverse bioactive metabolites, and their enormous diversity in different natural habitats make them more interesting to study. However, microalgae are under-investigated despite their several potential biotechnology applications. But times are changing and slowly microalgae are given more attention in recent years. In studying microalgae in the laboratory, isolation, cultivation and purification of microalgal species of interest are the crucial primary steps that must be done in order to advance in microalgal research. This chapter has discussed a number of methods that aimed to help budding phycologists on how to achieve **unialgal and axenic cultures in the laboratory**. The specific methods usually employed are collection of the sample from the field followed by enrichment of cultures using various medium. Once the target microalgae species is abundant, the isolation and purification can be performed next using streak-plate method, micropipette and microdilution techniques.

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Conflict of Interest
The authors declare that there is no conflict of interest.

Contributions of authors
Pintor KL and Vital PG conceptualized the paper, prepared the draft, and finalized the manuscript.

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Chapter 3. Culture of Freshwater Algae

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I. Introduction

Freshwater algae are found in freshwater habitats, of strictly aquatic habitats that are of four general types: 1) flowing waters, 2) ponds and lakes, 3) pools and ditches, and 4) bogs and swamps. This chapter will loosely include those from brackish waters, like Laguna de Bay. There is no general method by which collections of algae from the field may be kept alive in the laboratory indefinitely. Hence, it is essential to culture algae in order that the specimen is readily available when needed. Other basic reasons for algal cultivation are: (1) to allow the expression of their usual subtle features, (2) to reveal the extent of polymorphism of the organism under different culture conditions that bears importance in taxonomy; for example, the euryhaline green alga, *Scenedesmus obliquus*, became solitary and broadly ellipsoid to spherical at 10-15 g salt.L^{-1}, losing its usual spindle form in a coenobe in a freshwater inorganic medium (Martinez and Krauss 1977) , (3) to observe their life cycles, and (4) to study closely their physio-biochemical features (Martinez-Goss 2005).

An extensive review on the cultivation of algae has been done earlier by Bold (1942). Over time there has been more extensive compilation of culture media for freshwater algae (Nichols 1973; George 1976; Thompson et al. 1988; Watanabe 2005). However, this compendium that is being presented differs from the rest by including the concentrations of the chemical compounds in stock solution and in the final medium, recommends alternative cheap glassware as culturing containers, presents alternative undefined or artificial media, such as the use of agricultural fertilizers and animal manure extracts.

Preparation for the stock solutions was done at 100-1000x greater than the concentrations in the final medium. Prior mixing of stock solutions is advantageous because it avoids repetitive weighing. All units of measurement are uniformly done in g.L^{-1} or mg.L^{-1} medium for ease in comparison. Furthermore, some of the media presented here were tested for Philippine microalgae.

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II. Materials

A. Chemicals

The chemical compounds used in the defined media should be of the analytical reagent (AR) grade. Another consideration in preparing the media is accuracy in weighing the chemicals, especially the micronutrients or the trace elements. Hence, it is suggested that an analytical balance be used in weighing, such as the Mettler balance.

Agar is a neutral polymer of galactose used as a solidifying agent. The purer the agar is, the better, and this is based on the washing procedures. Chelating agents, such as EDTA (ethylenediaminetetraacetic acid), are usually combined with a heavy metal, such as iron to prevent the iron from precipitating making the element unavailable to the organism. The chelating agent is usually added with the metal at a 1:1 molar ratio, although excess chelate is usually added to ensure adequate suspension. EDTA is usually available in the form of a disodium salt ($Na_2EDTA•2H_2O$) to make it more freely soluble because the free acid is insoluble in water.

B. Glassware

There is a variety of types and sizes of glassware for culturing, including disposable ones. Sterilizable glassware manufactured by Corning or Pyrex are usually recommended. However, locally available recycled bottles can also be used, such as those that used to contain catsup, wine, gin or rum because they are sterilizable. One popular container is the 350 mL used rum bottle (20 cm x 8.5 cm, length x width), locally known in Filipino dialect as “lapad” (broad and flat) because it is observed to be more resistant to frequent sterilization. These alternative containers also come in a variety of sizes. They are easily accessible from junk shops at a minimal cost of Php 0.25 – 1.00 per piece, depending upon the size, the clarity of the glassware, and the place of the junk shop in relation to the center of the municipality.

Screw-capped glassware is the best container for stock solutions and is preferably kept in the refrigerator, at about 4°C, to minimize evaporation, since evaporation alters initial concentrations. This is especially true for trace elements because of the small quantity of the compound in the stock solution. Compounds with an iron component should be kept in ambered containers or wrapped in aluminum foil or several layers of carbon paper to avoid photodecomposition.

C. Water

Distilled water is usually the solvent used in preparing media. Glass-distilled water is preferred over copper-distilled water, especially in places where the tap water has a very high content of calcium salts, such as in Los Baños, Laguna. Membrane-filtered water (0.45 µm) or deionized water can also be used.

III. Methods

Media are usually prepared from pre-mixed stock solutions. Aliquots from these stock solutions are measured using volumetric pipettes to a given volume of water in a volumetric flask. Accuracy in measuring liquid aliquots from stock solutions or water, and weighing of chemicals, are essential.

Sterilization of liquid media is usually done in an autoclave or an All American pressure cooker of 41.5 quarts (77L) capacity, up to 121ºC at 15 psi (pounds per in^2) for 15 min. In some cases, some chemicals have to be sterilized separately and mixed aseptically with the other nutrients while still hot to avoid any precipitation. Some organic compounds and vitamins are heat-sensitive, and hence, are sterilized by membrane filtration with at least 0.22 to 0.45 µm pore size filter paper, under aseptic conditions and are added to the media aseptically while the latter is still hot (please see Chapter 1 for details on sterilization and disinfection). Specific instructions in the preparation of some of the media are indicated in their respective tables.

The pH of the medium is best adjusted after sterilization because the media tend to change in pH after sterilization.

IV. Media Classification

Culture media can be classified in various ways depending upon the purpose of the researcher and the type of microalgae the researcher wishes to isolate or culture. One of the ways of differentiating media is on the basis of the **state of the media**, i.e., whether in the **liquid** or **solid state**. Another way of differentiating the media is based on the composition of the nutrients in the media, which can either be **undefined** or **defined**. **Undefined media** have nutrient compositions that are not quantifiable, while **defined media** have definite amounts of nutrients. The latter media can be made up of inorganic or organic chemical components or a combination of both. The **inorganic chemical component** is usually made up of: 1) macronutrients, and 2) micronutrients or trace elements. The macronutrients are usually needed in large amounts, as in $g.L^{-1}$ of the medium, e.g., $NaNO_3$ (in BG-11 medium) has 1.5 $g.L^{-1}$, while the micronutrients are needed in small quantities, as $mg.L^{-1}$, such as H_3BO_3 (in BG-11 medium) which has only 2.86 $mg.L^{-1}$. The **organic compounds** include all carbon-containing compounds, such as citric acid, acetic acid, and vitamins.

On the basis of the target organisms, media may be classified as **general** or **specific** or **selective**. The culture medium may also be a **nutrient-poor medium** or **an enriched medium.**

A. On the Basis of the State of the Medium

1**.** Solidified media

Solidified media are media that have been hardened with agar for cultivating algae. In this case, agar is used as a solidifying agent, at concentrations of 1-2%. Agar is a neutral polymer of galactose. The purer the agar is, the better, and this is based on the washing procedures. The brand that usually has a high degree of purity is the Difco type. In this case, the algae will grow on the surface of the agar. However, some algae may not grow on the surface of agar, but they will grow if embedded in the agar or by using the agar pour plate method (Skinner 1932). Solidified media are usually used to isolate algae in a unialgal or axenic state.

Preparation:

- Heat the culture media up to the boiling point and slowly add the desired quantity of agar until well-dispersed and in solution, then sterilize, although it is suggested to sterilize the agar plus distilled water and the culture media separately (both at double strength) and mix them aseptically while hot in a laminar flow hood to prevent further decrease of the medium (Hutner et al. 1966). Then, to homogenize the mixture very well, put it in the microwave oven for about 5 min.
- Then the sterilized, solidified media can now be dispensed either in pre-sterilized Petri dishes or in pre-sterilized test tubes. Agar plates are cooled in a laminar flow hood with the lid slightly ajar to avoid too much steam condensation. Once the agar has gelled, the plates should be inverted (agar up) and stored in air-tight containers (such as sterilized plastic bags) and kept in the refrigerator (at about 4ºC).
- The **solidified media in Petri dishes** are usually used to isolate microalgae, either by streaking with the use of a wire loop or by spread-plating with the use of an L-shaped glass rod (see chapter 2).
- On the other hand, solidified media in test tubes should be placed at an appropriate angle (for the slant) and cooled in the laminar flow hood. **Agar slants** are usually used for long storage of usually unialgal or axenic microalgae.

2. Liquid media

Liquid media are culture media in the fluid state. This is the state of the medium that is usually used for the enriching and initial cultivation of the microalgae. The algae grow throughout the depth of the culture medium. Hence, the culture medium usually occupies only 1/3 of the volume of the culture vessel to allow proper mixing and exchange of gases. Preparation of the media and culturing of the algae is as mentioned above.

B. On the Basis of Composition of the Media.

1. Undefined Media

An **undefined medium** is a type of medium wherein the nutrients added are not quantifiable. An extensive review on the development of this type of medium has been done by Provasoli, et al. (1957) and Kinne (1976). Some examples are presented below.

a. Soil Water Medium (SWM) is one of the easiest culture media to prepare, especially for a laboratory that is still in its infancy. Soil extract was originally introduced by Pringsheim (1912). Later it became known as the Erd-Schreiber medium with the original recipe derived from Schreiber (1927). Since then, a number of variations to this recipe have evolved, including the one below which has been formulated for culturing common freshwater algae.

Materials needed:
Sieved garden soil
Catsup bottles (250 mL; 5 cm bottom dia.)
Cotton plugs
Dried green peas, as source of Mg
Tap water or distilled water
pH meter

Procedure
Place the sieved garden soil and water into the catsup bottle at a ratio of 1:3. Plug the bottle with cotton and sterilize or steam. Steaming is done for one hour for three consecutive days to kill the spore formers. Steaming is also advantageous because it does not change the nutrient composition of the soil drastically. In both the steamed and sterilized soil, the soil remains at the bottom of the bottle. This is what is called the **soil water medium (SWM).**

A portion of a green pea, ½ or ¼ size, is added to the still warm steamed or sterilized soil. Adjust the pH of the medium to 7.5 after it has cooled down.

A variation of the above formulation is to let the soil-water mixture stand overnight, then decant or filter the supernatant into a sterilized vessel, and this is called the **soil water extract (SWE) medium.** Some authors vary this medium by adding some salts, and it is now called **enriched SWE or SWM** (Thompson et al.1988; Provasoli 1968). Hence, the salts added have enriched the SWE or SWM.

2. The Agricultural fertilizer medium in Table 3.1 is another artificial medium that has been used to culture *Chlorella vulgaris* Beijerinck (Chlorophyta) (Santiago et al. 2013; Matanguihan et al. 2020).

Prepare stock solutions of Urea-NPK and EDTA-iron separately in one liter distilled water. Keep refrigerated.

Table 3.1: Agriculture fertilizer medium (Santiago el al. 2013; Matanguihan et al. 2020).

Compound	Stock Solution ($g.L^{-1}$ tap or distilled H2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ tap H_2O or distilled H_2O)
Urea	17.192	10	0.17192
NPK (16-20-0)	2.073	10	0.02073
$FeCl_3 \cdot 6H_2O$	1	10	0.01
Na_2EDTA	1	10	0.01

3. Animal manure, from chicken, carabao, hog, horse, and cattle, was used in culturing microalgae (Martinez 1979). Of these six animal manures, hog manure supported the growth of a variety of microalgae in the laboratory. It was successful in growing *Chlorella vulgaris*, at a concentration of $2g.L^{-1}$ of tap water, wet wt., in four open ponds (about 7,500 L capacity/pond) for one year.

b. Defined Medium

A **defined medium** uses a certain amount of nutrients, such that each of the components may be quantified. Some defined media can be used for **cultivating a wide range** of freshwater microalgae, while some media are meant for **cultivating a specific alga or group of algae**.

1. Some of the media meant for cultivating **a wide range of freshwater microalgae** are presented in Table 3.2. They include six commonly used media. **Modified Chu no. 10 medium** (Table 3.8; Watanabe 1960) is being used extensively for a variety of algae including green algae, diatoms and cyanobacteria (Chu 1942, Nalewajko et al. 1995) and the glaucophycean alga, *Glaucocystis nostochinearum* Itzigsohn (Hall and Claus 1967). Of these media, only Bristol's medium and Modified Detmer's medium are made up of purely inorganic compounds. **BG-11** has citric acid as an organic component and Na·EDTA (sodium ethylene diamine tetra-acetic acid), a chelating agent and an organic compound. Among these media, **Bristol's medium** is the simplest to prepare because it has the least components (six macronutrients). It is also one of the most popular media used as an **enriched medium.** For example, if we want to enhance the population density of the microalgae in Laguna de Bay, the usual technique is to mix any defined inorganic media with the filtered bay water at a ratio of 1:1 and use this as the **enrichment culture medium**. On the other hand, **BG-11** has numerous components, and is usually used for culturing diazotrophic or nitrogen-fixing cyanobacteria because it has optimum amounts of iron (Fe) and molybdenum (Mo) that are needed components of the nitrogenase enzyme. The latter is the enzyme needed for nitrogen fixation. It is for these reasons that among these media in Table 3.2, **Bristol's medium** is not a good **selective medium** for cultivating diazotrophic cyanobacteria even if we remove the nitrogen source in the medium because it lacks Mo and Fe. **Bold's basal medium** is a useful medium for many microalgae, especially the green algae (Table 3.6). However, it is not suited for algae that require vitamins.

Table 3.2: Some defined media for culturing a wide range of freshwater microalgae.

Medium	Reference	Table no. in this chapter
BG-11 Micronutrients or trace metals (A5)	Stanier et al. 1971	3.4 3.5
Bold's basal medium	Bold 1949; Bischoff and Bold 1963	3.6
Bristol's medium	Trainor 1978	3.7
Chu no.10, modified	Watanabe 1960	3.8
Detmer's medium, modified	Bold 1942	3.9
Tris buffered- inorganic medium (TBIM)	Smith and Wiederman 1964	3.10

2. There are some **defined media** that are suited for cultivating **selective o**r very specific freshwater microalgae. Table 3.3 presents 16 media for this purpose. Hence, these media can also be called **selective media** because they enhance the population density of a particular group of microalgae or a specific alga.

Table 3.3: Some defined and selective media specifically for culturing an alga or groups of freshwater microalgae.

Medium	Target microalga/groups of algae	Reference	Table no. in this chapter
CYANOBACTERIA			
BG-11 minus N medium	Diazotrophic cyanobacteria when N source is removed	Stanier et al. 1971	3.4 and 3.5
Anabaena variabilis medium	Diazotrophic cyanobacterium, *Anabaena variabilis*, when N source is removed. *Anabaena variabilis*	Watanabe 1960	3.11

Anabaena variabilis A5 micronutrients		 Stanier et al. 1971	 3.5
B-12 medium	*Microcystis aeruginosa*	Nakagawa et al. 1987; Shirai et al. 1989	3.12
CB medium	*Microcystis aeruginosa*	Shirai et al. 1989	3.13
Spirulina medium B6 solution A5 trace metals	*Spirulina/Arthrospira*	Zarrouk 1966; Vonshak, ed. 1997 Stanier et al. 1971	3.14 3.15 3.5
Tolypothrix tenuis medium A5 trace metals	Diazotrophic cyanobacterium, *Tolypothrix tenuis,* when N source is removed.	Watanabe 1960 Stanier et al. 1971	3.16 3.5
RHODOPHYTA			
Allen's Cyanidium medium Allen's Cyanidium micronutrient solution (trace metals)	*Cyanidium*	Allen 1959; Watanabe et al. 2000	3.17 3.18
PYRRHOPHYTA and **BACILLARIOPHYTA**			
Carefoot's medium A5 trace metals	dinoflagellates	Carefoot 1968 Stanier et al. 1971	3.19 3.5
Tañada's medium	diatoms	Tañada 1951	3.20
EUGLENOPHYTA			
Maeda's medium	euglenoids	Watanabe 1960	3.21
Modified AF6 medium	*Colacium;* organisms with preference for acidic pH	Kato 1982; Watanabe et al. 2000	3.22
CHLOROPHYTA			
Chlorella ellipsoidea medium A5 trace metals	*Chlorella ellipsoidea*	Watanabe 1960 Stanier et al. 1971	3.23 3.5
Modified C (Closterium) medium Trace metals Vitamins	*Closterium*	Ichimura 1971; Watanabe et al. 2000 Provasoli and Pintner 1960; Watanabe et al. 2000 Watanabe et al. 2000	3.24 3.25 3.26
Volvox medium	*Volvox*	Provasoli and Pintner 1960	3.27
MIXED			
Jaworski's medium	Green algae and diatoms	Thompson et al. 1988	3.28
Rodhe medium	Green algae and diatoms	Rodhe 1948	3.29

Among these recipes, six culture media are suited for cyanobacteria. Included in the group is BG-11 minus N (nitrogen) because it favorably allows good growth of diazotrophic cyanobacteria when the nitrogen source, such as $NaNO_3$, is removed from the recipe. **BG-11 medium** is especially suited for cultivating diazotrophic cyanobacteria because it is rich in iron and molybdenum that are needed components of the nitrogenase enzyme. Other media suited for culturing nitrogen-fixing cyanobacteria, when nitrogen is removed, are **Fogg's medium** (Fogg 1949), **Anabaena variabilis** medium (Watanabe 1960), and **Tolypothrix tenuis medium** (Watanabe 1960) because they all have optimum amounts of iron, molybdenum, calcium, and magnesium in their recipes for good growth of these types of cyanobacteria.

On the other hand, **B-12 and CB media** are used for culturing the bloom-forming cyanobacterium, *Microcystis aeruginosa*. Both media have vitamins in them (Nakagawa et al. 1987; Shirai et al. 1989), which can be explained by the tendency of *Microcystis aeruginosa* to be found in eutrophic habitats. **Spirulina medium,** more popularly known as **Zarrouk's medium** (Zarrouk 1966), is very specific for growing *Spirulina*/*Arthrospira* mainly because of its preference for high pH, and by the addition of high amounts of salts, particularly the weak base $NaHCO_3$ (16.8 $g.L^{-1}$), aside from the

other trace elements that are added which are unique for this medium. This medium selectively eliminates the growth of other microalgae except *Spirulina/ Arthrospira.*

Cyanidium medium is used for cultivating the red alga*, Cyanidium caldarium*, and related genera such as *Cyanidioschyzon* and *Galdieria* (Watanabe et al. 2000). Carefoot's medium was developed to grow the freshwater dinoflagellate (*Peridinium cincutum* f. *ovoplanum* Lindeman) (Carefoot 1968). A number of media have been prepared for culturing diatoms, and one of them is the **Tañada's medium** (Table 3.20; Tañada 1951). In particular, diatoms require some silica in order to induce them to undergo sexual reproduction and in the end restore their original vegetative size. Silica is also needed because it is a major component of the cell walls of diatoms. Hence, media with sufficient amounts of silica will support the growth of freshwater diatoms, like **Modified Chu no. 10** (Table 3. 8; Watanabe 1960). **BG-11 medium** can be used to grow diatoms when enriched with $Na_2SiO_3 \cdot 9\ H_2O$ (to give a final concentration of 0.1 g/ L medium). Sometimes, some vitamins are also added, such as thiamine·HCl, biotin and vitamin $B_{12,}$ both for freshwater and marine types for the proper development of some diatoms (Patrick and Reimer 1966; Round et al. 1990).

The media for growing the photosynthetic euglenophytes usually have vitamins and other organic compounds (**Maeda and AF6**; Table 3.21 and Table 3.22, respectively) (Watanabe 1960; Kato 1982; Watanabe et al. 2000) because of their common habitat in eutrophic waters, or attached to some substrates (*Colacium* on some zooplankton), hence they have a tendency to be heterotrophic to auxotrophic. AF6 medium is also meant for growing microalgae that require an acidic medium.

The media for cultivating the green algae are mostly made up of inorganic compounds (except for the presence of EDTA), as those mentioned in Table 3.2. **Chlorella medium** is a medium that is formulated for *Chlorella ellipsoidea* (Watanabe 1960). However, some of the fastidious and bloom-forming green algae, such as the desmids, *Closterium*, and the volvocalean green alga, *Volvox*, require some vitamins in their media for their optimum growth (Table 3.23-3.26). This requirement for vitamins may be attributed to the fact that these algae are usually found in freshwaters of low conductivity and poor nutrient conditions (Gerrath 1993; Ngearnpat and Peerapornpisal 2007). Hence, to support and enhance their growth, the authors of these media added vitamins (Provasoli and Pintner 1960; Ichimura 1971; Watanabe et al. 2000). Some media that have vitamins and organic compounds in them that are meant to culture more fastidious freshwater green algae and diatoms include **Jaworski's** and **Rodhe media** (Tables 3. 28-3.29).

Defined media can be further classified on the basis of their composition as: 1) purely inorganic compounds or 2) a combination of organic and inorganic compounds. Among the 22 defined media, only three are made up only of inorganic compounds, such as Bristol's medium (Table Table 3.7), Modified Detmer's medium (Table 3.9), and Tañada's medium (Table 3.20).

V. The Defined Media Recipes

Defined media-wide range types

Table 3.4: BG-11 medium (Stanier et al. 1971).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration (g/L^{-1} distilled H_2O)
$NaNO_3$	150	10	1.5
*K_2HPO_4	4	10	0.04
$MgSO_4 \cdot 7H_2O$	7.5	10	0.075
$CaCl_2 \cdot 2H_2O$	3.6	10	0.036
*Citric acid	0.6	10	0.006
*Ferric ammonium Citrate	0.6	10	0.006

EDTA (disodium magnesium salt)	0.1	10	0.001
Na_2CO_3	2	10	0.02
A5 micronutrient solution (Table 3.3)	(See below)	(See below)	

*Yellow solution should be autoclaved separately and should be added to the other salts after autoclaving.

Table 3.5: A-5 micronutrient solution for BG-11 medium (Stanier et al. 1971).

Compound	Stock solution (g/L^{-1} distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration (g/L^{-1} distilled H_2O)
H_3BO_3	2.86	1 mL	0.00286
$MnCl_2 \cdot 4H_2O$	1.81		0.00181
$ZnSO_4 \cdot 7H_2O$	0.22		0.00022
$Na_2MoO_4 \cdot 2H_2O$	0.39		0.00039
$CuSO_4 \cdot 5H_2O$	0.08		0.00008
$Co(NO_3)_2 \cdot 6H_2O$	0.05		0.00005

Table 3.6: Bold's basal medium (BBM; Bold 1949; Bischoff and Bold 1963).

Compound	Stock solution (g/L^{-1} distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration (g/L^{-1} distilled H_2O)
Major stocks			
$NaNO_3$	25.0	10	0.250
$CaCl_2 \cdot 2H_2O$	2.5	10	0.025
$MgSO_4 \cdot 7H_2O$	7.5	10	0.075
K_2HPO_4	10.0	10	0.100
KH_2PO_4	17.5	10	0.175
NaCl	2.5	10	0.025
Alkaline EDTA solution		1	
EDTA	50.0		0.050
KOH	31.0		0.031
Acid solution		1	
$FeSO_4 \cdot 7H_2O$	4.98		0.00498
H_2SO_4			
Boron solution		1	
H_3BO_3	11.42		0.01142
Trace elements		1	
$ZnSO_4 \cdot 7H_2O$	8.82		0.00882
$MnCl_2 \cdot 4H_2O$	1.44		0.00144
MoO_3	0.71		0.00071
$CuSO_4 \cdot 5H_2O$	1.57		0.00157
$CO(NO_3) \cdot 6H_2O$	0.49		0.00049

Adjust pH to 6.8. For toxicity testing, use ½ strength BBM (500 mL of above working medium + 500 mL distilled water).

Table 3.7: Bristol's medium (Trainor 1978).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$NaNO_3$	25.0	10	0.250
$CaCl_2$	2.50	10	0.025
$MgSO_4 \cdot 7H_2O$	7.50	10	0.075
K_2HPO_4	7.50	10	0.075
KH_2PO_4	17.50	10	0.175
NaCl	2.50	10	0.025

Table 3.8: Modified Chu's No. 10 medium (Chu 1942; Watanabe 1960; Nalewajko et al. 1995).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$Ca(NO_3)_2 \cdot 4H_2O$	4.0	10	0.040
K_2HPO_4	1.0	10	0.010
$MgSO_4 \cdot 7H_2O$	2.5	10	0.025
Na_2CO_3	2.0	10	0.020
Na_2SiO_3	2.0	10	0.020
Ferric citrate	0.3	10	0.003
Citric acid	0.3	10	0.003
A-5 micronutrient solution (Table 3.5)	(See Table 3.5)	1	(See Table 353)

Adjust pH to 6.0.

Table 3.9: Composition of modified Detmer's medium (Bold 1942).

Compound	Stock solution (g.L-1 distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
Macronutrients			
KNO_3	100	10	1
$CaCl_2$	1	10	0.01
K_2HPO_4	25	10	0.25
$MgSO_4 \cdot 7H_2O$	25	10	0.25
NaCl	10	10	0.1
$FeSO_4 \cdot 7H_2O$	2	10	0.02
Micronutrients			
H_3BO_3	2.86	1 mL	0.00286
$MnCl_2 \cdot 4H_2O$	1.81		0.00181
$ZnSO_4 \cdot 7H_2O$	0.22		0.00022
$MoO_3[Na_2MoO_4].2H_2O$	0.021		0.000021
$CuSO_4.5H_2O$	0.079		0.000079

Table 3.10: Tris buffered-inorganic medium (TBIM) (Smith and Wiederman 1964).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
KNO_3	10.10	20	0.202
Na_2HPO_4	14.20	10	0.142
$MgSO_4 \cdot 7H_2O$	24.60	3	0.0738
$CaCl_2 \cdot 2H_2O$	14.60	1	0.0146
Trihydroxy micronutrients	4.84	25	0.121
EDTA	31.00	1	0.031
H_3BO_3	11.44		0.01144
$FeSO_4 \cdot 7H_2O$	4.98		0.00498
$ZnSO_4 \cdot 7H_2O$	8.82		0.00882
$MnCl_2 \cdot 4H_2O$	1.44		0.00144
MoO_3	0.71		0.00071
$CuSO_4 \cdot 5H_2O$	1.57		0.001570
$Co(NO_3)_2 \cdot H_2O$	0.49		0.00049

Defined selective media types

Table 3.11: Anabaena variabilis medium (Watanabe 1960).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
KNO_3	202	10	2.02
K_2HPO_4	38	10	0.38
$MgSO_4 \cdot 7H_2O$	25	10	0.25
KCl	30	10	0.3
NaCl	12	10	0.12
$FeSO_4 \cdot 7H_2O$	2	10	0.02
A5 micronutrient solution (Table 3.3)	(See Table 3.3)	1	(See Table 3.3)

Phosphate and iron solutions should be autoclaved separately and mixed aseptically. Adjust pH to 8.0.

Table 3.12: B-12 medium for *Microcystis aeruginosa* (Nakagawa et al. 1987; Shirai et al. 1989).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$NaNO_3$	10	10	0.100
K_2HPO_4	1	10	0.010
$MgSO_4 \cdot 7H_2O$	7.5	10	0.075
$CaCl_2 \cdot 2H_2O$	4	10	0.040
Ferric citrate	6	1	0.006
Disodium EDTA$\cdot 2H_2O$	1.0	10	0.010
Vitamin B_{12}	0.1	1	0.0001

Table 3.13: CB medium for *Microcystis aeruginosa* (Shirai et al. 1989).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$Ca(NO_3)_2 \cdot 4H_2O$	15	10	0.150
KNO_3	10		0.100
$MgSO_4 \cdot 7H_2O$	4		0.040
B-disodium glycerophosphate	5		0.050
bicine	50		0.500
*biotin			0.0001
*Vitamin B_{12}			0.0001
*thiamine hydrochloride			0.0100
PIV metals	Mg/100mL distilled H_2O	Amount of the stock solution used to make 1L medium (mL)	
$FeCl_3 \cdot 6H_2O$	19.6	3	0.586
$MnCl_2 \cdot 4H_2O$	3.6		0.108
$ZnSO_4 \cdot 7H_2O$	2.2		0.066
$CoCl_2 \cdot 6H_2O$	0.4		0.012
$Na_2MoO_4 \cdot 2H_2O$	0.25		0.007
Disodium EDTA$\cdot 2H_2O$	100.00		

*Sterilize through millipore filtration and add into the major compounds while the latter is still warm after sterilization.

Table 3.14: Spirulina medium (Vonshak 1997; Zarrouk 1966).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
NaCl	100	10	1.0
$CaCl_2$	4	10	0.04
$NaNO_3$	250	10	2.5
$FeSO_4 \cdot 7H_2O$	1	10	0.01
EDTA (Na)	8	10	0.08
K_2SO_4	100	10	1.0
$MgSO_4 \cdot 7H_2O$	20	10	0.2
$NaHCO_3$	1,680	10	16.8
K_2HPO_4	50	10	0.5
A5 micronutrient solution (Table 3.5)	(See Table 3.5)	1	(See Table 3.3)
B6 solution	(See Table 3.16)	1	(See Table 3.16)

Table 3.15: B6 Solution for Spirulina medium (Vonshak 1997; Zarrouk 1966)*.

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
NH_4VO_3	0.02296	1	0.000023
$K_2Cr_2(SO_4)_4 \cdot 24H_2O$	0.09600		0.000096
$NiSO_4 \cdot 7H_2O$	0.04785		0.000048
$Na_2WO_4 \cdot 2H_2O$	0.01794		0.000018
$Co(NO_3)_2 \cdot 6H_2O$	0.04398		0.000044
$Ti_2(SO_4)_3$	0.04		0.00004

*Adjust the pH of the medium to pH 10 before sterilization.

Table 3.16: Tolypothrix tenuis medium (Watanabe 1960).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
KNO_3	300	10	3.0
$Na_2HPO_4 \cdot 12H_2O$	50	10	0.5
$MgSO_4 \cdot 7H_2O$	50	10	0.5
$CaCl_2$	2	10	0.02
$FeSO_4 \cdot 7H_2O$	2	10	0.02
A5 micronutrient solution (Table 3.5)	(See Table 3.5)	1	(See Table 3.5)

Table 3.17: Allen's Cyanidium medium (Allen 1959; Watanabe et al. 2000).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$(NH_4)_2SO_4$	132	10	1.320
KH_2PO_4	27.2	10	0.272
$MgSO_4 \cdot 7H_2O$	24.6	10	0.246
$CaCl_2 \cdot 2H_2O$	7.4	10	0.074
Allen's metals (Table 3.8)	(See below)	0.1	(See below)

Adjust pH to 2.5 using 1N sulfuric acid. Autoclave.

Table 3.18: Micronutrient solution (Trace elements for Allen's medium (Watanabe et al. 2000).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
Fe-Na-EDTA.3 H_2O	301.6	0.1	0.03016
H_3BO_3	28.6		0.00286
$MnCl_2 \cdot 4H_2O$	17.9		0.00179
$ZnSO_4 \cdot 7H_2O$	2.2		0.00022
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.3		0.00013
$CuSO_4 \cdot 5H_2O$	0.8		0.00008
NH_4VO_3	0.23		0.000023

Table 3.19: Carefoot's medium (Carefoot 1968).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration
$NaNO_3$	25	10	0.25
K_2HPO_4	0.97	10	0.00970
KH_2PO_4	2.27	10	0.02273
$MgSO_4 \cdot 7H_2O$	0.49	10	0.00490
$CaCl_2 \cdot 2H_2O$	1.65	10	0.01654
NaCl	1.65	10	0.01648
PIV trace metals solution (Table 3.5)	(See Table 3.5)	1	(See Table 3.5)

Adjust pH to 7.5 and autoclave.

Table 3.20: Tañada's medium for diatoms (Tañada 1951).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
KNO_3	62.5	10	0.625
K_2HPO_4	34.8	10	0.348
Na_2SiO_3	10	10	0.10
$MgSO_4 \cdot 7H_2O$	6.1	10	0.061
$Ca(NO_3)_2 \cdot 4H_2O$	0.14	10	0.0014
$FeSO_4$	0.11	10	0.0011
$MnSO_4 \cdot 4H_2O$	0.075	10	0.00075
$ZnSO_4 \cdot 7H_2O$	0.05	10	0.0005
H_3BO_3	0.05	10	0.0005
$CuSO_4 \cdot 5H_2O$	0.0015	10	0.000015

Table 3.21: Maeda's medium for *Euglena* (Watanabe 1960).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
Component A		100	
K-citrate	6.0		0.6
$MgSO_4 \cdot 7H_2O$	4.0		0.4
$FeSO_4 \cdot 7H_2O$	0.4		0.04
$CaCl_2$	2.0		0.2
A5 micronutrient solution (Table 3.3)	(See Table 3.3)		(See Table 3.3)
Component B		100	
Na-butyrate	1.1		0.11
$NH_4H_2PO_4$	0.4		0.04
Component C		100	
Na-glutamate	20.0		2.0
Casamino acids	20.0		1.0
Vitamin B_2	0.002		0.0002

Table 3.22: Modified AF6 medium (Kato 1982; Watanabe et al. 2000).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$NaNO_3$	14.0	10	0.140
NH_4NO_3	2.2	10	0.022
$MgSO_4 \cdot 7H_2O$	3.0	10	0.030
KH_2PO_4	1.0	10	0.010
K_2HPO_4	0.5	10	0.005
$CaCl_2 \cdot 2H_2O$	1.0	10	0.010
Ferric citrate	0.2	10	0.002
Citric acid	0.2	10	0.002
Glucose	10	10	0.100
Sodium acetate	10	10	0.100
PIV trace metals (Table 3.5)	See Table 3.6	1	See Table 3.6
Vitamins solution	See Table 3.7	1	See Table 3.7

Adjust pH to 6.6 before autoclaving.

Table 3.23: Chlorella ellipsoidea medium (Watanabe 1960).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
KNO_3	125	10	1.25
KH_2PO_4	125	10	1.25
$MgSO_4 \cdot 7H_2O$	125	10	1.25
$FeSO_4 \cdot 7H_2O$	2	10	0.02
A-5 micronutrient Solution (Table 3.5)	(See Table 3.5)	1	(See Table 3.5)

Table 3.24: Modified C (*Closterium*) medium (Ichimura 1971; Watanabe et al. 2000).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
TRIS base	5	100	0.50
KNO_3	1	100	0.10
$Ca(NO_3)_2 \cdot 4H_2O$	1.5	100	0.15
Na2 β-glycerophosphate$\cdot 5H_2O$	50	1	0.05
$MgSO_4 \cdot 7H_2O$	40	1	0.04
Trace metals solution	(See below)	3	(See below)
Vitamins solution	(See below)	1	(See below)

Autoclave. Final pH should be 7.5.

Table 3.25: Micronutrient solution for modified C medium (Provasoli and Pintner 1960; Watanabe et al. 2000).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
Na_2EDTA	1	3	0.003
$FeCl_3 \cdot 6H_2O3$	0.194		0.00058
$MnCl_2 \cdot 4H_2O$	0.036		0.00011
$ZnCl_2$	0.01044		0.00003
$Na_2MoO_4 \cdot 2H_2O$	0.01262		0.00004
$CoCl_2 \cdot 6H_2O$	0.00404		0.00001

Table 3.26: Vitamins solution for modified C medium (Watanabe et al. 2000).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
Thiamine·HCl (vitamin B_1)	0.01	1	1×10^{-5}
Biotin (vitamin H)	0.0001		1×10^{-7}
Cyanocobalamin (vitamin B_{12})	0.0001		1×10^{-7}

Filter-sterilize and store frozen.

Table 3.27: Volvox medium (Provasoli and Pinter 1960).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
Glycylglycine	5	100	0.5
$Ca(NO_3)_2 \cdot 4H_2O$	1.178	100	0.1178
Na_2 β-glycerophosphate$\cdot 5H_2O$	50	1	0.05
$MgSO_4 \cdot 7H_2O$	40	1	0.04
KCl	50	1	0.05
PIV micronutrient solution	(See Table 3.5)	1	(See Table 3.5)
Biotin (vitamin H)	0.0001	1	1×10^{-7}
Cyanocobalamin (vitamin B_{12})	0.0001	1	1×10^{-7}

Table 3.28: Jaworski's medium (Thompson et al. 1988).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$Ca(NO_3) \cdot 4H_2O$	2.00	10	0.02
KH_2PO_4	1.24	10	0.0124
$MgSO_4 \cdot 7H_2O$	5.00	10	0.05
$NaHCO_3$	1.59	10	0.0159
EDTA FeNA	2.25	1	0.00225
EDTA Na_2	2.25	1	0.00225
H_3BO_3	2.48	1	0.00248
$MnCl_2 \cdot 4H_2O$	1.39	1	0.00139
$(NH_4)6Mo_7O_{24}44H_2O$	1.00	1	0.04
Cyanocobalamin (Vitamin B_{12})	0.04	1	0.00004
Thiamine HCl (Vitamin B_1)	0.04	1	0.00004
Biotin	0.04	1	0.00004
$NaNO_3$	80.0	1	0.080
$Na_2HPO_4 \cdot 12H_2O$	36.0	1	0.036

Table 3.29. Rodhe medium (Rodhe 1948).

Compound	Stock solution ($g.L^{-1}$ distilled H_2O)	Amount of the stock solution used to make 1L medium (mL)	Final concentration ($g.L^{-1}$ distilled H_2O)
$Ca(HO_3)_2$	0.160	10	0.0016
K_2HPO_4	0.005	5	0.000025
$MgSO_4 \cdot 7H_2O$	0.025	25	0.000625
Na_2SiO_3	0.020	20	0.0004
$MnSO_4$	0.00003	0.3	9×10^{-9}
Ferric citrate	0.01	10	0.0001
Citric acid	0.01	10	0.0001

VI. Summary

This chapter presented three undefined media and 22 defined media in uniform, consistent units, including stock solutions; user friendly instructions; and equipment available to many laboratories in the Philippines. There were six media that were broad and general in range, being able to support many freshwater microalgae. On the other hand, there were 16 media for specific/selected microalgae or groups of microalgae, including the Anabaena variabilis medium, for culturing *Anabaena variabilis*, Carefoot's medium, for the freshwater dinoflagellate *Peridinium cincutum* f. *ovoplanum*, and Tañada's medium, which is an inorganic medium for diatoms. In this chapter BG-11 medium, with nitrogen source, was grouped under general media when it is used in culturing most microalgae. It is classified under selected media, called BG-11 minus N or the nitrogen source is removed, when it is used in culturing nitrogen-fixing cyanobacteria.

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Chapter 4. Culture Media and Propagation of Marine Microalgae

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I. Introduction

Natural seawater (NW) is a complex medium containing more than 50 known elements and many different organic compounds (Harrison and Berges 2004). However, NW is rarely used for algal culture because it does not contain all the needed nutrients for growth, and needs further enrichment. In addition, variations in the quality of NW throughout the year, the need to control nutrient and trace element concentrations, and the limited availability of seawater at inland locations make the option of artificial seawater (AW) attractive.

McLachlan (1973) compared today's marine culture media with those made 30 years ago, and noted that a lot of progress has led to the formulation of several media that are quite broad-spectrum, indicating that most algal cultures can be grown by using only a few different media.

The most challenging phytoplankton to culture is still the marine species. The culture of marine microalgae may utilize suitable media used by other culture collections. However, in microalgae culture, there are other media that can be used which are as suitable as those used in some culture collections. Since thousands of algal species with variable growth needs, exist in marine environments, the culture cost and sustainable production are important. Thus, a simple nutrient medium is often preferred. It is always the goal of researchers to sustain the production and obtain a continuous supply of microalgal species. This chapter will discuss the different culture media that can be applied for most marine microalgae species. Culture procedures in indoor and outdoor set-up will also be presented.

II. Nutrient Enrichment/ Culture Media Preparations

A. Importance of Preparation and use of Culture Media

The objective of using nutrient media in microalgae culture is to sustain production of cells having

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the highest cell densities in the shortest period of time using seawater. Several nutrient enrichment media containing soil extract, nitrates, phosphorus, trace elements, and vitamins have been formulated for marine algae to enhance culture propagation. The succeeding culture media are some of the nutrient preparations and formulations used in the University of the Philippines Visayas in the Institute of Aquaculture laboratory for microalgae culture.

B. Different Marine Culture Media

Walne's Medium
One of the popular culture media used for marine microalgae propagation is Walne's medium which is commonly used in the culture of microalgae in the laboratory (Villegas and Dela Peña 1989).

To prepare, first we begin with the Stock Solution A: Add into 1L distilled water (dH2O), the pre-weighed 100.0 g Nitrate (as $NaNO_3$); 45.00 g Disodium salt EDTA (Na_2EDTA); 33.60 g Boric acid (H_3BO_3); 1.30 g each of Ferric chloride ($FeCl_3.6H_2O$) and Manganese chloride ($MnCl_2.4H_2O$). Stir and mix. Then, separately, in 100 mL dH2O, mix vitamins composed of 0.1 g Thiamine (B1) and 0.005 g Cyanocobalamine (B12) to make-up 100mL Vitamin Stock Solution B. For Stock Solution C, pre-weighed trace metals composed of 2.1 g Zinc chloride ($ZnCl_2$); 2.0g Cobalt chloride ($CoCl_2.6H_2O$); 0.90g Ammonium molybdate $(NH_4)6Mo_7O_{24}.4H_2O$; 2.00g Copper sulfate ($CuSO_4.5H_2O$). Mix together all components, then dissolve to make-up 1L of Trace Metal Stock Solution C. Take note that this solution is normally cloudy. Acidify with a few drops of concentrated HCl to give a clear solution. Final medium is prepared by dispensing 1.0 mL of Stock Solution A and 0.1 mL of Vitamin Stock Solution B and 1.0mL of Trace Metal Stock Solution C in a container to make a final volume of one L with distilled water.

Erd-screiber's Medium (McLachlan 1973)

Erd-schreiber's medium has been used successfully for many years and for a wide variety of organisms, both unicellular and benthic marine species. Other species have not been grown in other media.

To prepare Erd-screiber's Medium, one must initially weigh all ingredients composed of 1.18-2.35 mM Nitrite ($NaNO_4$); 0.056-0.14 mM Phosphate (NaH_2PO_4); and 50mL soil extract. All these components are mixed and dissolved in distilled water to make-up 1L Erd-screiber's medium.

The sea water and additives are sterilized separately and combined aseptically. The concentration of nitrogen and phosphorus is high, especially for an unbuffered medium. In many instances, addition of iron and other trace metals and vitamins can replace soil extract.

Enriched sea water (ES) Medium (McLachlan 1973)

The enriched natural sea water medium (ES Medium) as proposed by McLachlan (1973) has in addition to nitrite and phosphate, Tris buffer, trace metals and vitamins in place of soil-water extract. This is a good general-purpose medium to culture numerous unicellular and benthic marine species. However, there are some references that appeared totally different from McLachlan's.

To begin preparation, all ingredients are pre-weighed first with the major elements composed of 0.66 mM Nitrite ($NaNO_4$); 0.025 mM Sodium glycerophosphate (Na_2. Glycerophosphate); 7.2 mM Iron chelate (FeEDTA). Again, in place of soil-water extract, Tris buffer, Trace metals and Vitamins are used. To prepare this Trace Metal Solution, we need 0.0016 mM Cyanocobalamin; 0.0008 mM Biotin; 0.02 mM Thiamine.HCl and 0.66 mM Tris.

Sea water is sterilized by filtration or steaming. The enrichments are assembled into a single solution, then the sterile (filtered) sea water is added aseptically to the enrichment medium to make-up into a one L ES medium.

Provasoli's Enriched Seawater (PES) Medium (Provasoli et al.1957)

Provasoli's enriched seawater medium (PES) is a general purpose marine medium for xenic cultures of microalgae. This is prepared by adding one tube (20 mL) of ES-enrichment to 1000 mL of pasteurized, filtered sea water. To prepare for the PES-enrichment solution, add the following to 100 ml glass-distilled water:

In 100 ml glass-distilled water, add 350mg of NaNO3; 50mg Na2glycerophosphate.5 H2O; 25mL Fe-solution; add 25mL of PII metal-solution; 10 µg vitamin B12; 0.5 mg thiamine; plus 5 µg biotin and 500mgTris buffer. Adjust to pH 7.8, dispense in tubes (20 mL/tube) and autoclave. Store at 10°C. To prepare Fe-solution: Dissolve 351 mg of Fe (NH4)2(SO4) 2. 6H2O and 300 mg of Na2EDTA in 500 mL of de-ionized or distilled water.

For PII metal solution preparation: in 100 ml glass-distilled water add 100mg Na2EDTA; 114mg H3BO3; 4.9mg FeCl3 6H2O; 16.4mg MnSO4 H2O; 2.2mg ZnSO4; 7 H2O; 0.48 mg CoSO4. 7 H2O.

For marine strains that require salinities less than 30 ppt, the basic Provasoli´s enriched sea water medium is combined with de-ionized or distilled water prior to inoculating into the new culture.

Enriched sea water medium (ESM) (Okaichi et al. 1982)

Enriched sea water medium (ESM) as formulated by Okaichi et al. (1982) is prepared by first diluting seawater with distilled water (30 mL seawater: 70mL distilled water) instead of using full strength sea water.

Then to prepare the medium, the major nutrients are pre-weighed and mixed, such as: 12 mg Nitrate ($NaNO_3$); 0.5 mg Potassium phosphate (K_2HPO_4); 0.0001 mg Vitamin $B_{12;}$ 0.0001 mg Biotin; 0.01 mg Thiamine HCl; 0.0259 mg Fe – EDTA; 0.0332 mg Mn – EDTA; 100.0 mg Tris (hydroxymethyl) aminomethane. These nutrients are made into solution with 2.5 mL soil extract and 97.5 mL diluted sea water.

Soil extract is prepared by adding 200 g of soil (soil from undisturbed deciduous woodland) into 100 mL distilled water. Autoclave the mixture for 1 h at 105°C for two times. Pass the supernatant through a GF/C filter and Celite, and then pass the filtrate through a GF/F filter. Adjust to 1000 mL by adding distilled water. Dispense 10 mL of the final filtrate into each test tube and sterilize by autoclaving for 20min at 121°C. Keep in a cool place.

Guillard and Ryther's F/2 Medium (Guillard 1975)

Among the nutrient media formulations used to culture marine microalgae in laboratories and hatcheries, Guillard and Ryther's F/2 medium is one of the most widely used. In this nutrient media you add Nitrate, Phosphate, Silicate, Trace Metals* and Vitamins**. But before mixing, a little preparation has to be made. Initially, pre-weighed the major ingredients: 75.0 g Nitrate ($NaNO_3$); 5.0 g Phosphate ($NaH_2PO_4.H_2O$); 30.0 g Silicate ($Na_2SiO_3.9H_2O$). Dissolve in 900 ml of distilled H_2O. Make final volume up to 1.0 L with dH_2O. Utilization is 0.5ml per liter.

Then prepare the other ingredients, trace metals and vitamins. When preparation is completed, store primary stock solutions in freezer. Store completed vitamin solution in the refrigerator or place small aliquot in the freezer.

In mixing ingredients, add nitrate and vitamin solution directly to seawater and autoclave. Autoclave separately aliquots of the phosphate, trace metals and silicate. Add these aseptically to your media once it has cooled to room temperature. Take note that F/2 Medium contains extensive silica precipitate and should be used only when growing diatoms.

*For the Trace Metal Solution, components include 3.50 g Ferric chloride ($FeCl_3$.6H2O); 4.36g Sodium EDTA ($Na_2EDTA.2H_2O$); 0.98g/100ml dH_2O Copper sulfate ($CuSO_4.5H_2O$); 0.63g/100ml dH_2O $Na_2MoO_4.2H_2O$; 2.20g/100ml dH_2O $ZnSO_4.7H_2O$; 1.00g/100ml dH_2O $CoCl_2$ $6H_2O$; 18.00g/100ml dH_2O $MnCl_2.4H_2O$. Add 1 ml of each of the trace metal solutions. Solution will initially be cloudy. Add 1N NaOH to adjust pH to about 4.5. Solution should clear unless too much NaOH has been added. Bring final volume to one liter.

**For F/2 Vitamin Solution, add 10 mg/10ml dH_2O Vitamin B_{12} and 10 mg/10ml dH_2O Biotin. Dissolve Biotin in a little 0.1N NaOH first. Add 200.0 mg Thiamine HCl.

Liao and Huang's Modified Tung Kang Marine Research Laboratory (TMRL) Medium

Liao and Huang's Modified Tung Kang Marine Research Laboratory (TMRL) medium is a common medium in hatcheries in the Philippines because it is simple and not too laborious to prepare. The simple preparations for stock solutions followed the work of Villegas and De la Peña (1989) for the culture procedures of marine microalgae.

Four major nutrients are used in the preparations of Stock solution composed of 100g Nitrate (KNO_3); 10g Phosphate ($Na_2HPO_4.6H_2O$); 3g Ferric chloride ($FeCl_3.6H_2O$); 1g Silicate ($Na_2SiO_3.9H_2O$). Each major nutrient is dissolved in 1 liter sterile, filtered sea water and ready for use. Utilization is 1.0 ml per liter culture.

C. Water
Distilled water is usually the solvent used in preparing media. Glass-distilled water is preferred over copper-distilled water, especially in places where the tap water has a very high content of calcium salts, such as in Los Baños, Laguna. Membrane-filtered water (0.45 µm) or deionized water can also be used.

III. Culture Techniques for Marine Microalgae

A. Equipment/Supplies
Most required equipment comprises standard items in laboratories. The autoclave is one of the most expensive items. The other pieces of equipment that may be needed are the microscope, analytical and top-loading balances, pH meter, and hot-plate-magnetic stirrer.

B. Chemicals
Reagent grade salts (e.g., American Chemical Society grade) should be used if possible (Harrison and Berges 2004). Organic chemicals such as vitamins, buffer and chelators are available from Sigma Chemical Company, Chemline and other chemical suppliers. Many of them now issue more efficient and reliable chemicals using modern technologies, and any changes in brand should be noted because different brands have different amounts of contaminants or impurities.

C. Glassware
Boro-silicate glassware should be used exclusively for all glassware, including stock bottles, beakers, and cultural tubes and flasks (examples of brand names are Pyrex and Kimex). Teflon or plastics are recommended because they reduce breakage. Tap water often contain high amounts of nutrients, trace metals and heavy metals, and if it is to be used for washing and rinsing, make sure that de-ionized water is used for the final rinse. Furthermore, domestic detergents leave a residual film on glassware. The detergents from most large chemical supply companies are satisfactory, but labels should be read to determine if the contents meet your requirements. New glassware and plastic ware should be degreased in dilute NaOH, soaked in dilute HCl, and then soaked in deionized water for several days before use. Glassware should not be cleaned in chromic acid, because chromium is toxic to many phytoplankters (McLachlan 1973). Teflon is useful only for stock bottles and is not suitable for culture vessels because of its reduced light transmission properties. Polycarbonate is good for culture vessels, especially for experiments involving trace-metal limitation. Polypropylene may yield toxins from stocks, notably silicate stocks

(Brand et al. 1986). Glassware should be autoclaved, and clean glassware and plastic ware should be stored in closed cupboards.

For general purpose culturing, flasks and test tubes made of borosilicate glass and tissue culture-grade polycarbonate or polystyrene plastic ware are recommended. Teflon-lined caps are recommended for screw-top glass test tubes, and black caps should be autoclaved several times in changes of seawater because new caps may release toxic phenolics when heated (McLachlan 1973). For studies on silicon limitation, polycarbonate is recommended. However, borosilicate glassware may be used as long as it has not been rinsed with any acid that causes severe leaching of silicate from the glass. Likewise, rubber stoppers (or anything that releases volatile compounds when heated) should be autoclaved separately from media. Older autoclaves with copper tubing should be avoided because excess copper is toxic to algae. The autoclave steam may be contaminated with metals or chemicals used to inhibit corrosion of the autoclave. In the absence of the glassware mentioned, a dextrose bottle is also a good alternative that can be used as a culture vessel.

D. Air Tubings

Early studies conducted using 50 types of culture materials revealed that type of culture materials have inhibitory effects on phytoplankton and zooplankton (Bernhard and Zattera 1970; Bernhard 1977). Later study showed that latex tubing was toxic to phytoplankton, zooplankton, and bacteria (Price et al. 1986). Even using latex tubing to siphon water from one bottle to another rendered the water toxic for phytoplankton growth. Preliminary results indicated that the toxic compounds may be penta-chlorophenols and tetra-chlorophenols used to preserve the latex tubing (Price et al. 1986). Tygon tubing is generally safe, provided that the powder inside the new tubing has been carefully removed by rinsing before use. Silicone tubing is completely safe to use. Colored or black rubber stoppers may be toxic, and therefore silicone stoppers are recommended, especially those from Cole-Palmer that are made by injecting small air-bubbles into the polymer, and are thus lighter and easier to work with than the solid silicone stoppers. All containers and tubing used for cultures and media stocks should be carefully selected to avoid toxic compounds.

E. Sea water

Sea water as the base for culture media must be clean and free from any toxic elements or unwanted particles. Chlorinated and filtered seawater is recommended to avoid contamination with other organisms. Sea water can be treated with 10 ppm chlorine for disinfection. Filtration can be done using fine filters and sand filtrations for large volume of seawater (Fortes et al. 2002).

F. Collection, isolation and purification of sample

The same techniques are utilized for microalgae, particularly the unicellular forms, as those employed for bacteria, protozoa, yeasts and molds. Phytoplankton may be collected by towing through the water using special plankton nets made of fine silk bolting cloth (180 meshes/inch^2) (Figure 4.1). A small vial is attached to the end of the net which serves as a collector (Garrett 2011). Collected samples may be centrifuged or filtered. Samples for examination may be preserved in 8-10% formaldehyde.

Sampling bottles should be uncovered and illuminated promptly upon reaching the laboratory since natural collections kept in the laboratory immediately begin to alter their state and composition. Competition among species is one of the main problems in maintaining a mixed population. Successful cultures can therefore be obtained only after their separation. Two conditions must be fulfilled to obtain unialgal or pure cultures: (1) the relevant or desired microalgal species must be isolated, and (2) the said species must be induced to multiply.

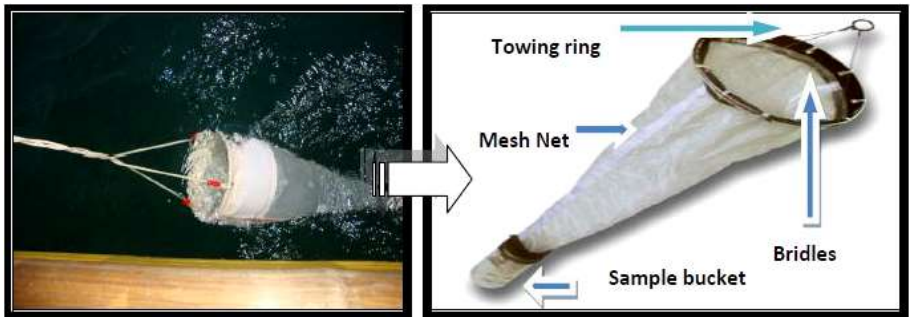

Figure 4.1: A complete plankton tow net with a single net ring and towing bridle, filtration net and sample bucket. The net concentrates the plankton from the water that passes through it and it is towed by boats.

G. Culture of marine microalgae

1. Indoor cultures

These are cultures that commenced using small vessels that once an increased in algal density or log phase was reached, can be harvested either to be used directly as feed in hatcheries or as starter for the next batch culture for gradual scale-up of culture to bigger volume. See steps in doing indoor microalgae culture in Figure 4.2.

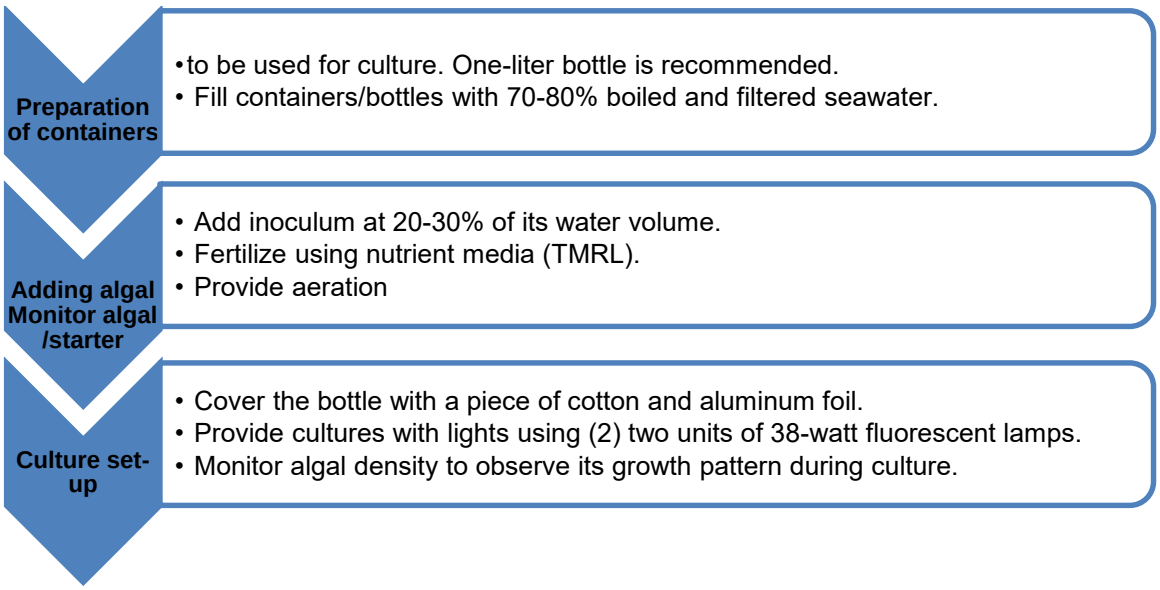

Figure 4.2: Steps in doing indoor microalgae culture.

NOTE: Once this algal culture reached its peak or an increase in algal density, then the culture can be utilized as feed or starter for the next culture.

2. Outdoor Culture

This refers to microalgal propagation done in large outdoor facilities either with a natural bottom, ponds, raceways, concrete or canvass-lined tanks, PVC sheets or HDPE's. Outdoor culture depends mainly on natural light for illumination. The nutrient medium for outdoor culture is based on the one used in indoors, but agricultural grade fertilizers are used instead of the laboratory or analytical grade reagents.

Compared with the indoor culture, the microalgal production cost in outdoor facilities is relatively cheaper, but it is only suitable for a few, fast-growing species particularly those that are highly resistant to the changing weather conditions. Sustaining continued culture of live microalgae is highly favorable especially in aquaculture as microalgae serves as primary food for the smaller aquatic organisms. Below are the steps followed in doing outdoor production of microalgae (Figure 4.3).

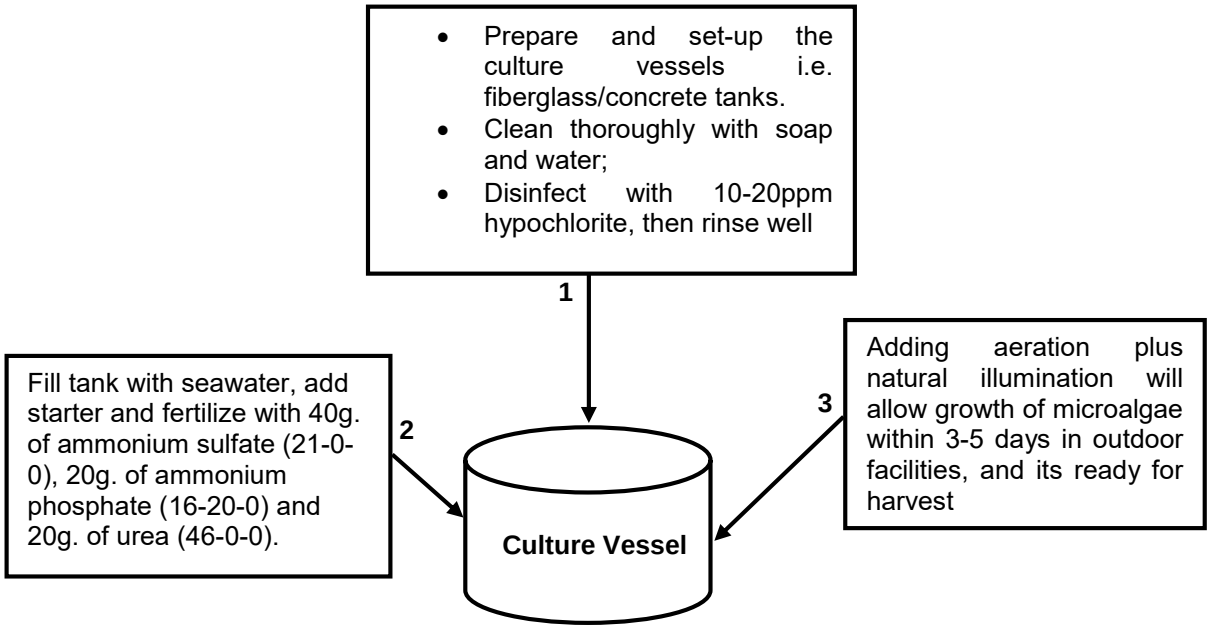

Fig. 4.3: Steps in doing outdoor microalgae culture.

Once peak of algal density is reached, microalgae can be collected. The harvested microalgae is ready for use in aquaculture as direct feed or for any other practical purposes. The above procedure can be repeated if there is a requirement to scale-up the production.

IV. Summary

Culture media preparation for marine microalgae production and propagation requires a number of mineral elements that include macronutrient such as N, P, K, S, Mg and micronutrients such as Fe, Mn, Cu, Zn, Mo and Si. The marine microalgae are probably the most challenging species to culture because of the great diversity of their kinds that will require different nutrient formulation for each kind. The culture of marine microalgae may utilize suitable media used by other culture collections which may just be as suitable for other species as well. This chapter has presented the different nutrient preparations and formulations used in the culture of marine microalgae. The formulations provided were originally formulated by experts, and modified by other authorities based on local conditions. All of the culture media are important, but their proper utilization should take into consideration the target species, culture conditions, availability of nutrient media, and other relevant factors. Microalgae production achieved both in indoor and outdoor facilities have only one main goal that is to maintain it live to sustain the requirement of aquatic commodities being fed.

Compared with the indoor culture, the outdoor microalgal production cost is relatively cheaper than the former, but it is only suitable for a few, fast-growing species, particularly those that are highly resistant to the changing weather conditions. Sustaining continued culture of live microalgae is highly favorable especially in aquaculture as microalgae serves as primary food for the smaller aquatic organisms. Hence, having both an indoor and outdoor culture facilities are more beneficial. Sustained production ensures continuous supply and availability of microalgae.

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Chapter 5. Maintenance and Preservation of Microalgal Cultures

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I. Introduction

Microalgae are a diverse group of organisms that carry out photosynthesis (Madigan et al. 2015). They may exist as single cells or as loosely organized clumps of cells, viz., colonies, and are found in both marine and freshwater ecosystems. These organisms contribute to more than half of the total primary production at the base of the food chain. The ability of microalgae to grow rapidly over a broad range of environmental conditions causes the production of a wide array of secondary metabolites, which are of considerable value in several biotechnology fields including health, aquaculture and food industries **(**Andersen 2005; Arguelles 2018). The collection of microalgal and cyanobacterial cultures has a similar purpose with that for the collections of other microorganisms, i.e., to assure long-term maintenance of strains without changing their identity for future use.

It is necessary to consider in algal culture and preservation, some important characteristics arising from the autotrophic nature of microalgae and cyanobacteria, in contrast with those for heterotrophic microorganisms. In addition to this, algae are sensitive to changes in environmental conditions, causing the maintenance of strains, specifically, the stability of all their morphological and physiological characteristics and properties, a more difficult task than that for heterotrophic microorganisms. The laboratory techniques in the preservation of these organisms are similar, yet the use of new and progressive methods for algae is considerably limited and selective (Puncocharova 1988; Rhodes et al. 2006; Youn and Hur 2009).

Cultures of microalgae (prokaryotic and eukaryotic) are generally maintained by four methods of preservation namely **periodic transfer, freeze drying, lyophilization (L-drying) and cryopreservation**. Each of these methods will be discussed in the following section of this chapter.

II. Techniques in Microalgal Culture Preservation

There are various preservation techniques used for the long-term storage of microalgae, viz., periodic transfer, freeze-drying, lyophilization or liquid (L)-drying, and cryopreservation. The method of choice depends largely upon the capability of the laboratory, type and number of

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cultures to be preserved. Other considerations in choosing a method of preservation are longevity, genetic stability, cost of materials, and labor cost (Snell 1991; Green 2000). A comparison of the different methods of algal culture preservation and their corresponding applicability is presented in Table 5.1. It must be emphasized that there is no universal method for the successful preservation of all species of microalgae. Different taxonomic groups and even strains within a species exhibit significant differences in response to the stresses imposed by culture preservation and resuscitation. Relatively little research has been carried out on the development of long-term algal culture preservation methods as compared with other groups of microorganisms (Day and Deville 1995). One preservation technique used to preserve microalgal strains in the Philippines is cell immobilization in alginate beads (see Chapter 6). It has been successfully used to preserve Philippine microalgal isolates such as *Chroococcus* sp., *Anabaena variabilis*, *Oscillatoria tenuis*, *Chlorella vulgaris*, *Chlorococcum humicola*, *Scenedesmus bijuga*, and *Selenastrum bibrainum* (Perez 2001).

Microalgal response to the different methods of long-term preservation, i.e., to freeze-drying, L-drying and cryopreservation, varies with different strains, too. Some strains are successful for long-term storage using freeze-drying and cryopreservation while others are not (Day and DeVille 1995; Day et al. 1997). For this reason, it is imperative to have at least two methods of preservation for the perpetual maintenance of microalgae to be assured of having back-up cultures and to minimize or eliminate losses of these resources (Snell 1991).

Of these four methods, lyophilization and cryopreservation protocols will be discussed in detail. These methods are the least practiced in the Philippines probably due to the unavailability of the equipment required for these procedures.

A. Periodic Transfer/ Sub-culturing

Cyanobacteria (blue-green algae) and eukaryotic microalgae are traditionally maintained by **routine serial sub-culture (or periodic transfer to fresh media)**. The frequency of transfer is largely determined by the growth characteristics of the strain. Conventional serial sub-culturing is conducted using aseptic technique and involves moving an inoculum from a late log/stationary phase culture into a pre-sterilized, fresh culture medium. The aim of this preservation technique is to retain morphologically, physiologically, and genetically active representative populations of the species or strain.

Continuous maintenance of actively growing algal and cyanobacterial strains over long periods of time is relatively complex, time-consuming and costly (Day 2007; Arguelles and Martinez-Goss 2019), as well as labor intensive. However, given the many limitations to this practice, it is often the only avenue for the preservation of microalgae that are recalcitrant to preservation by other methods, such as cryopreservation or lyophilization. It is the main preservation method employed to date by the Philippine National Collection of Microorganisms (PNCM) and the Museum of Natural History (MNH), University of the Philippines Los Baños (UPLB), Laguna, Philippines, as well as, by the National Institute of Technology and Evaluation, Biological Resource Center (NBRC), Japan for their microalgal collection.

In a large service culture collection, which functions as a repository for cultures, this approach is sub-optimal since it cannot assure the long-term conservation and preservation of viable, healthy, and stable cultures of the collection. It is also labor-intensive. A principal limitation of perpetual transfer is the selective and artificial nature of the media and incubation regimens in contrast to the natural ecological conditions of the species. Laboratory conditions can, in extreme cases, lead to the loss of important morphological features and physiological traits. Some examples of instability include the size reduction of diatom frustules (Jaworski et al. 1988), retention or loss of spines in *Micractinium pusillum* Fresenius, and loss of normal pigment composition in numerous algae (Warren et al. 2002). Other concerns include the possibility of contamination of axenic cultures and mislabeling or other handling mistakes. Routine serial sub-culturing is a labor- and consumable-intensive process and certainly limits the capacity of workers to maintain large numbers of strains.

Table 5.1: Common methods of microalgal culture preservation*.

Method of Preservation	Microalgal species	Shelf life of the microalga	Availability of the microalga to recover	Advantages	Disadvantages
1. Periodic transfer (sub-culturing)	All eukaryotic microalgae and cyanobacteria	1-3 months depending on the strain	Good	Applicable even for recalcitrant strains	Prone to genetic drift, high possibility of contamination and mislabeling, laborious, with large space requirement
2. Freeze-drying	Cyanobacteria and eukaryotic microalgae (diatoms, chrysophytes, and chlorophytes	25-30 years	Fair	Genetic stability, low possibility of contamination, cheaper, less labor intensive, small storage space requirements, ease of shipment	Not applicable to all microalgae; requires special equipment (freeze-dryer)
3. Lyophilization (liquid drying/ L-drying)	Cyanobacteria and eukaryotic microalgae (diatoms, chrysophytes, and chlorophytes)	25-30 years	Fair	Genetic stability, low possibility of contamination, cheaper, less labor intensive, small storage space requirement, ease of shipment	Not applicable to all microalgae; requires special equipment (lyophilizer)
4. Cryopreservation	Cyanobacteria, pigmented euglenophytes and eukaryotic microalgae (chlorophytes, diatoms)	15 years or longer	Fair	Genetic stability, low possibility of contamination and less labor intensive	Not applicable to all microalgae, requires special equipment and reagents, cost intensive to maintain, i.e., liquid nitrogen supply

*Adapted from McLellan et al.1991; Day 2007.

B. Freeze-drying

Freeze-drying requires the elimination of water from a frozen algal suspension by sublimation under reduced pressure wherein water is evaporated without passing through the liquid phase. The process occurs under vacuum with the algal sample completely frozen. During the process, water inside the cell slowly vaporizes under the low air pressure and ends in a condenser. Freeze-dried microalgae maintain their original shape and texture. This method of preservation has been found to be successful in preserving only a few species of cyanobacteria, particularly the filamentous forms which produce heterocysts or akinetes (Corbett and Parker 1976). McGrath et al. (1978) recovered viable cells from 39 of 106 strains tested. Freeze-dried *Nostoc muscorum* was reported with no observable reduction in viability after five years of storage (Day 2007). This technique has been adopted by some researchers to preserve those cyanobacterial strains with which this technique has proven to be effective. Some local strains of *N*. *commune* maintained at BIOTECH-UPLB, in the Philippines, have been successfully preserved by freeze-drying because the cyanobacterium is capable of reviving after about forty years.

C. Lyophilization (L-Drying) Technique

Lyophilization (L-drying/liquid drying) is a preservation technique wherein the microalgae to be preserved are not frozen but are maintained at temperatures just above freezing and at the liquid state, while moisture from the sample is quickly removed under vacuum (Tan Gana et al. 2014). It is a method of choice for preserving several microalgae since it has the advantage of longevity, i.e., 25-30 years for some bacterial and fungal strains, genetic stability, and ease of application. It only requires a small space for storage and is ideal for shipping. A study by Tan Gana et al. (2014) showed the suitability of liquid drying (L-drying), a modification of freeze-drying, wherein the pre-freezing stage is bypassed. The cell suspension is immediately dried in vacuum. L-drying had been successfully carried out in preserving nine prokaryotic and eukaryotic algal strains, viz., *Anabaena laxa* (BIOTECH 4003), *Anabaena variabilis* (BIOTECH 4004), *Aphanothece pallida* (BIOTECH 4005), *Fischerella ambigua* (BIOTECH 4006), *Gloeotrichia natans* (BIOTECH 4007), *Hapalosiphon welwitschii* (BIOTECH 4008), *Spirulina platensis* (BIOTECH 4012), *Hapalosiphon welwitschii* (BIOTECH 4017), and *Chlorella sorokiniana* (BIOTECH 4016), using fresh culture medium and 12% saccharose as the suspending medium. L-drying was found to be suitable for preserving some cyanobacteria, viz., *Anabaena laxa* BIOTECH 4003, *A. variabilis* BIOTECH 4004, *Aphanothece pallida* BIOTECH 4005, and *Gloeotrichia natans* BIOTECH 4007 using either fresh culture medium or 12% saccharose as the suspending medium. On the other hand, some strains of algae such as *Fischerella ambigua* BIOTECH 4006, *Spirulina platensis* BIOTECH 4012 and *Chlorella sorokiniana* BIOTECH 4016 failed to revive after L-drying. Results indicate that not all species of algae can be preserved by L-drying and that strains within a species may exhibit different reactions to the process.

Lyophilization (liquid drying or L-drying) minimizes the hazard of genetic drift, contamination, and accidental mislabeling, which may occur with continuous routine passage. The advantages of using this method include smaller storage space requirement, relative affordability, and reduced labor. It is also the best mode for shipping cultures.

Materials/Equipment:

Microalgal culture
Soft glass ampoules
Pasteur pipette (long-nose)
Air-gas crossfire torch
Cotton
Suspending medium (i.e., lamb serum)
Freeze dryer (VirTis SP Scientific Sentry 2.0)

Methodology:

Preparation of ampoules:

The glass ampoules must first undergo acid washing to remove organic residues and other chemical contaminants. The acid-washed ampoules will be rinsed several times using distilled water to remove excess acid. The ampoules will then be set aside for drying for 30 minutes. After drying, cotton plugs are placed in each ampoule, and will be sterilized using an autoclave at 15 psi (121°C) for 15 minutes. The sterile ampoules will be placed immediately in a drying oven set at 50-60°C for 24 hours to remove excess water and moisture.

Preparation of algal cell suspension:

The microalga is grown in 50-100 mL of appropriate medium such as BG-11 or Bold's Basal medium in a sterile 250 mL flask. The culture is incubated at 20-25°C for 10 days, or until the late log to stationary phase under continuous illumination (light intensity of 120-130 µmol photons m^{-2} s^{-1}) with periodic agitation. After incubation, the algal cells will be harvested via centrifugation at 3,500 rpm for 10-15 minutes. An aliquot of the harvested alga is suspended in a small amount of fresh culture medium to make a thick suspension of cells. Alternatively, the cells may be suspended in lamb serum (Corbett and Parker 1976) or skim milk (McGrath et al. 1978). Using a long-nose sterile Pasteur pipette, ~0.2 ml of thick algal suspension is aseptically transferred in sterile ampoules. The cotton plugs in each ampoule will be replaced with a new one and excess cotton to the level of the glass ampoule will be cut off using a sterile pair of scissors. The cotton plugs are then pushed down (~1 cm deep) the ampoule using a sterile glass rod. All the prepared ampoules must be kept and maintained at -60°C before lyophilization.

L-drying procedure:
Initially, the condenser of the freeze dryer is drained, and the valves are closed. Any excess condensate from the previous run of the equipment is wiped off to avoid contamination. The ports in each manifold of the freeze dryer are closed before plugging the equipment in the voltage regulator and to the main power supply. The condenser of the refrigeration unit is turned on and set aside until the condenser temperature falls below 4°C (indicated by the LED light for “OK”). The ports of the manifolds of the freeze dryer are examined for leaks before opening the vacuum pump. The drying process of the prepared organisms (in ampoules) will initiate after sufficient vacuum is achieved (100 m Torr). Each prepared ampoule will be placed in rubber stoppers attached to each port. After completing a cycle of the drying process, each port in the manifold is opened one at a time. Initially, a single port is opened to allow the vacuum to go down (<100mTorr) before opening the other port containing the other ampoules. To ensure that adequate vacuum is attained, the attached ampoules and ports will be monitored for any leak. The ampoules are then allowed to dry (~6 hours) at room temperature. After drying, the ampoules are sealed using an air-gas crossfire torch under vacuum and stored at 4°C in the dark. The freeze dryer is turned off by first closing the condenser, followed by the opening of a single port to relieve the vacuum.

Revival of cultures:
Aseptically open an ampoule by heating (using a Bunsen burner) for a few seconds at the end point, which is away from the dried algal cells. The heated end is immediately sprayed with 70% ethanol until it cracks. The glass ampoule is gently tapped on the mouth of a sterile beaker or the edge of an open sterile Petri dish to open the ampoule. Alternatively, the middle portion of the ampoule is scoured with the aid of a file or ampoule cutter. The surface of the ampoule is sterilized with 70% ethanol-dampened gauze. The tube is wrapped with sterilized layered gauze and the ampoule is broken gently. The dried cells in the ampoule are suspended using an aliquot of appropriate fresh sterile medium. Using a sterile long-nosed Pasteur pipette, the suspended cultures will be transferred in a test tube or flask with the fresh medium (~10 ml). Also, a portion of the suspended culture (about a drop) is spread on a suitable agar plate. Both the culture set up will be incubated at conditions appropriate for the growth of the algae.

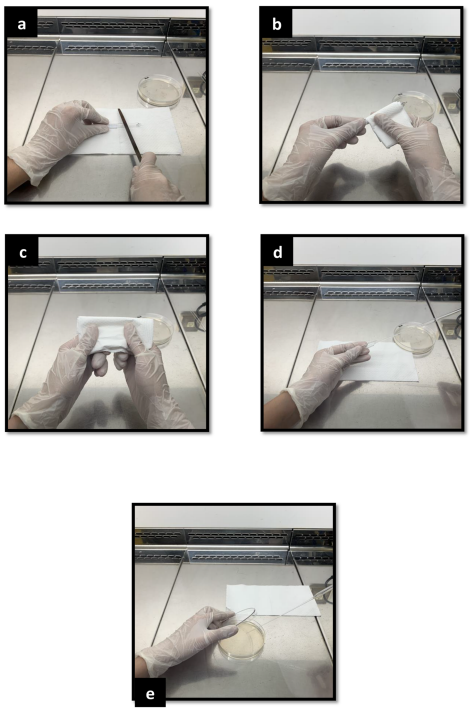

Figure 5.1: Reviving lyophilized cultures. a) Place ampoule on a flat surface and file at the neck; b) Wipe ampoule with cotton soaked in 70% ethyl alcohol; c) Wrap the ampoule with thick sterile cotton pad and break carefully at the neck; d) Suspend culture by adding 0.2-0.3 mL of suitable medium using a sterile Pasteur pipette; e) Spread an aliquot (about a drop) on a suitable agar plate and then put the remaining suspension in 10 mL of liquid culture medium. Incubate the cultures under prescribed conditions.

D. Cryopreservation Technique

Cryopreservation may be defined as the storage of a living organism, or a portion thereof, at an ultralow temperature, typically colder than -130°C, such that, it remains capable of survival upon thawing. Cryopreservation is still largely an empirical science because the underlying biological mechanisms of cell injury during freezing and thawing are not fully understood (Baust 2002; Rhodes et al. 2006; Day 2007; Youn and Hur 2009). Despite this limitation, hundreds of species of cyanobacteria and eukaryotic microalgae have been successfully cryopreserved. Table 5.2 presents a list of some cryoprotective additives (CPAs) used when preserving organisms by freezing.

Table 5.2. Cryoprotective additives (CPAs) used in freezing organisms*.

Blood serum or serum albumin	Peptone
Dextran, mannan	Polyvinylpyrrolidone (PVP)
Dimethylsulfoxide (DMSO) or (Me_2SO)	Polyethylene glycol
Ethanol	Proline
Ethylene glycol	Propylene glycol
Glutamic acid	Skimmed milk
Glucose	Saccharose
Glycerol	Sucrose
Glycine	Sorbitol
Malt Extract	Trehalose
Mannitol	Xylose
Methanol	Yeast extract

*(adapted from Day and Deville 1995; Day 2007)

Three penetrating **cryoprotectants (CPAs)** have been utilized quite extensively for algal cryopreservation, viz., methanol (MeOH), dimethylsulfoxide (DMSO; Me_2SO), and glycerol (Taylor and Fletcher 1998). Freshwater and terrestrial strains of microalgae cryopreserved at the Culture Collection of Algae and Protozoa (CCAP) and University of Texas Culture Collection of Algae (UTEX) have responded better to MeOH and DMSO than glycerol with MeOH as the more preferred CPA. Many marine phytoplankters are most effectively cryopreserved with DMSO, while glycerol is effective for *Tetraselmis* (Day and Fenwick 1993). A penetrating CPA lowers the temperature at which intracellular water freezes (Franks 1985) and reduces osmotically driven decreases in cell volume. In addition, permeating CPAs may confer cryoprotection by altering membrane properties such as solute permeability (Santarius 1996). Ethylene glycol and formamide, which are rarely used as algal CPAs, as well as DMSO, may decrease the cell membrane permeability for ions and lower the membrane potential (Chekurova et al 1990). Penetrating **cryoprotectants** may also act as free radical scavengers (Benson 1990). Other effective CPAs are methanol, ethylene glycol, and serum or serum albumin. On the other hand, glycerol, polyethylene glycol, PVP, and sucrose are less effective. Glycerol is sometimes chosen over DMSO because it is less toxic.

The specific procedure and CPA used for a particular species or even a strain may be varied. Some trial and error may be required for new isolates to determine which procedure will give the best results.

Materials/Equipment:

Microalgal culture
DMSO (10%)
Membrane filter (0.22 µm)
Cryogenic vials
Pasteur pipettes (long-nose)
Tong
Thick gloves
Liquid nitrogen tank
Biofreezer

Methodology:

Initially, the microalga is grown in a 50-100 ml of appropriate medium such as BG 11 or Bold's Basal Medium in a sterile 250 ml Erlenmeyer flasks. The culture is incubated at 20-25^{o} C for 10 days (or until the late log to stationary phase), under continuous illumination (light intensity of 120-130 µmol photons m^{-2} s^{-1}) with periodic agitation. Before starting the preservation, the cryoprotectant (10% DMSO) is filter sterilized using a 0.22 µm pore size membrane. An aliquot (0.5 mL) of the algal

culture is placed in a sterile 2.0 ml cryogenic vial. The 10% DMSO solution (100 µL) is added in intervals of 10 minutes, with gentle agitation after each addition, for a total of 500 µL in the vial. The vials are immediately closed and kept in in the dark to allow the alga-CPA mixture to equilibrate. The algal-CPA mixture is transferred to an ultralow temperature chamber for preservation.

Several variations on cryopreservation procedures are available. The success in applying each procedure depends on the species or strain for cryopreservation. Two or more procedures may work well for certain strains. Some variations in cryopreservation steps include:

A. directly placing 1.0 ml of the culture at the desired temperature of choice, either a -20°C, or, -80°C (biofreezer), or -170°C (vapor phase of liquid nitrogen). This is without CPA.
B. directly placing the cryogenic vial at the desired temperature of choice, either at -20°C, or -80°C (biofreezer), or -170°C (vapor phase of liquid nitrogen). This is with CPA.
C. placing cryogenic vials into a pre-chilled freezing canister (4°C) and transferring it into a -80°C **biofreezer**. After 90 minutes or when the algal culture has reached -55°C, plunge the cryogenic vial into -170°C (vapor phase of liquid nitrogen). The cultures can also be kept directly in the -80°C biofreezer after being slow-cooled to -55°C if liquid nitrogen tanks are not available.

Revival of cultures:
For the revival of the preserved algal cultures, the cryogenic vials are placed in a 35°C water bath for 5 minutes to allow thawing. The contents of the cryogenic vial are then transferred into 50 ml of appropriate growing medium and incubated for 2-3 weeks with continuous illumination at 20-25°C.

III. Summary

Four methods were presented for long-term maintenance and preservation of microalgae, viz., periodic transfer, freeze-drying, lyophilization (liquid drying or L-drying), and cryopreservation. Among these methods, periodic transfer is the most commonly used and the least expensive. Lyophilization and cryopreservation are the least popular, mainly due to the high cost of the equipment required. It must also be emphasized that there is no universal method for the successful preservation of all species of microalgae. Different taxonomic groups and even strains within a species exhibit significant differences in response to the stresses imposed by culture preservation and subsequent revival. Thus, in choosing the best technique for long-term preservation of microalgae, it is important to note the characteristics, kind, and type of microalgae to be preserved.

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Chapter 6. Immobilization of Microalgal Cells

Teresita R. Perez

I. Introduction

The ability of microorganisms to go into a state of dormancy has generated interest in immobilized cell technologies. This innovation implies the prevention of free movement of a biological material and has been applied to bacteria, yeasts, molds, algae, to cellular fragments and organelles, and to pure enzymes (Kierstan and Bucke, 1977; Lukavsky,1986; Hameed and Ebrahim, 2007). Studies have also shown entrapment of bacteria in alginate beads (Garde et al., 1981). More recently, immobilized algae have been developed (Hameed and Ebrahim, 2007; Moreno-Garrido, 2008).

Cell immobilization has been shown to be useful in the following: a) to present in a convenient form for commercial use, a single enzyme produced by and contained within the cells; b) to provide a highly concentrated source of viable cells; c) to bring together biochemical systems that would not normally coexist; d) to allow the medium-term storage of cells without further growth for commercial application; and, e) to preserve cell lines otherwise stored with difficulty through conventional means (Bucke, 1983). This technology has been gaining popular demand for application in several biomedical and industrial technologies (Santaella et al., 2013).

Cell immobilization appears to be particularly useful when individual enzymes cannot be extracted in an active form (Cheetham et al., 1979). Single cell enzymes of immobilized cells have application in industry for sugar transformation or production of organic acids, amino acids, or alcohol (Bucke, 1983).

Cell immobilization is a simple, cheap technique, and easier to accomplish than most enzyme immobilization processes, and yet, it is capable of producing materials suitable for use in large-scale industrial reactors. Successful immobilization criteria must meet a set of standards. First, the process, including the materials used, should not harm the operator and the consumer of the product. The process has to be simple in such a way that even non-skilled personnel can handle it. Second, cell viability must be maintained during the process of immobilization. These criteria ensure the immobilized cells are long-lived with a sustained low level of metabolic activity (Bucke, 1983). The process is inexpensive, requiring only sodium alginate, serving as the immobilization medium with minimal maintenance energy thus affording this process with a low carbon footprint. Various techniques have been utilized for immobilization, such as, covalent coupling, affinity

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immobilization, adsorption, confinement in liquid-liquid emulsion, capture behind a semi-permeable membrane, and entrapment (Mallick, 2002). Cells may also be immobilized by aggregation, adsorption onto a support material, or entrapment in gels. This involves various materials such as polyacrylamide, K-carrageenan and agar, calcium alginate, cellulose acetate and collagen. Cells usually retain their viability in such matrices (Travieso et al., 1996).

II. Algal immobilization

Immobilization of algal cells came about in the mid-1980s (Robinson et al., 1986). A limited number of studies using microalgae has been reported in literature wherein most of the studies were on freshwater microalgae (Lukavsky et al., 1986; Lukavsky, 1986; Bozeman et al., 1989; Abel Hamid, et al. 1991; Travieso et al. 1996; Hameed and Ebrahim, 2007; Moreno-Garrido 2008).

Applications proposed for immobilized algae include hydrocarbon production, removal of nitrogen and phosphorous from wastewater, and photo- production of ammonia and hydrogen (Robinson et al., 1986; Shi et al., 2007). Additionally, microalgae have also been immobilized for the removal of toxic substances from effluents (Travieso et al., 1996; Mallick, 2002; Moreno-Garrido, 2008) and for toxicity testing (Bozeman et al., 1989).

Immobilization of microalgal cells generally results in great improvement in the functional and storage stability of photosynthesis (Bucke, 1983). According to Lukavsky (1986), the metabolic activity of cells can remain constant and cells do not easily senesce. Their low metabolic activity can continue for a long time, consequently blocking other processes in their generation cycle. Entrapped cells are however subjected to a microenvironment wherein ionic strength or chemical composition may be different from free cells (Hameed and Ebrahim, 2007). The favorable storage characteristics of immobilized algae (Bucke, 1983) could allow distribution from central culturing facilities so as to relieve small laboratories of the burden of maintaining cultures of microorganisms in the traditional manner. Thus, this technology is cost effective, useful in culture management (Hertzberg and Jensen, 1989), and could enhance further standardization of different protocols.

The chloroplast content of immobilized cells has generally been found to be higher than that of free cells, probably because of self-shading and subsequent reduction in incident light in the immobilized state (Robinson et al., 1986). Photosynthetic oxygen evolution (Bailliez et al., 1985) was about three times higher in immobilized *Botryococcus* spp. than in free cells. Robinson et al. (1986) observed the mean respiratory rate of entrapped *Chlorella* to be lower than that of free cells. Immobilization might have some positive effect on the stability of protein-chlorophyll complexes of *Botryococcus* (Bailliez et al., 1985).

Although several carriers can be used for immobilization of microalgae, agar and alginate are more commonly utilized (Lukavsky, 1986). In using the agar, different forms may be produced, such as, cubes, pellets, layers with an inner framework, or homogenous agar films with immobilized algae in tubes. Alginate is however preferred for the entrapment of cells. Different forms of alginate carriers have been tried by Lukavsky (1986) and among them, the bead-like shaped pellet is more commonly used (Bozeman et al., 1989; Hertzberg and Jensen, 1989; Abel Hamid et al., 1991).

Cell immobilization in calcium alginate gels is a safe, fast, mild, simple, inexpensive, and versatile technique. Alginate is acceptable as a food additive and no toxic nor corrosive materials are utilized (Bucke, 1983). Alginates of high viscosity appear to be ideal for gel entrapment (Hertzberg and Jensen, 1989) since there is gelification without covalent bonding (Bucke, 1983). The alginate is mixed with an appropriate amount of medium and then, sterilized. The algal suspension is transformed into pellets which are rinsed with water and stored in the appropriate medium. Recovery of the algae involves suspending the pellets in a dissolving medium, viz., sodium polyphosphate (Abel-Hamid et al., 1991).

Methodology
Following below is the protocol adopted at the Department of Environmental Science, Ateneo de Manila University, Loyola Heights, Philippines.

A. Culture of microalgae
Microalgal isolates are maintained in the Algal Culture Laboratory of the Department of Environmental Science, Ateneo de Manila University, Loyola Heights. The microalgae are separately grown in Erlenmeyer flasks containing BG-11 medium at a temperature of 28°C-30°C under a 12 dark and 12 light cycle with an illumination of 600 *u*mol/m^2/sec.

B. Algal growth monitoring
The growth curve of each isolate can be monitored using a UV-vis spectrophotometer. The wavelength to be used for each species will depend on the highest peak of absorbance of the microalgae on the tenth day of its growth. The observed absorbance will be used to monitor the growth curve of the microalgae for 20-25 days or until it reaches the death phase.

An alternative protocol in algal growth monitoring is through cell counting using either a haemocytometer or a Sedgewick counting chamber. This method of monitoring growth can also check the status of the microalgae and examine for possible contamination.

C. Immobilization of microalgae
Alginic acid (Sodium salt, Sigma No. A-7128) is used as the carrier of the microalgae. Five grams of sodium alginate is dissolved in 100 ml of BG-11 medium and mixed thoroughly using a clean spatula to form a homogenous solution. The alginate solution is then autoclaved for 15 minutes at 10 psi and cooled down to room temperature.

The selected microalga in its exponential phase is centrifuged to come up with a cell density of 3 x 10^7 cells/ml. The microalga is then added to the sterile alginate solution and mixed thoroughly to attain an even distribution of the organism.

A 0.6 M $CaCl_2$ solution is prepared in two flasks, with the temperature maintained at 4°C. These flasks are placed in a small clean basin with ice water to further maintain the temperature at 4°C.

The algae-alginate solution is then poured into a sterile 20 ml syringe and its plunger slowly pressed down so that the alginate solution would form drops of beads in the cold $CaCl_2$ solution. The alginate beads are then transferred to a fresh solution of the cold $CaCl_2$ and kept in it for about 15 minutes. The beads are then transferred into a sterile flask with BG-11 medium, sealed with cotton plug, covered with a piece of aluminum foil, and stored in the dark at 4°C.

D. Recovery of immobilized microalgae
The flask with alginate beads is decanted. A 2% sodium polyphosphate solution is then poured into the flask with alginate beads to dissolve the beads. The flask is placed on a magnetic stirrer to hasten the dissolution of the beads and to eventually release the immobilized microalgae. This step takes about 20 minutes.

The microalgae are then separated from the solution using a vacuum pump. If the alga is small, e.g., *Chlorella*, a 0.4*u*-in pore size filter paper should be used. After separation of the microalgae from the solution, rinse twice with distilled water to completely remove the sodium polyphosphate solution.

Distilled water is used to wash off the microalgae from the filter paper and transferred to a flask with freshly autoclaved BG-11 medium. Expose the microalgae to a temperature of 28-30°C under a 12 dark and 12 light regime with an illumination of 600 *u*mol/m2/sec.

III. Results and Discussion

Algal beads were successfully formed from the developed protocol. The immobilized algae were tested for its viability by exposing them under continuous illumination for three days after their transfer in flasks with BG-11 medium. A distinct green color of the beads was observed and the greenness of the beads intensified on the sixth day with continuous exposure to light (Fig.6.1-Fig. 6.3).

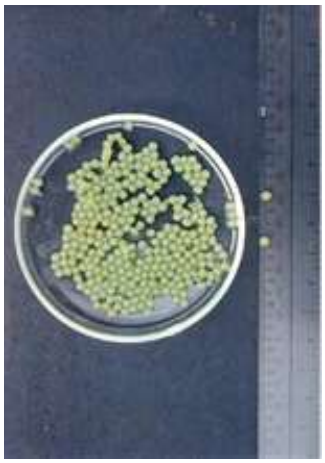

Figure 6.1: Algal beads containing *Chlorella.*

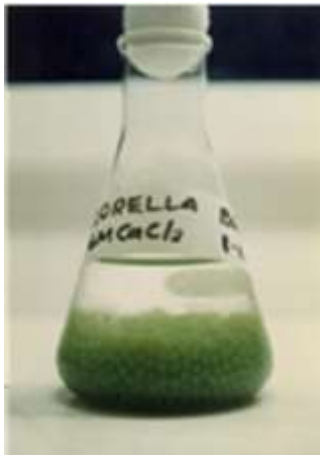

Figure 6.2: Cells of *Chlorella* immobilized in sodium alginate beads.

A. *Chroococcus*

B. *Scenedesmus*

Figure 6.3: A and B: *Chroococcus* (A) and *Scenedesmus* (B) in sodium alginate beads.

Upon dissolution of the algal beads in sodium polyphosphate and thoroughly rinsing the freed algae in distilled water, all the isolates, except for *Anabaena variabilis*, were viable. This strain of *Anabaena* was observed to be sensitive to sodium polyphosphate.

This immobilization technology using sodium alginate is an inexpensive and simple technique. Sodium alginate extracted from kelps, that grow in temperate waters, is a polymer of D-mannuronic acid and L-glucuronic acid. In the Philippines, the source of alginate is the genus *Sargassum*. *Sargassum*-based alginate is processed at the Seaweed Section of the Marine Science Institute of the University of the Philippines in Diliman.

Immobilization of algae in an inert matrix for a long period will not require illumination and continuous change of medium. This frees the laboratory from the tedious task of maintaining cultures of several stocks of microalgae. This also reduces the manpower requirement in algal culture room. This protocol not only provides readily available organisms for taxonomic purposes or for toxicity tests, but also, for culture collection because of the simplicity of the immobilization

procedure (Lukasvky, 1986). Immobilized algae compared favorably to alternative microbial testing (Bozeman, (1989).

The beads were relatively firm outside with a jelly-like consistency inside. This condition allows space for the microalgae to further undergo cell division. The motile *Chlamydomonas globosa* was transformed into the palmella stage when immobilized using sodium alginate. The inner jelly-like consistency allowed further cell division of the microalgae (Abel-Hamid et al., 1991).

Sodium polyphosphate proved to be a strong dissolving medium for *Anabaena variabilis*, an isolate from Agos River, Quezon Province, Luzon, Philippines. It survived immobilization but upon dissolution of the algal beads in sodium polyphosphate, *A. variabilis* was not viable. An appropriate concentration of this dissolving medium should be studied per species as there can be differential sensitivities per algal species. The minimum concentration and exposure time to sodium polyphosphate should be taken into consideration, too. All the other algal species, with the exception of *Anabaena variabilis*, remained viable after release from the beads.

IV. Summary

The immobilization of microalgal cells was successfully demonstrated using sodium alginate as a carrier in BG-11 medium. Algal beads were formed following the earlier described procedure. The immobilized algae for all of the species tested, except *Anabaena variabilis*, were found to be viable after their release from the alginate beads.

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Chapter 7. Production of Microalgal Paste in the Philippines

Milagros R. de la Peña[1]* and Annie V. Franco[1]

I. Introduction

Microalgae are used as **live feed** in the early life stages of mollusks and crustaceans. In some cases, the microalgae are fed to zooplankton, like rotifers and copepods, while the latter serve as food for the early life stages of finfishes. In aquaculture hatcheries, the use of fresh live microalgae is a major constraint because it involves 30–50% of the cost and labor of hatchery operations. It is a basic component of the hatchery system that covers more than 50% of tank facilities since a ratio of 1:2 larval rearing and algal food tank is followed. The culture of live food remains the bottleneck of hatchery operations since it is affected by the season because culture crashes are often encountered during the rainy season. The transition from a multi-step batch culture to a semi-continuous culture system requires a culture period of one month prior to larval rearing operations. This system is costly and requires a longer period to reach the desired volume of culture in the hatchery. Also, the continuous culture of live food is done even during the off-season to avoid a shortage of live food for the succeeding operations. Due to the difficulty of producing good quality algae, alternatives were searched, such as **microalgal concentrates** in **paste form**, **dried** and **preserved microalgae,** and algal substitutes in the form of **dried diets**.

The use of the **microalgal paste** is a new approach in hatchery systems to avoid the logistical problems encountered in the continuous culture of algae. Because of the wide application of microalgal concentrates in aquaculture, several methods to concentrate microalgae have been tried to simplify hatchery operations. The use of the **centrifugation method** to produce microalgal paste was successfully tried by Brown (1995), D' Souza et al. (2000); Bonaldo et al. (2005) and Nunes et al. (2009). The application of *Chaetoceros calcitrans* paste to shellfishes was done by Nell and O'Connor (1991); McCausland et al. (1999); Heasman et al. (2000) and Poniset et al. (2003). **Chemical flocculation** of microalgae by adjusting the pH was tested by Aujero and Millamena (1979); Millamena et al. (1990); Brown et al. (2002); and Harith et al. (2009). Furthermore, the use of **concentrated diatoms** was successfully tested on *Penaeus monodon* larvae (Millamena et al. 1990). Naorbe et al. (2015) showed that *C. calcitrans* paste could replace the protein, vitamin and mineral component of a *Penaeus monodon* diet. De la Peña et al. (2018) optimized the mass culture of *C. calcitrans* before paste production to attain a lower cost of USD 8.24 kg^{-1}. The cost and return of paste production were presented based on Samonte et al. (1993).

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The use of **microalgal paste** is advantageous in remote hatcheries with no microalgal culture facilities. It is convenient because it is categorized as “off the shelf” and can be stored in refrigerated conditions.

About nine species of microalgae belonging to three classes, i.e., Bacillariophyceae, Prasinophyceae, and Chlorophyceae, are being used in the preparation of algal paste (Table 7.1; Fig. 7.1).

Table 7.1: Common microalgal species used in the preparation of algal paste.

Class	Species	Description
Bacillariophyceae	*Chaetoceros calcitrans*	Cells are solitary with a siliceous cell wall. Cell size 5–6 µm wide, 8 µm long; cultured in great quantity as larval feed for shrimps, shellfishes and copepods
	Skeletonema costatum	Cells occur in chains formed by strutted tubular processes arranged in marginal ring. Cell size 5–6 µm wide; cultured as larval feed for shrimps
Prasinophyceae	*Tetraselmis tetrathele*	Cells are solitary, motile and with four flagella; 10–12 µm in length. They appear grass green due to the presence of chlorophyll a and b in the chloroplasts; used as feed for shrimps, shellfish, rotifers and cladocerans
Chlorophyceae	*Nanochlorum* sp.	They are small coccoid algae that morphologically resemble *Chlorella* sp. but lack pyrenoid; 2–3 µm in dia; used as food for rotifers and can be easily cultured under tropical conditions.
	Nannochloropsis sp.	This is a marine coccoid form that resembles *Chlorella* with small cells (2–4 µm in dia), spherical to slightly ovoid in shape; has a high content of the polyunsaturated fatty acid eicosapentaenoic acid useful for marine animals. *Nannochloropsis oculata* is the most studied microalga used for rotifer culture.
	Chlorella spp.	Cells globular/ spherical or ellipsoidal unicells (2–12 µm in dia) found in fresh and marine waters. *C. pyrenoidosa*, *C. vulgaris*, *C. virginica*, and *C. sorokiniana* are the common cultured species; extensively cultured as food for rotifers.

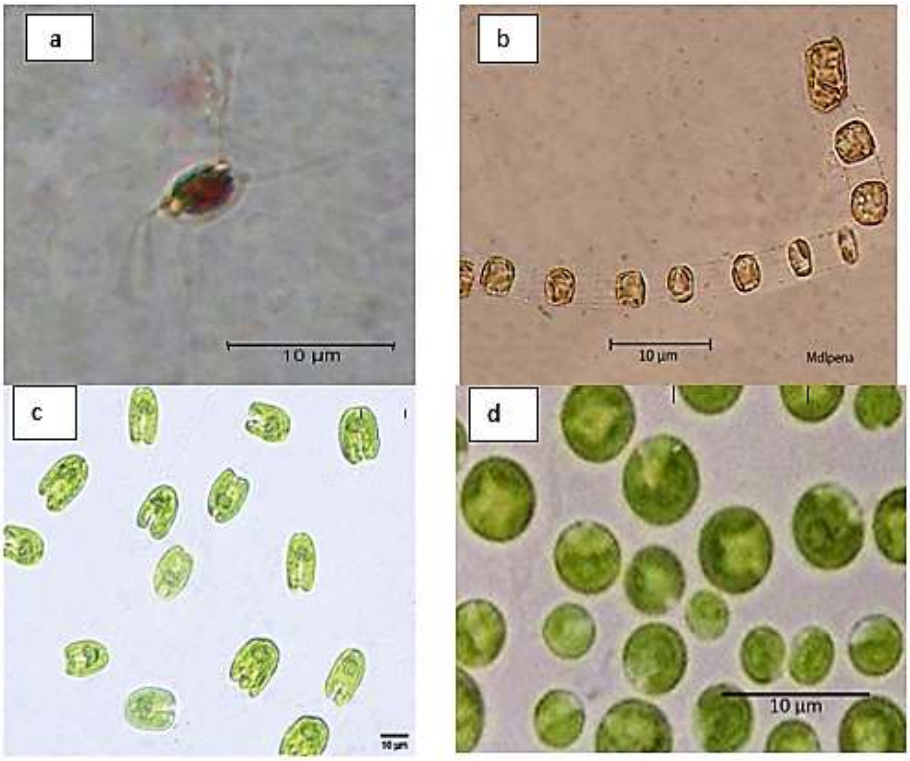

Figure 7.1: (a). *Chaetoceros calcitrans* (1000x), (b) *Skeletonema* sp. (1000x), (c) *Tetraselmis tetrathele* (1000x), (d) *Chlorella* sp. (100x)
[Photos 7.1a, b, d were taken from MR de la Peña and AV Franco (2013); while photos 7c was provided by MR de la Peña]

II. Methods of Concentrating the Microalgae

Initial studies on concentrated microalgae in the Philippines were done by Aujero and Millamena (1979) at the Aquaculture Department of the Southeast Asian Development Center (SEAFDEC/AQD) in Tigbauan, Iloilo. Several methods for the recovery of algae have been developed. These include **centrifugation**, **electric flocculation,** and **flotation** with **flocculants**. The application of techniques to concentrate algae varies depending on the quality of algal cells and operational cost.

Centrifugation
Concentrates are prepared by **centrifugation** with the use of a **cream separator** or **continuous flow centrifuge**. This method produced a concentration of 1:500 (Brown 2002). The use of a cream separator is limited due to a lower paste production capability. On the other hand, the continuous centrifuge is ideal since the quality of algae is retained, and it can process bigger volumes of culture. The high cost of acquiring this piece of equipment is one drawback in concentrating microalgae (Fig. 7.2).


MR dela Pena

Figure 7.2: Algal paste produced by centrifugation (left) and by flocculation (right)

Chemical Flocculation

Chemical flocculation is defined by D'Souza et al. (2002) as the coalescence of finely divided suspended solids into larger, loosely packed conglomerates. This technique is commonly used to remove unwanted particulate matter and can be used to concentrate algae. In the Philippines, chemical flocculation of microalgae was initially tried by Millamena and Aujero (1978) and Millamena et al. (1990) by adjusting the pH of the solution. **Flocculation** may be induced by the addition of floc-forming chemicals that allow the development of floc particles capable of removal by settling. **Alum** as hydrated aluminum sulfate and lime as calcium hydroxide are the two chemicals used for concentrating microalgae. Alum and lime are used as chemical coagulants because they are cheaper and have good floc-forming properties (Oswald and Golueke 1968; Pantastico 1976 in Millamena et al. 1990). The pH is adjusted prior to the addition of the coagulant. Using 100 mg L^{-1}alum, the pH is adjusted to 6.5 with the use of 1N H_2SO_4, and then pH is adjusted to 9.5 using 100 mg.L^{-1} lime and 1 N NaOH. After the addition of the chemicals, the alga is rapidly mixed to distribute the coagulant, followed by slow stirring to enable flocculation to take place. The algal floc is allowed to settle until a clear supernatant is observed. The supernatant is then discarded, and the algal slurry neutralized to the initial pH with 1 N NaOH (alum flocculation) and 1 N lime HCl (lime flocculation). The algal concentrates are then placed in plastic bags and stored in a refrigerator (5–10°C) or in a biofreezer (-20 to -22 °C) without a cryoprotectant. Chemical flocculation is effective for *Chaetoceros*, *Tetraselmis* and *Skeletonema*, but not for *Isochrysis*. The use of NaOH successfully maintains the viability of *Chaetoceros calcitrans* after 18 months of storage in a bio-freezer. The use of alum results in poor growth of *Skeletonema costatum* after two months of storage (Aujero and Millamena 1981). This method can concentrate microalgae up to 100x (Brown, 2000).

Electrolytic Flocculation

Electrolytic flocculation or **'electrolysis'** is based on the principle of the movement of electrically charged particles in an electric field. The microalgae have a negative surface charge, which causes them to be attracted to the cathode during the electrolysis of the algal suspension. Once they reach the cathode (+), they lose their charge, which makes them able to form algal aggregates (Aragon et a1. 1992; Poelman et al. 1997). The electrolysis of water refers to the production of hydrogen and oxygen gas at the electrodes. The bubbles produced at the anode (oxygen) rise to the surface, taking with them the algal aggregates (flocs), which can be skimmed off easily. This method has many advantages over the conventional process of concentrating the algae. It is cheaper, and unwanted particles found in chemical flocculation are eliminated. Harvest and concentration of microalgae are carried out in 200–1000 L fiberglass (FGT) oval tanks. For the 200 L setup, six flat rectangular cathodes consisting of lead (52 cm L x 16 cm W) are installed along the two opposite sides of the tank, while the three aluminum cylindrical tubes (61 cm H x 2.5 cm R) as the anode are placed in the middle portion of the tank supported by a wooden frame (Fig. 7.3). Both the anodes and electrodes are installed in the tank after 48 h when the cultures reach the logarithmic phase of growth. They are connected to a 12-volt car battery (Fig.7.4).

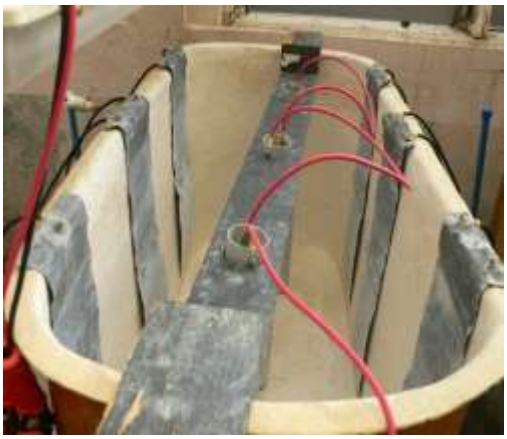

Figure 7.3: Electrolytic flocculation FGT set-up (200L) with lead sheets as cathode and aluminum tubes as anode.

Figure 7.4: Car battery as a source of power for electrolytic flocculation setup.

After one h, the algal aggregates rise to the surface and will be collected using a plastic dipper and allowed to settle in a 35 L capacity cylindrical plexiglass tank (Figs 7.5 - 7.6). The excess water will be removed by passing the concentrate through a 45µm mesh net lined with a wooden box. This method can concentrate microalgae up to 200x (de la Peña et al. 2018).

Figure 7.5: *Chaetoceros calcitrans* aggregates in plexiglass for settling.

Figure 7.6: Settled *C. calcitrans* aggregates in plexiglass.

II. Preservation of Concentrated Algae

The usefulness of microalgal paste lies in its acceptability as a larval food in the hatchery. The quality of concentrated paste must be retained to preserve its nutritional value and cell viability. The nutritional value of *C. calcitrans* paste concentrated through high-speed centrifugation was retained beyond eight weeks of storage (Heasman et al. 2000). The shelf life of algal paste produced from electrolytic flocculation can be extended by one to three months by storing it in a refrigerator with a temperature range of 4–10°C (Fig. 7.7).

Figure 7.7: *Chaetoceros calcitrans* paste stored in a refrigerator (4°C).

Low-temperature storage slows down the metabolic process but retains the viability of cells, including oxidative denaturation of essential vitamins and highly unsaturated fatty acids (HUFAs), autolysis, and microbial degeneration (Heasman et al. 2000). The recovery and activation of cells are delayed by a longer period of storage. The algal paste produced from chemical flocculation can be frozen at 20-22°C using a bio-freezer. The concentrate can be neutralized to the initial pH before freezing. The use of non-toxic preservatives, as butylated hydroxytoluene (BHT), can preserve *C. calcitrans* concentrate for one to two months (de la Peña and Franco, pers. com). The algal slurry can be treated with a cryoprotectant like glycerol and dimethyl sulfoxide (DMSO). The use of glycerol maintains the viability of *Tetraselmis chuii* after four months of storage but was not successful for *Isochrysis galbana* (Aujero and Millamena 1981).

After storage, viability testing will be conducted by culturing the concentrates under laboratory conditions at a temperature of 21-24°C, with continuous lighting and aeration. Cells that will be able to undergo cell division are considered viable. The algal flocs should be capable of being resuspended to be acceptable to planktonic feeders.

IV. Application of Algal Paste

Numerous studies were conducted on the use of algal concentrates in the early 1970s. The commonly cultured diatoms like *Skeletonema* and *Thalassiosira* were concentrated, frozen, and successfully fed to penaeid shrimp (Mock and Murphy 1970; Millamena and Aujero 1978), while the Centre Oceanologique du Pacifique (AQUACOP) in Tahiti used the concentrated green phytoflagellate, *Tetraselmis* sp. (AUACOP Tahiti 1977). Lime-flocculated and frozen *Chaetoceros* and *Tetraselmis* fed to *P. monodon* larvae resulted in good survival at the zoea stages (Millamena et al. 1990). Concentrates fed to larvae and spat of Sydney rock oyster (Heasman et al. 2000) and Pacific oyster (McCausland et al. 1999; Brown and Robert 2002) were effective as partial diets (e.g. up to 80%), with growth rates similar to, or marginally inferior to complete live diets. Concentrated *Chaetoceros calcitrans* in combination with *Pavlova lutheri* increased the length of Sydney rock oyster (*Saccostrea commerciales*). The diatom was stored for 7 to 14 d at 4°C before feeding (Nell and O'Connor 1991). Recently, the study of D'Souza et al. (2002) used flocculated algae concentrate as feed to tiger prawn *Penaeus* larvae. Due to the high concentration of algal paste, feeding is done by diluting the paste with seawater and exposing the suspension to strong aeration to reduce the aggregate forms, whereas live (non-concentrated) microalgae are fed directly to zooplankton or other crustaceans, which is similar to the dried form of microalgal feed.

V. Future Research

The wide application of concentrated microalgae in aquaculture hatcheries can be viewed under different perspectives: as a source of starter for remote hatcheries with a shorter start-up cultivation process or as direct live feed to reduce hatchery culture facilities. During the off-season, culture can be stopped, and the algae can be concentrated, stored and used for the next operating cycle. All of these can increase efficiency in running the hatchery and at the same time reduce the cost of operation.

However, there is a need for further study on how to retain the nutritional quality of algal concentrates for longer storage periods. The growth of the Pacific oyster (*Crassostrea gigas*) was adversely affected when fed with 8-36-day old algal paste of the following microalgae: *C. calcitrans, Pavlova lutheri* and *Isochrysis galbana* (Brown 1995). The ascorbic acid content of the microalgae was reduced by 29% when stored at 4°C for 21 days (Brown 1995). However, *C. calcitrans* concentrates, stored at a shorter period of time than 36 days, were successfully used as artificial diets (D'Souza et al. 2000). Furthermore, the use of cryoprotectant needs further evaluation due to its high cost and toxicity.

VI. Summary

Technical advances have made possible the production of microalgal concentrate in paste form. *Chaetoceros calcitrans* was concentrated and made into an algal paste through centrifugation, chemical flocculation, and electrolytic flocculation. The paste can be stored up to three months when refrigerated, and is diluted with seawater for feeding to the tiger prawn, *Penaeus monodon*. This can also be done with other cultured diatoms, and they can be fed to other penaeid shrimp and other organisms such as the Sydney rock oyster and the Pacific oyster. The use of algal paste can simplify hatchery operations and reduce costs among hatcheries with no microalgal culture facilities.

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Chapter 8. Preparation of Diatoms for Light and Electron Microscopy

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I. Introduction

Diatoms are unicellular to colonial forms of algae that belong to division **Bacillariophyta** (Round et al. 1990). One of their unique features is that their cell walls (or **frustules**) are made up mostly of silica, with fine, delicate and intricate markings and other structures that are constant for a species. Therefore, it is necessary to bring out the best of their cell wall features in doing diatom taxonomy. Hence, this chapter will emphasize various methods of removing the cell's protoplast to view clearly the frustules of the diatoms, aside from presenting the various methods in preparing the diatoms for viewing under the light microscope and the scanning electron microscope.

II. Preservation of Samples

Immediately after collecting field samples of diatoms, it is best to preserve the diatoms either in the dried or wet (liquid) state. Diatoms can be preserved in dried form by putting a drop of the

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sample on a mica or plastic sheet and be kept in an envelope. They should be stored in a dark, cool, low-humidity place and in aseptic conditions. The diatom collection of Walter Arnott stored in envelopes from the middle of the 19th century was observed to be still in good condition in 2013 (Mann 2013). An important consideration in storing these samples for a long period of time is to place them in containers of minimal contamination. One of the reasons for such a precautionary measure is that diatoms are microscopic forms, hence there is greater danger of their being contaminated. Handling the samples should be in the same manner as the handling of axenic cultures.

The most common **liquid preservative** for diatoms is to add a small amount of formalin (a 37% aqueous solution of formaldehyde) to make a final concentration of 4% solution using filtered field water sample as the solvent. The samples may also be preserved in 70% ethanol (Patrick and Reimer 1966) except for the danger of dissolution of the siliceous parts of the cell walls, especially for the slightly silicified ones, and also after a long period of storage. Glass bottles or vials are preferred over plastic ones for long storage for the same reason, that there is an alleged dissolution of the samples in plastic containers.

III. Cleaning of Diatoms for Light and Scanning Electron Microscopy

Taxonomy of diatoms has long been done based on the morphology of their siliceous **exoskeleton (cell walls** or **frustules)** mainly because of the constancy of these markings for each species. However, to examine the cell walls, it is usually necessary to remove the protoplast, a process called **cleaning**. One of the common methods of removing the protoplast is with the use of oxidizing agents, hence, the process is sometimes known as **oxidation of the protoplast**. The process is also known as **acid treatment** because some of the oxidizing agents used are strong acids, such as sulfuric acid. **Digestion of the protoplast** is also used to define this process because bacteria can also decompose and in turn “digest” the protoplast. Four steps are usually followed in the cleaning of diatoms in preparation for light and electron microscopy: a) washing off the preservative, b) cleaning the protoplast, c) washing away the cleaning reagents with distilled water, and d) dehydration.

A. Washing Off the Preservative

If the diatom samples are stored in preservative, the preservative must be decanted and the diatom samples washed with several changes of distilled water until no smell of the preservative is observed or until the pH of the solution becomes neutral. If the acids in the preservative are left, the mounting medium might decay prematurely. Another way of washing away the preservative is by adding concentrated hydrochloric acid or nitric acid very slowly by means of a medicine dropper (keeping the end out of touch with the diatom sediment), and leaving the sample until frothing ceases. The samples should be washed with several changes of distilled water. This latter method removes calcium salts which could become insoluble calcium sulfate if left in the sample. Washed samples should be placed in small glass containers.

B. Cleaning or Digestion of the Protoplast

The siliceous cell walls of diatoms are insoluble in acids (pH 1-7) but are soluble in alkaline solutions (≥pH 7.0). However, some diatoms, like the tropical species, have very thin siliceous cell walls. In this case, they may not be **cleaned** but instead, the diatoms will just be washed several times with distilled water to remove some of the clinging debris, and the salt solution in which they were collected. They can then be mounted as soon as possible, either for light or electron microscopy.

Cleaning may be done using three different methods, such as: **1) biological**, **2) burning or incineration**, and the **3) use of chemicals**. Table 8.1 summarizes the differences among these three methods.

Table 8.1: Distinguishing features of the three major cleaning methods of the protoplasts of diatoms.

Method	Definition	Advantages	Disadvantages
1. Biological	The use of decomposing bacteria to mineralize the protoplast	Useful in cleaning delicate diatoms; keeps the frustules intact.	A slow and unsure process
2. Incineration	Burning of the diatoms on a glass cover slip in a hot flame or on a hot surface	Fast method; intended for small and delicate diatoms; preferably used for unialgal diatoms.	Needs considerable skill in getting good mounted diatoms
3. Chemical	Cleaning the protoplast with the use of strong acids; hence it is an oxidative process	Wide selection of chemicals to use for different diatoms and for whatever purpose the diatom sample is required; chemicals are easily available.	Some of the strong acids may be explosive.

In the **biological method**, decomposing bacteria are involved in the mineralization or digestion process of the protoplast (Round et al.1990). Actually, this usually happens when the samples are left unpreserved for some time, and what remains in the samples are only intact frustules. This method has the advantage of leaving the frustules intact.

Burning or incineration involves heating the diatom material on the glass slide or on the cover slip in a muffle furnace to 500-600^{o}C for about 10-60 min depending on the thickness of the frustules (Round et al. 1990). This method needs considerable skill. It is usually employed to clean small and delicate diatoms and those that are not mixed with other algae and do not contain bits of the substrate.

Procedure:
The specimens from which preservatives were washed off are air-dried on the cover slip or glass slide. The cover slip, with the diatom samples, is now placed on a piece of tin plate (usually the cover of a powdered milk or powdered chocolate milk container may be used). The above setup is heated carefully on a hot plate or a Bunsen flame until it turns an ash gray or brown in color and ceases to smoke or bubble. This sample is now ready to be mounted. The sample in this incineration method can be pretreated with chemical oxidation, like perchloric acid, if separation of the frustule portion is necessary (Von Stosch 1982).

The cleaning method using **chemicals** utilizes chemicals that are usually **strong mineral acids** or **oxidizing agents** or **strong acids**, such as **sulfuric acid, nitric acid, hydrochloric acid,** or **oxidizing agents**, such as **hydrogen peroxide.** This method of cleaning is also known as **oxidation of the protoplast.** Below are some of the chemicals used and the procedures in using them. Table 8.2 summarizes the distinguishing features of three common oxidizing agents in cleaning diatoms.

a) Concentrated sulfuric acid (H_2SO_4)
Concentrated sulfuric acid may be used in combination with potassium dichromate ($K_2Cr_2O_7$). This combination is the harshest cleaning reagent meant for diatoms that are heavily silicified. It also gives an explosive reaction, so it is necessary to be very cautious in using it. Fig. 8.1 shows the flowchart process in cleaning up to the mounting of diatoms for light microscopy, using this method.

Concentrated sulfuric acid can also be used with crystals of potassium permanganate ($KMNO_4$). The chemicals used in this combination are not as strong oxidizing agents as those used with potassium dichromate. However, it has the disadvantage of making some sulfates like $CaSO_4$, insoluble, and this could interfere in the observations.

b) Concentrated nitric acid (HNO_3)
An advantage of nitric acid is that it makes all nitrate compounds soluble, thus lessening the interference of these sediments with observation. The protocol used with this strong acid is to boil for a few minutes a mixture in the ratio 1:1:1 of the sample, nitric acid and sulfuric acid (Round, et al. 1990). Another method used with this acid is to leave the diatom samples in 70% HNO_3 in an oven at 60ºC for 24 hrs. Another variation is to add concentrated HNO_3 into washed diatom samples. The diatom samples are left overnight in capped centrifuge tubes in an oven at 60ºC. During this period, the samples should be disturbed occasionally.

c) Concentrated hydrochloric acid (HCl)
Hydrochloric acid (HCl) is another oxidizing agent used in cleaning diatoms. The diatom sample is mixed with 30% hydrochloric acid (HCl) at a ratio of 1:1. Let the mixture stand for a day, with occasional stirring to mix the sample (Franchini n.d.). Following the acid treatment, the sample must be washed thoroughly with distilled water to remove all traces of acid. HCL is useful in dissolving any calcium and iron compounds in the sample.

d) Peroxides - Hydrogen peroxide (H_2O_2)
This is a gentle oxidizing agent, when mixed with water, used especially for delicate and slightly silicified diatoms. It can also be used for cleaning siliceous cysts of chrysophytes and the loricas of the euglenophyte, *Trachelomonas* (John et al. 2011). Concentrated hydrogen peroxide (peroxide) (~30% v/v) at a ratio of 1:1, sample: peroxide for a 6 hr oxidizing reaction was the optimum protocol observed in cleaning diatoms by Blanco et al. (2008). However, in the Philippines the usual concentration of peroxide from drug stores is 3%. If this is the case, the ratio of sample to peroxide is adjusted, based on the type of diatoms, between 1:10 to 1:100. This procedure can also be made stronger by UV treatment (Round et al. 1990). There are two variations on the use of this oxidizing agent, i.e., 1) ordinary peroxide treatment, and 2) hot peroxide treatment.

1) Ordinary Peroxide Treatment, a method that is suitable for delicate diatoms (Belcher 1999).
Procedure (to be done in a fume hood):
A portion of the washed diatom sample is air-dried on the cover slip then further heated on a hot plate. A small amount of hydrogen peroxide (3% v/v) is added on the diatom sample (1:10-1:100, diatom sample: H_2O_2) using a glass eyedropper. The sample is covered with a Petri dish and left to dry for at least 6 hrs.

2) Hot Peroxide Treatment (modified from Bellinger and Sigee 2010)
Procedure (to be performed in a fume hood):
The diatom sample is mixed with commercial hydrogen peroxide at a ratio between 1:10 to 1:100, sample: H_2O_2, in a 250mL beaker. The mixture is left standing for 20 min, after which it is heated on a hot plate (at about 90°C) for 2 hours, keeping it topped with H_2O_2. The beaker is removed from the hot plate, after which a few drops of 50% HCl are added. This will release chlorine, and at the same time release surplus H_2O_2 and carbonates. The samples are allowed to cool at room temperature, then transferred to centrifuge tubes to wash away the oxidizing agents. Fig. 8.2 shows the flowchart process in cleaning up to the mounting of diatoms for light microscopy, using this method.

Table 8.2: Distinguishing features of some common oxidizing agents in the cleaning of the diatom's protoplast.

Chemical	Characteristic	Advantages	Disadvantages
1. Sulfuric acid	A strong oxidizing agent, especially when combined with $K_2Cr_2O_7$; Another combination is with $KMnO_4$.	One of the harshest cleaning reagents meant to clean diatoms that are heavily silicified. The addition of $KMnO_4$ is not as a strong oxidizing agent as with $K_2Cr_2O_7$.	Great care in using it because it is explosive; makes some sulfates insoluble, such as $CaSO_4$, when $KMnO_4$ is added;
2. Nitric acid	It is not as strong an oxidizing agent as sulfuric acid.	It makes all nitrates soluble.	
3. Hydrogen peroxide	A strong oxidizing agent but gentle when mixed with water.	Easily accessible chemical; relatively cheap; a gentle oxidizing agent when mixed with water, used for delicate and slightly silicified diatoms; can be made a stronger oxidizing agent with the addition of HCl.	Longer method than with the use of sulfuric acid

C. Washing after the Cleaning the Protoplast

After oxidizing the sample, it must be washed thoroughly to remove any trace of the oxidizing agents by repeatedly centrifuging and re-suspending in distilled water until a pH of about 7.0 is obtained. A few drops of weak ammonia may be added before the final wash to help in the removal of clay particles (Bellinger and Sigee 2010). Sometimes, for samples with delicate cell walls, like those from the tropics, the sample is repeatedly sedimented and washed with distilled water. However, this may take longer, so the samples must be covered to avoid contamination.

D. Dehydration

The water from the sample is removed by repeatedly centrifuging and re-suspending in a series of increasing alcohol solutions, 75% then 95% ethyl alcohol. The diatom sample solution should be clear but has many fine white particles (of diatoms) in suspension. It is often advisable to store the samples in small glass vials, adding a crystal of thymol to prevent fungal growth. The diatom samples can also be stored dry on pieces of mica or plastic. This sample is now ready for mounting and observation for light and electron microscopy.

IV. Preparation of Diatoms for Light Microscopy

A. Check for Density of diatoms in the sample.

The cleaned sample is checked for density by examining a temporary mount under the low power objective of any light microscope. The sample can be concentrated by decanting the suspending solution, or diluted with the suspending solution.

B. Mounting or Slide Making

Materials to prepare: glass slides and cover slips, mounting medium, heating agent, cardboard "fig", slide labels.

Procedure:

Glass slides and cover glasses or cover slips should be thoroughly cleaned with a cleansing reagent such as nitro-chromic acid solution for 2-3h. Chromic acid solution is a saturated solution of $K_2Cr_2O_7$ in a 1:1 mixture of nitric acid : sulfuric acid (H_2SO_4). These are then rinsed thoroughly with distilled water. They are immersed in 20% KOH for 1-2h, then rinsed thoroughly with distilled water. Slides are stored in acid alcohol (1 ml 1 N HC1 in 99 ml of 70% CH_3CH_2OH). If they are freshly taken from an unopened box they can be immersed in 70-95% ethanol to remove the oil film from their surfaces. Cover slips must be handled with forceps.

Mounting media are usually adhesives and at the same time sealants and preservatives, and are usually resins that are easy to handle with a high refractive index. Table 8.3 shows some of the natural and synthetic resins used as mounting media, together with some of their properties and current cost in the Philippines.

Table 8.3: Natural and synthetic mounting media for diatoms.

Mounting medium	Refractive Index	Solvent	Cost in the Philippines (Php)/Source, year	Reference
Natural 1. Canada balsam	1.522	Xylene	10,000.00/100mL, (BP Integrated, Calamba, 2018)	CANADA BALSAM.2019. Retrieved from https://www.sigmaaldrich.com/catalog/product/sial/c1795?lang=en&region=PH
2. Styrax	1.59			GUM STORAX, STYRAX.2019. Retrieved from https://www.sigmaaldrich.com/catalog/product/aldrich/w303704?lang=en&region=PH
Synthetic 1. Pleurax*	1.73	Isoprop-anol and acetone	Not easily available locally.	Hanna, 1949; HIGH-REFRACTIVE MOUNTING MEDIA (PLEURAX / NAPHRAX / ZRAX.2019. Retrieved from http://www.mikrohamburg.de/Tips/TE_Mountingmedia.html
2. Hyrax	1.65	Toluene, xylene, benzene	Not easily available locally.	Hanna, 1930
3. Naphrax= Zrax	1.65	Toluene	Not easily available locally.	HIGH-REFRACTIVE MOUNTING MEDIA (PLEURAX / NAPHRAX / ZRAX.2019. Retrieved from http://www.mikrohamburg.de/Tips/TE_Mountingmedia.html
4. Eukitt	1.5	Xylene	9,000.00/500mL (Nicolie, Los Baños,Laguna, 2019)	EUKITT ® QUICK-HARDENING MOUNTING MEDIUM.2019 Retrieved from https://www.sigmaaldrich.com/catalog/product/sial/03989?lang=en&region=PH

*Unsuitable as a mounting medium for UV-microscopy because wavelengths below 400nm are blocked.

The heating medium can be an alcohol lamp (spirit lamp), Bunsen burner or hot plate. An electric iron that is inverted and held in a bench stand makes an excellent hot plate as its heat can also be easily adjusted. It also has a flat surface where the cover slips can be placed directly.

A cardboard “fig” is built to mark the center of slides and cover glasses. Making centers coincide and uniformly prepared improves neatness and encourages careful work.

Types of slides that can be prepared

There are two types of slides that can be prepared, i.e. **“strewn” slides** and **“selected” slides**. **Strewn slides** or **strewn mounts** contain a mixture of diatoms from a sample. This procedure is the one commonly done in mounting diatoms, as discussed above. On the other hand **“selected slides”** are slides with specific kinds / species or parts of a frustule.

"Selected" slides
[Procedure based mainly on the personal experience of the scientists in the Diatom section of the British Museum of Natural History]

A small amount of the material is spread on a clean glass slide. The specimen to be mounted should be located under a low power objective of a light compound microscope. The specimens are then transferred from the glass slide to a cover glass with a bristle or a pig's eyelash glued to the wooden rod about the size of a pencil, then the point of the eyelash is used to manipulate the diatoms. Transferring can be easily done in a microscope with an erect image, e.g., a binocular dissecting scope.

The cover glass (slip) on which the diatom will be placed may be thinly applied with a coating medium to hold the specimen in place. Table 8.4 shows a sample recipe of a coating medium and its preparation (Beats 2015).

Table 8.4: Recipe of a coating medium adopted from Beats (2015).

I. Composition:	
Glacial acetic acid	25 mL
Isopropyl alcohol	12 mL
High quality white bovine gelatin, fine powder	0.3 g
II. Procedure: The coating medium is prepared by first adding the acid to the gelatin and heating in a warm bath. Then alcohol is added. The mixture is filtered through an ordinary filter paper while warm. A small amount is spread in a very thin layer over the cover glass and allowed to dry.	

After the diatoms have been transferred to the dry, coated cover glass, it is necessary to gently breathe on them to sufficiently melt the gelatin and hold the diatoms in place. The cover glass is inverted on the mounting medium and pressed gently, but not enough to disarrange the diatoms. If a harder mount is desired, the slide is heated.

C. Two Flowcharts from Cleaning to Mounting Diatom Frustules

1. I. Washing of preserved samples

3% buffered formalin solution + diatoms

↓

Wash several times in distilled water by centrifugation and alternately decanting the supernatant until no odor is noticeable.

↓

II. Oxidation/Cleaning of Samples

↓

Wash sample's sediment in a small test tube

↓

Slowly add concentrated sulfuric acid (H_2SO_4) until the frothing stops

↓

Heat in a water bath,10-20 minutes until solution turns brown to black

↓

Add a pinch of potassium dichromate ($K_2Cr_2O_7$) with a bamboo spatula until brown to black

↓

Heat in water bath, 10-20 minutes until brown to black

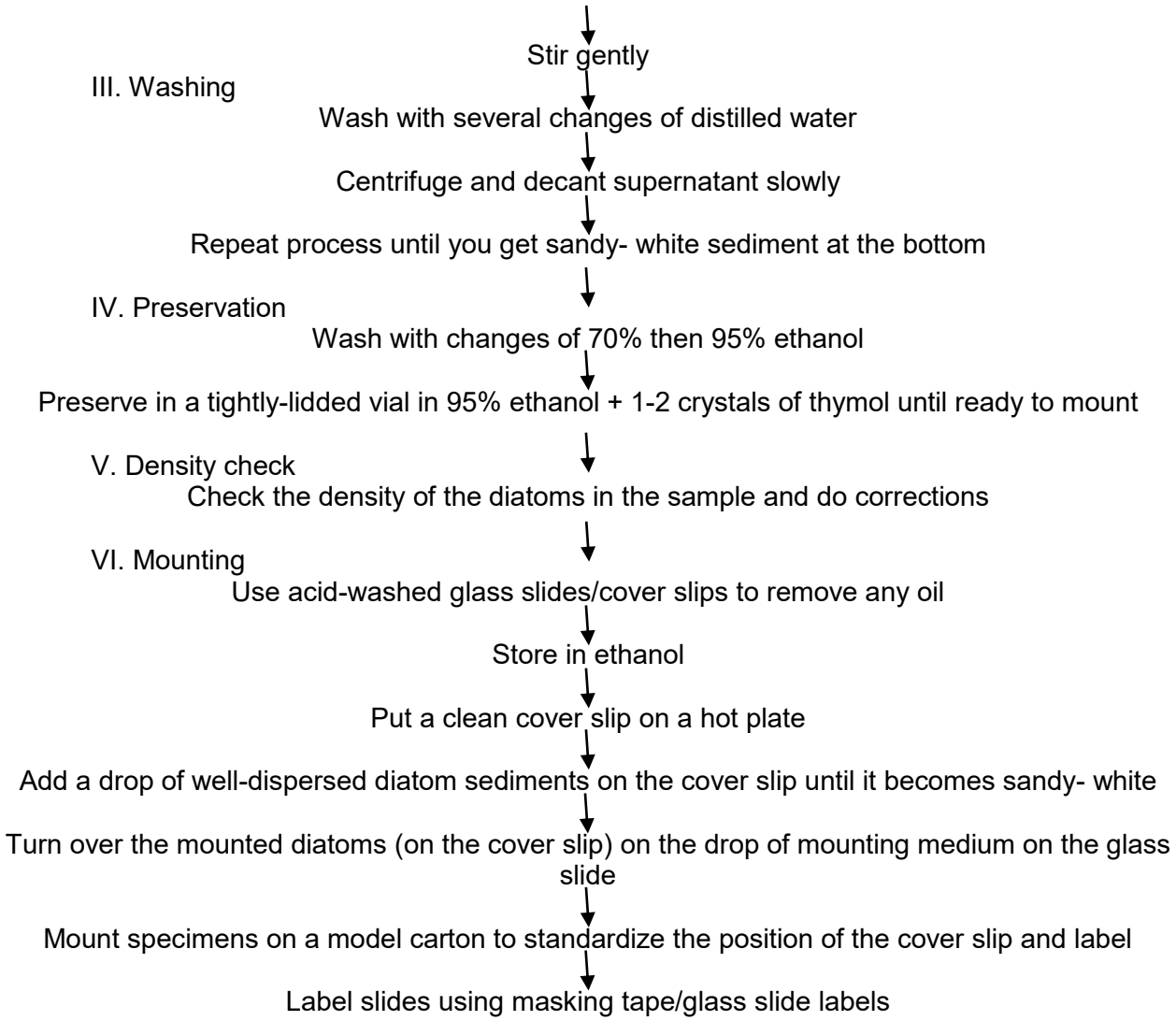
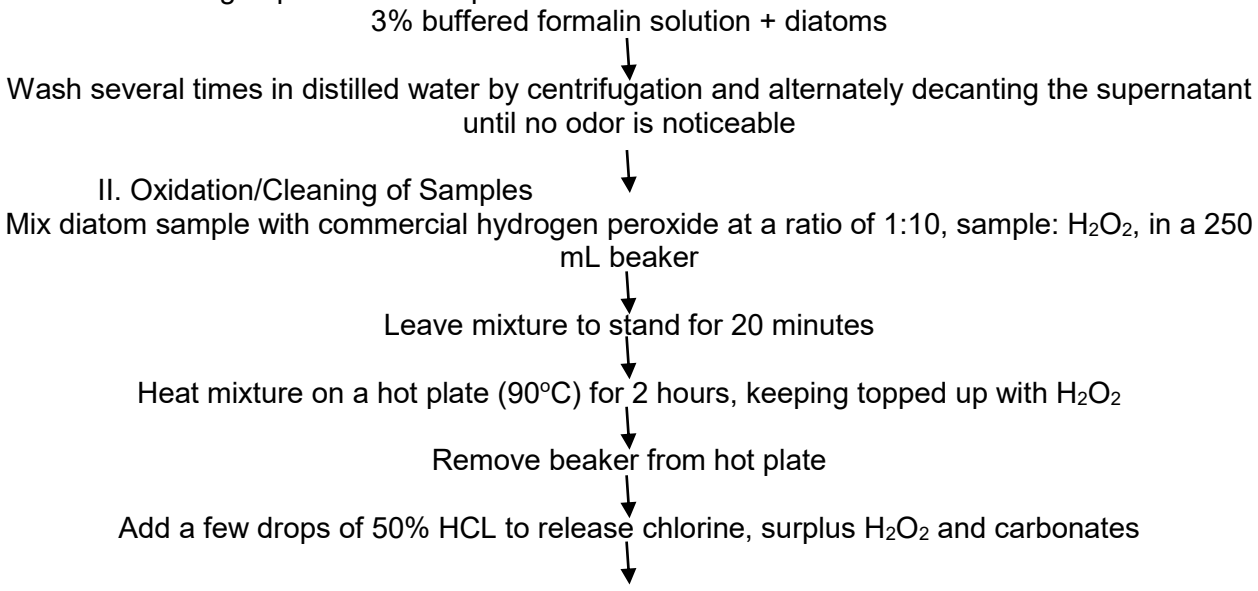


Figure 8.1: Flowchart in preparing diatom (frustules) slides for light microscopy using the sulfuric acid and potassium dichromate method of cleaning or oxidizing the protoplast.

2. I. Washing of preserved samples



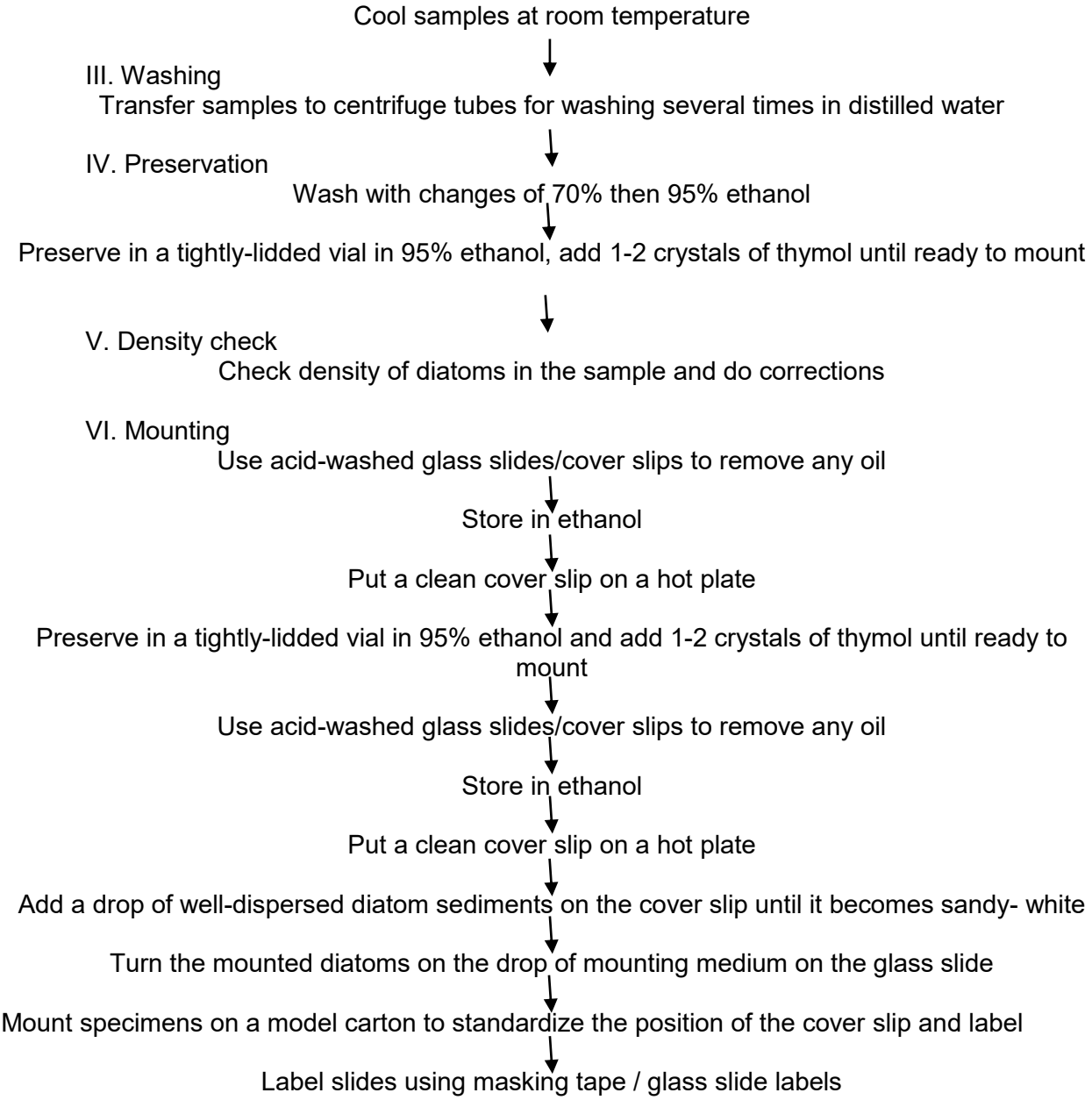


Figure 8.2: Flowchart in preparing diatom slides for light microscopy using the hot peroxide treatment in cleaning or oxidizing the protoplast (adopted from Bellinger and Sigee 2010).

Figure 8.3 shows the light micrographs of a pennate, heterovalvar diatom, *Achnanthes coarctata* (Brébisson ex Wm. Smith) Grunow. A live specimen of this diatom (uncleaned sample) shows the siliceous cell wall (cw) enclosing its protoplast (pr) that is filled with many oil globules (A). A partially cleaned sample (B). shows two cells in their girdle view attached at their valve faces. In girdle view the cells are longitudinally bent or appear geniculate. This figure shows the cell wall (cw) enclosing the chromoplasts (cr). The raphe valve or hypotheca of the cell is shown in C with the true raphe (a) and the stauros (b). In Fig. D the epitheca or pseudoraphe valve is in focused showing the pseudoraphe (a) and the striae (st).

D. Light micrographs showing differences between uncleaned and cleaned diatom cells.

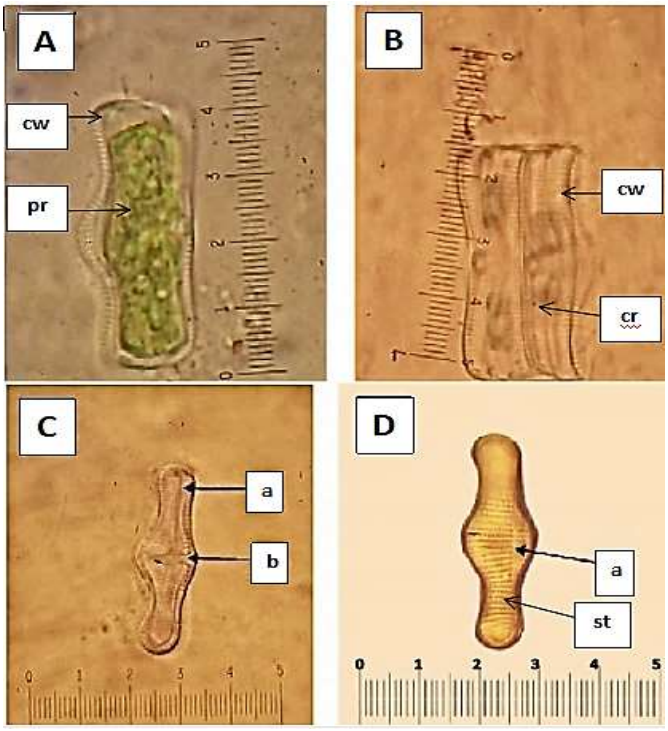


Figure 8.3: *Achnanthes coarctata* (Brébisson ex Wm. Smith) Grunow.
A. Live cell; B-partially cleaned cells;
C-D. Cleaned cells showing the frustules.

A. Solitary live cell in girdle view showing the cell wall (cw) enclosing the protoplast (pr);
B. Two cells, in their girdle view, united at their valve faces, showing the cell wall (cw) enclosing the chromoplasts (cr);
C. Cleaned cell in valve view showing the raphe valve or the hypotheca with conspicuous true raphe (a) and stauros (b);
D. Cleaned cell in valve view showing the pseudoraphe (a), hence, called the epitheca and the striae (st)

Light micrographs taken with an American Optical Microscope, Phycology Laboratory, University of the Philippines Los Baños (UPLB), College, Laguna, Philippines; (10 micrometer spaces= 10µm; x1000).

V. Preparation of Diatoms for the **Scanning Electron Microscope**

The diatoms mounted for the scanning electron microscopy (SEM) should be thoroughly cleaned, washing away the oxidizing agents with distilled water, dehydrated and kept in 95% ethyl alcohol as summarized above in the procedure for cleaning diatoms. The protocol of the Electron Microscopy Service Laboratory (EMSL) of the National Institute of Microbiology and Biotechnology (BIOTECH), University of the Philippines Los Baños, was followed in the preparation for SEM of the diatoms. For example, the cleaned, preserved samples in 95% ethyl alcohol were dried overnight in a vacuum oven and mounted on aluminum stubs using carbon tape. However, large specimens, like *Mastogloia*, do not need to be placed on supporting film. They can be selected with a bristle and placed by hand directly on the surface of the grid. Then the diatoms were subjected to ion coating using a gold-palladium target by sputtering or by evaporation in JEOL JFC 1100 prior to SEM imaging at 10kv, accelerating voltage using the secondary electron detector of Hitachi SEM S 510.

Fig. 8.4 shows a comparison of a light micrograph (A) and a scanning electron micrograph of *Achnanthes coarctata* (B) with its magnified parts in girdle view. One distinctive difference between the two micrographs is that under the light microscope the striae appear as rough lines (A-a) while under the electron microscope each line (stria) is actually made up of uniseriate poroids (B-b). The girdle bands (c) are observed in both micrographs except that in the electron micrograph (B) the girdle bands (c) appear to be made up of overlapping bands, which was not clearly shown in the light micrograph (A-c).

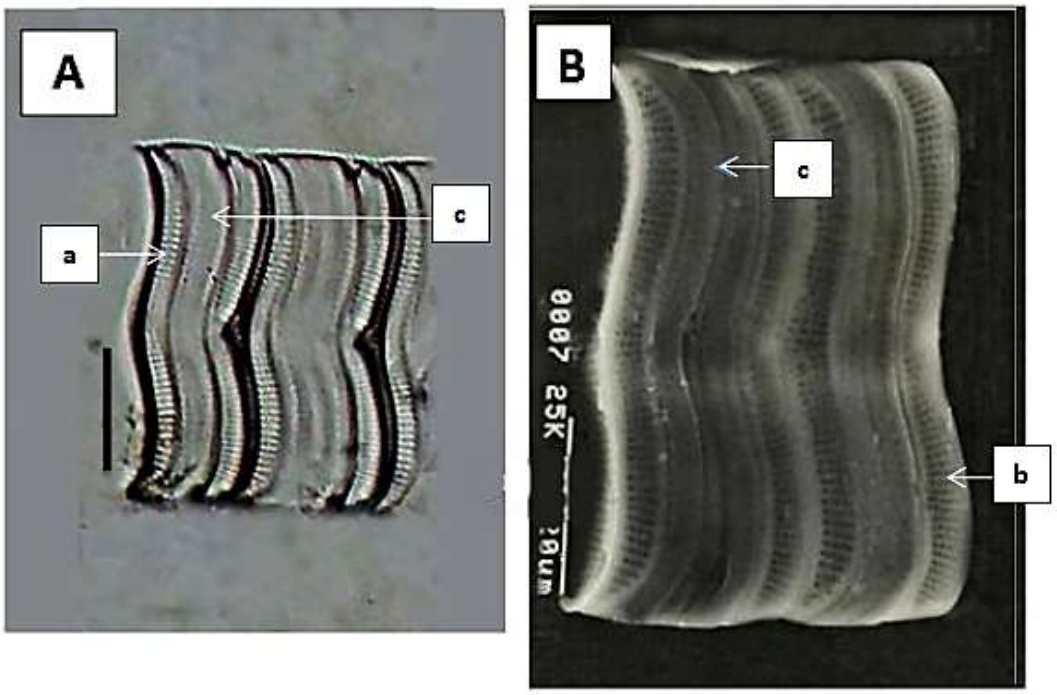


Figure 8.4: The diatom cell, *Achnanthes coarctata*, in girdle view when viewed under the light microscope (A) and under the scanning electron microscope (B).
The light micrograph (A) shows the transverse striations (a), like a continuous rough line (1000x), but under the scanning electron micrograph (B) the transverse striations are really made up of uniseriate poroids (b); c = girdle bands.
Scale bar in A=20µm.

VI. Summary

Taxonomic studies of diatoms have been based mainly on the fine structures of their siliceous cell walls (frustules). Hence, it is important to clean the diatom cells or remove the organic matter within them and/or the substrates with which they are associated to have a clearer view of the fine features of the frustules both under the light and the electron microscope. Three general methods of cleaning diatoms are: 1) biological means, 2) incineration, and 3) chemical treatment. A combination of any of the three can be employed depending upon the diatom material and the purpose of the study. After the diatom samples have been cleaned, they can either be separately processed for light microscopy or electron microscopy. A distinction was made between images taken of uncleaned and cleaned diatoms, and diatoms taken under the light microscope and the scanning electron microscope.

VII. Acknowledgments

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Chapter 9. Preservation in Fluids

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I. Introduction

Preservation in liquid or fluid means storage of specimens in liquid state of matter to protect them from subsequent deterioration. A colloquial term for this method is to **"pickle"** the specimens because the usual component in the solution is an acid, e.g., glacial acetic acid. Another common term is to have **"spirit" collections** because ethyl alcohol e.g., 70% ethyl alcohol, is one of the components. Preservation and fixation are terms that are often used interchangeably because some solutions can be used as both fixative and preservative. This chapter will attempt to differentiate these two terms. **Preservatives** are solutions that have germicidal effect and prevent autolysis, hence, providing the specimens an environment that is free from bacteria, zooplankters, and other contaminants so that in this way, the specimens are maintained in their near "normal" appearance for a long period. **Preservatives** are solutions that do not alter tissues, nor do they protect them from any changes. They, however, provide mechanical protection to the specimens during handling (Stoddard 1989).

On the other hand, **fixatives** are usually used in "killing" tissues or specific parts of cells or tissues. Fixatives have deep penetrating ability and, thus, their action is immediate. These solutions tend to coagulate and stabilize proteins in specimens, preventing distortion during preservation, study, and storage (Martin 2004). Additionally, fixatives usually raise the refractive index of the tissue, making it more susceptible to staining. However, they usually cannot be used for long-term storage, probably the longest acceptable storage time for the fixation process would be a maximum of 24 hrs (Martin 2004) otherwise, the specimen becomes brittle. Such is more likely the case when using the fixative, Bouin's solution which contains picric acid. A fixative and a preservative may be used together, for instance when processing diatoms for a glass slide preparation. Wherein, a 4% buffered formalin, the fixative, and 70% ethyl alcohol, the preservative, are both applied (see chapter 8 in this book). Some advantage of this type of preservation is that the chemicals are readily available, usually from local drugstores, and the procedure is simple to follow. However, the preserved samples require a large storage space. The specimens tend to deteriorate with prolonged storage. It is possible to prevent its deterioration with regular maintenance check and topping up the specimens with the preservative fluid.

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Specimens **preserved in fluids**, if done in a satisfactory manner, are readily available, as near as possible to freshly collected samples, for examination by students and researchers. They are also excellent materials for embedding in paraffin for sectioning. They can easily be stained to give contrasting colors of the different parts of a cell. In this manner, the internal parts of a thallus can be observed under the light or electron microscopes. If ethyl alcohol is used as a preservative it will even allow for DNA extraction. Some of the disadvantages in using this type of preservation are the need for a large storage space and a need for regular checking and topping up the fluid levels of the specimens.

II. Preserving Fluids

The most common types of fluid preservatives are formalin (formaldehyde), ethyl alcohol, iodine solutions, or a combination of any of these chemicals.

A. Formalin

One of the most common preservatives is **formalin**. Commercial formalin is sold as 37% formaldehyde in bulk, in most drug stores in the country. Formalin is an aqueous mixture of formaldehyde gas in water, with a little amount of methyl alcohol to prevent the formaldehyde from forming a solid mass through polymerization. It is considered to be the best preservative for freshwater algae (John et al. 2011). This is what is commonly used in the Phycology Laboratory at the Institute of Biological Sciences, University of the Philippines Los Baños (UPLB) because it is readily available and relatively inexpensive. It is also stable in less than 10% water solution (Kawanobe and Ikeya 2016). However, formalin emits gas and prolonged exposure to this may pose a health hazard. There are reports that it could be carcinogenic and may cause infertility in female human beings (Duong et al. 2011).

Oxidative decomposition during storage leads to an acidic condition when formic acid forms. At this low pH condition, the DNA of specimens are denatured, making it difficult to use them for future molecular studies. It is for this reason that formalin has to first be neutralized (France and Kocher 1996). The commonly used neutralizers are calcium carbonate, sodium borate, sodium tetraborate, disodium tetraborate, or borax, which can be readily purchased in local drugstores. Approximately one tablespoon of borax is added to a liter of formalin solution. Another way of preparing buffered formalin is by adding borax into a liter of formalin until no more quantities of borax goes into solution. This is known as a saturated buffered formalin. Baking soda (sodium bicarbonate) may also be used as a neutralizer at a mixture of 40 g per L of formalin. However, one should be careful not to add too much buffer because this may cause some thalli to become brittle or even disintegrate. Buffered formalin is usually added to the sample fluid to give a final concentration of 4% , i.e., add about 10 mL of 37% buffered formalin in 100 mL of sample fluid.

Modifications to Formalin-based Preservatives

Some chemicals may be added to formalin to enhance some of the cytological features of the cells, One such chemical is copper nitrate, which is added at one g/L of formalin (37% formaldehyde) to help maintain the greenness of chloroplasts (John et al. 2011).

FAA (Formalin-acetic acid- alcohol) is one of the formalin-based preservatives for all types of algae, except for calcareous algae, e.g., *Chara* or *Acetabularia*. Different combinations of these chemicals have been formulated (Smith 1950; John et al. 2011). A combination of 10 mL 37% formaldehyde, 5 mL glacial acetic acid, 50 mL 95% ethanol, and 35 mL water was found useful for flagellated algae, particularly ideal for preserving the flagella (John et al. 2011). It is preferable that the water from the habitat of the sampled alga be used in preparing FAA to avoid abrupt changes in the osmotic pressure of the cells.

Another chemical that may be combined with formaldehyde is glutaraldehyde at the following combination: 1% formaldehyde (0.5 mL) + 0.04% glutaraldehyde (17 mL) to make a final volume of 50 mL using sample field water (Kawanobe and Ikeya 2016). It is best that the preservatives are prepared separately and fresh prior to its use, then they are kept in a cool, dark place. The

glutaraldehyde is always added to the formaldehyde. Glutaraldehyde is a colorless, viscous liquid that can penetrate cellular structures slowly, causing less damage to flagellates, and retaining *in vivo* fluorescence because of its colorless nature. It is less reactive to plastic storage containers. It is also used for industrial water treatment as a chemical preservative. However, it has a pungent odor and it is toxic which can cause severe irritation to the eyes, nose, throat, and lungs, which may be accompanied with headaches, drowsiness, and dizziness.

B. Alcohol

Alcohols are good preservatives, especially when used at high strength, because they kill bacteria and molds. However, they cause dehydration and dissolve certain pigments, proteins, and lipids. The most common concentration used as a preservative for algae is 70% ethyl alcohol (ETOH). Isopropyl alcohol may also be used at 55-70%, but the chances for shrinkage is greater than with ethyl alcohol. It is even more toxic than ethyl alcohol Commercially available hard liquors (e.g., gin, vodka, rum) in the appropriate strengths can be used for the temporary preservation of specimens (Martin, 2004). For example, a 150-proof rum (approximately 75% ETOH by volume) may be used as alternative and less expensive than a high grade ETOH.

Ethyl alcohol, however, is not usually used singularly as a preservative. However, it can be used singly at higher concentration and if only stored for a short period of time for future molecular studies. It is usually mixed with other chemicals such as in the preservative FAA. In some cases, the samples are initially preserved for 24hr in buffered formalin (to make a final concentration of 4%). After the samples were rinsed in filtered water sample, they are then transferred to 70% ETOH for prolonged storage.

C. Iodine

Iodine has long been known as **bacteriostatic**, thus, the use of tincture of iodine in treating wounds. It is for this reason that iodine-based solutions are good preservatives. They are also useful as staining solutions, mainly to indicate the presence of starch by turning the sample blue to black. Another advantage in using iodine-based preservatives is that the chemical can be easily obtained from pharmacies or drugstores. However, its incorporation in cells can obscure the presence of some cytological features, particularly if the cells contain starch granules. Iodine can also interfere with other stains and with fluorescence. Specimens which are darkly stained due to iodine, can be cleared with sodium thiosulfate (Strüder-Kypke et al. 2001).

Some iodine solution preparations

1) Tincture of Iodine

The tincture of iodine is prepared by dissolving 20 g iodine (I_2) plus 24 g sodium iodide (NaI) in 500 mL of 70% ethanol. This mixture is made to a final volume of 1000 mL with distilled water. As a preservative, this weak iodine solution is added drop by drop into a plankton water sample until it turns like a “weak tea.”

2) Lugol’s Iodine Solution

There are two ways to prepare a Lugol’s iodine solution. First, prepare by dissolving 50 g iodine in 100 mL glacial acetic acid, then dissolve 100 g KI in 1000mL distilled water (Throndsen 1978). Mix the two solutions together in a beaker and filter to remove any precipitates. Store in a dark colored glass-stoppered container with possibly with the least amount of air trapped in it. Store the samples in a dark, cool place. This solution is used as a preservative by adding to the plankton samples to make a final concentration of 2-10%. However, in the Phycology Laboratory (at IBS, UPLB), they observed that even at low 1% concentration of Lugol’s iodine, the form and organelles of the euglena cells were conserved.

Another variant of preparing Lugol’s iodine is based from the formulation from the Centre for Ecology and Hydrology/Freshwater Biological Association at Windermere, U.K. (John et al. 2011), wherein 96 g KI is dissolved in 100 mL distilled water, then 14 g I_2 is added to the solution,

resulting into a ratio of about 7:1. Then glacial acetic acid is added to the mixture to make a 10% concentration. In this manner the flagella were observed to be conserved. In order to use it as a preservative, add the preservative to make a final concentration in the sample of 0.5%. This preservative, being a weak preservative, or being relatively less toxic than the aldehyde-based preservatives, has the advantage of not losing cells, especially the delicate ones, like the ciliates, but must be used immediately after preparation, to be more effective. Furthermore, iodine enhances the sinking of cells in settling chambers. Materials fixed with Lugol's iodine can be processed for the scanning electron microscopy (SEM) (Montagnes and Taylor 1994), for DAPI staining (4', 6-diamino-2-phenylindole dihydrochloride) (Strüder-Kypke et al. 2001). Some disadvantages reported in using Lugol's iodine solution are that it does not preserve the cell shape and size of live specimens and the gas vacuoles of cyanobacteria were reported to be largely destroyed (John et al. 2011). It also tends to mask chlorophyll fluorescence that hinders counting of cells in mixotrophic species (Gifford and Caron 2000). Lugol's solution tends to dissolve hard structures such as coccoliths and diatom frustules and hence, it is not an ideal preservative for these taxa (Gifford and Caron 2000).

III. Fixing Fluids

Bouin's solution is a non-coagulant picrate solution named after the French biologist, Pol Andrė Bouin, who described the fixative in 1897 (Hidalgo 1992). It is a mixture of 15 g solid picric acid, 25 mL of formalin (37% formaldehyde) , and 1 mL glacial acetic acid to make a final volume of 1 L solution with distilled water (West and Fritsch 1968).

Ruzin (1999) formulated two ways of preparing this fixative, i.e., a strong and a weak one (Table 9.1). Strong Bouin's (SB) has picric acid, while weak Bouin's (WB) does not have picric acid. SB has been successfully used to preserve telophase figures of root tip and embryo sacs (Chamberlain 1932), while WB solution was used to preserve phospholipids for subsequent identification using Orange G and Aniline Blue (Baker 1958.) Bouin's solution does not make a good preservative because the picric acid content tends to shrink the specimens and renders them brittle with prolonged storage, i.e., longer than 24-48 hrs. Hence, it is best to replace Bouin's solution with an aqueous alcohol solution, especially for specimen with softer tissues. However, Bouin's solution has been used in the Phycology Laboratory, IBS, UPLB in preserving the shape of the photosynthetic euglenoid cells and provided good contrast for cellular organelles such as chloroplasts, including the outer pellicle of the cell (Fig. 9.1). Among the different preservatives tested on photosynthetic euglenophytes, it seems that the best combination was 2% strong Bouin's solution + buffered formalin (Fig. 9.1-C) because it showed the normal leaf-like form of *Phacus* cell, the cell organelles, and its striated pellicle. Weak Bouin's solution was not found effective in preserving the structure and shape of the cells.

Table 9.1: Comparative formulation between Strong and Weak Bouin's preservative (Ruzin 1999).

Chemical	Strong Bouin's mL/105 mL	Weak Bouin's mL/100 mL Check total/final volume between SB and WB
Formalin	25	10
Picric acid (1.3% in d water)	75	
Picric acid (5% in d water)		50
Glacial acetic acid	5	5
Distilled water	-	35

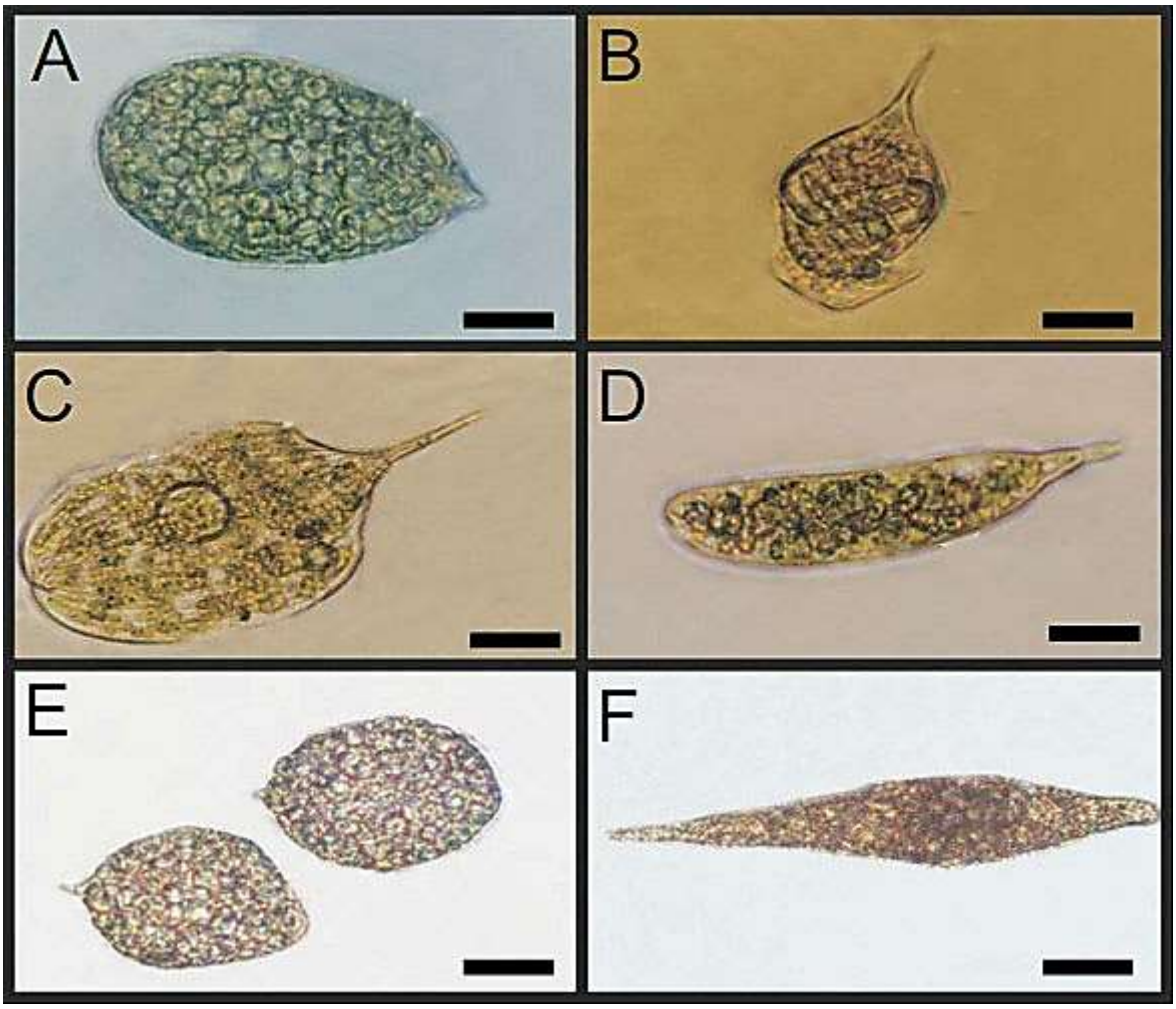

Figure 9.1: Various photosynthetic euglenophytes in different preservatives/fixatives.
All cells showed the enveloping pellicle.
(A) Euglenoid cell preserved in 3% buffered formalin showing the sub spherical shape of the cell, the numerous chloroplasts (a);
(B) A *Phacus* cell preserved in a strong Bouin's solution showing the cell could have "burst"; prominent tail (a), and striations in the pellicle (b);
(C) *Phacus* sp. in 2% strong Bouin's solution with 4% buffered formalin showing the normal leaf-like shape of the cell, the chloroplasts in rows (a), the paramylon body (b), the canal through which the flagellum emerges (c), striations in the pellicle (d);
(D) *Euglena* sp. in strong Bouin's solution showing normal spindle-shaped form, the chloroplasts (a), and the striations in the pellicle;
(E) *Euglena* sp. in 2% strong Bouin's solution with 10% buffered formalin showing the sub spherical shape of cells instead of the usual spindle-shaped form;
(F) *Euglena* sp. in strong 3% Bouin's solution with 10% buffered formalin showing the normal spindle-shaped form with rounded anterior end and a posterior end extended into a pointed tail piece, numerous chloroplasts. (Bar represents 10 µm; magnification 400x).

IV. Storage

Although **fluid-preserved samples** are easy to prepare, there are some points for consideration. First, is the **storage containers**, it is important to choose the correct size of containers that best fit the specimen. Large specimens, e.g., *Cladophora* and *Stigeoclonium*, are best stored in jars, while microscopic algae like *Chlorella* sp. are best kept in dram vials (5-10 mL capacity). However, dram vials cannot pack large specimens nor do they allow room for the exchange of gases. It is desirable to store specimens in several subsamples. This reduces contamination or oxidization of the specimen. Microalgae, which are usually stored in dram vials may be kept together in one jar,

preferably with rubber gaskets for an airtight seal, thus reducing chances for oxidation and dehydration. Capped vials or bottles may also be sealed with either parafilm or melted paraffin.

Glycerol (glycerin) may also be added to the preserved sample before sealing to prevent excessive evaporation. However, it is recommended to replace the original preserving fluid with a 5% solution of glycerin in 50% ethyl alcohol solution before permanent storage of collections (Smith, 1950).

Fluid-preserved specimens should be stored at a constant temperature of 20 ^{0}C (about 65 ^{0}F). A temperature below this may dissolve fats and may allow the formaldehyde in the preservative to solidify (Simmons, 1995). However, cold storage, i.e., in the refrigerator, at 4-10 ^{0}C, preserved marine diatoms better than at room temperature over a period of three months (Kawanobe and Ikeya 2016). Relative humidity (RH) is another important factor to be considered in the storage area. This should not be greater than 65% to avoid the growth of molds. Very low RH, i.e., lower than 20%, can dehydrate gaskets in the preserving containers. It is important to keep these two environmental factors at the constant optimum values to avoid excessive evaporation of the preservative fluid, which will ruin the specimens. It is very difficult to rehydrate preserved samples and restore them to their ideal preserved state.

The storage place should also be free from direct light penetration because light causes permanent, irreversible discoloration and brittleness. Photo damage is cumulative. It is best to keep the preserved specimens in the dark or in closed cabinets. Using ambered jars or vials or wrapping the clear containers with black or carbon paper will likewise prevent damage due to light. The storage area should be provided with rim-edged shelves to secure containers in place. This is especially true in earthquake-prone places.

V. Summary

Preservation in fluid or liquid means storage of specimens in this state of matter to protect them from possible deterioration. Fixatives and preservative fluids were differentiated from one another. There were three major types of preservative presented, i.e., formalin-based, ethanol-based, and iodine-based. For each type, the following were discussed: the preparation procedure, the probable combination with other chemicals, its advantages and disadvantages. Although the fixative and preservative fluids will cause chemical alterations in the microalgae, like discoloration, shrinking, and swelling, they enable the specimens to last longer compared to unpreserved samples. They are also useful for keeping specimens that are likely to be used in class demonstrations, laboratory studies as well as for future DNA studies. Fluid-preserved samples are also used for display in natural museum collections.

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Chapter 10. Preparation of Algal Herbarium Specimens

Milagrosa R. Martinez-Goss[1]* and Lawrence M. Liao[2]

I. Introduction

One of the oldest taxonomic studies on plants was done by a Greek naturalist-philosopher and a student of Aristotle named Theophrastus (372-288 B.C.) (Norstog and Long 1976). He devised the first classification of native Greek plants into a catalogue or book and produced detailed drawings and descriptions so that the plants could be identified. Some of the illustrations were done on woodcuts with great originality that presumably became the specimen records or preserved specimens for future studies. This and later Greek and Roman books on plants became models for botanical writings called **herbals**.

Today the study of taxonomy is not only based on books, illustrations of live specimens but also on preserved and prepared biological materials that are reasonably complete and well curated and which retain their information and scientific value by preserving them in a more permanent repository place, called the **herbaria** (**singular: herbarium**). Hence, a **herbarium** is a collection of preserved plant specimens, including algae, in its dried form together with some associated data used for scientific study. Since the specimens are in dried form and pressed or air-dried, they are most often referred to as **exsiccatae** (singular: **exsiccata**).

The concept of the herbaria started in Europe when collecting of medicinal plants and exotic plants became a fad among natural history enthusiasts a few centuries ago. In fact, it was Luca Ghini (1490-1556), an Italian physician and botanist, who is credited to be the creator of the first recorded herbarium by preserving plants under pressure then bound these specimens within a book (Findlen 2017). Algal herbaria may be found in **museums** or **research institutes** or even in **universities**. They may be catalogued in the Phycological Herbarium as in the Natural History Museum of Denmark (SNM). In some cases the algae are still included in the **Botanical Herbarium**, as in the Museum of Natural History at the University of the Philippines Los Baños (UPLB), or called the College of Agriculture Herbarium of the University of the Philippines (CAHUP); or they may be included in the **Cryptogamic Herbarium** (spore-bearing plants), as in the Museum National d'Histoire Naturelle (PC) in Paris, France. The various abbreviations cited above are standard and unique (ranging from a single upper case letter to a group of six upper case letters) assigned to various herbaria around the world and included in the Index Herbariorum (http://www. sweetgum.nybg.org/science/ih).

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Some of the large collection of algal herbaria are found in Europe and North America, e.g., the algal herbarium in the Museum National d'Histoire Naturelle (PC) found in Paris, France has about 500,000 algal herbarium specimens and 10,000 algal type specimens (http://www.mnhn.fr/en/ collections/collectiongrups/botany/ algae).

In the Philippines, some of the algal herbaria are the following: 1) the Gregorio T. Velasquez Phycological Herbarium in the Marine Science Institute of the University of the Philippines, Diliman, Quezon City, 2) the Phycological Herbarium at the University of the Philippines Los Baños (UPLB) is included with the Botanical Herbarium of the CAHUP of the Museum of Natural History at UPLB which is located at the third floor of the Bio-Science building of UPLB, College, Laguna, and 3) the Phycological Herbarium of the National Museum of the Philippines (NMP) is included in the Cryptogamic Herbarium of the NMP in Ermita, Manila.

One distinct advantage of herbarium specimens is that they provide constant and permanent records of botanical, in particular phycological diversity. Until recently, they have served the scientific community in the conduct of taxonomic studies. Samples, sometimes called **vouchers**, or some are **type specimens**, are preserved to allow for the verification and repeatability of experiments, as a means of determining the authenticity of taxonomic work (Culley 2013). But one has to search where the type specimens are stored. For example, the isotype of *Anabaena willei* N.L. Gardner (UC 463818) is found in Jepson Herbarium of the University of California in Berkeley, Calif., USA (http: //ucjeps. berkeley. edu/ina/ algal_types.html). Lately, however, the utility of herbarium specimens and herbaria has greatly expanded under the current era of rapid environmental changes (Liao 2013) to address research questions in phenology, to document erratic local temperatures, and even the survival of wildlife dependent on plant foods under changing climate patterns (Ziska et al. 2016). Herbarium specimens collected from the historical past, when compared to present-day collections, have also been useful in providing proxy data in tracking localized climate changes, among recent applications (Lavoie 2013; Liao and Bataan 2016). They are also useful in tracking the genetic variation of plants through time (Cozzolino et al. 2007).

Another distinct advantage of the herbarium method of preservation is that the specimen is kept as close as possible to its natural habit and color, especially if the preparation is done immediately on the freshly collected sample. Colors can be preserved for many years under ideal conditions of optimal humidity and darkness inside environmentally controlled herbarium cabinets. Lately, it is becoming standard practice to take photos of the samples in their freshly collected state, and to attach those photos on the herbarium sheet alongside the actual preserved samples. Furthermore, properly preserved herbarium samples, especially those that were dried directly without applying any DNA-damaging chemicals such as formalin, may be used for future molecular and phylogenetic studies (Bruns, et al.1990; Cano and Poinar 1993; Taylor and Swann 1994). Informative DNA has been obtained from as few as two algal spores extracted from algal herbarium specimens (Goff and Moon 1993). If preserved properly and stored well, some herbarium specimens like those of *Nostoc commune* Vaucher have been reconstituted for anatomical studies after 87 years of storage as herbarium specimens (Lipman 1941). However, the procedure is a long and a tedious process, although it is not a complicated process. In fact, it is a straight forward task. However, detailed instructions on the proper procedures have seldom been spelled out in writing, resulting in incompletely or improperly labeled, unsatisfactory or damaged specimens which are of limited use, and waste the efforts put into the collection. Although, the National Museum of the Philippines has published a Philippine Herbarium Manual in 1967, which dealt more on plants aside from the fact that it may now be out of print. Therefore, this chapter seeks to impart this microalgal herbarium specimen preparation, which will hopefully result in more and better herbarium specimens to give maximum benefit to phycologists, taxonomists and students, now and in future generations.

II. Methodology

The first step in the preparation of algal herbarium specimens is to ensure that the specimen is complete and freshly gathered. In this sense, specimens should ideally be undamaged and intact, with most parts attached and with verifiable key diagnostic vegetative and reproductive parts. The preparation of **herbarium sheets of larger (macroscopic) freshwater algae** involves adhering the whole part of the algal specimen naturally on a herbarium sheet. This preparation can be done with representatives of *Spirogyra, Stigeoclonium, Cladophora, Chara* and the like. Some colony-forming and mucilaginous cyanobacteria, like *Nostoc commune* Vaucher and *Gloeotrichia natans* Rabenhorst can be preserved with great ease in this manner. In some cases the algae can be air-dried under the shade, and put into **packets or improvised boxes** which are either glued into the standard herbarium sheets or separately catalogued in the herbarium, with complete field labels and museum labels.

A. Materials

Herbarium sheets (standard size 29 x 43 cm, acid free type, for final mounting)
Smaller herbarium sheets of various sizes (for initial mounting)
Plastic or enamel tray (preferably white)
Clear flat glass top or plastic slate (approx. 15 x 30 cm)
Roll of brown paper cut into small packets
Camel's hairbrush
Blotting paper or corrugated cardboard (cut into rectangles, approx. 25 x 40 cm)
Muslin cloth, "katsa" cloth or remnants from white flour sacks
Old newspapers and other absorbent materials
Herbarium press or plant press
Cordage or straps/belts for tying

B. Procedure

Algae, especially those with mucilaginous or gelatinous thalli, can be mounted easily because they naturally adhere on the herbarium sheet. The specimen is floated in a tray filled with clean tap water (use seawater if working with marine algae) to remove any debris and unwanted epiphytes manually or with the aid of tweezers. The sample can be spread apart gently with the use of a camel's hair brush. A piece of mounting paper (with code number or collecting information written on it in pencil) is then slid into the water under the floating sample and the specimen spread further using either a pair of tweezers, hair brush or even one's fingertips. Insert slowly the glass or plastic slate under the mounting paper with the least agitation on the floating sample. When the mounting paper is fully supported by the slate beneath it, one can slowly lift the slate to one side with both sample and mounting paper on top, allowing the excess water to drip from the other side, until the mounting paper is relatively free of water (Figs. 10.1A-C). This is important to avoid leaving watermarks on the dried paper later on (Figs. 10.2A-B).

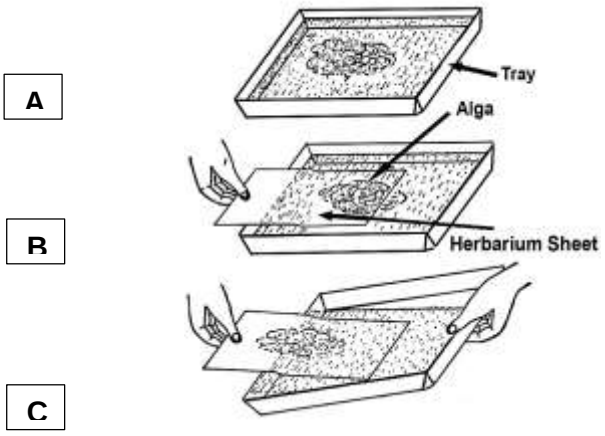

Figure 10.1.A-C: Preparation of algal herbarium sheet.

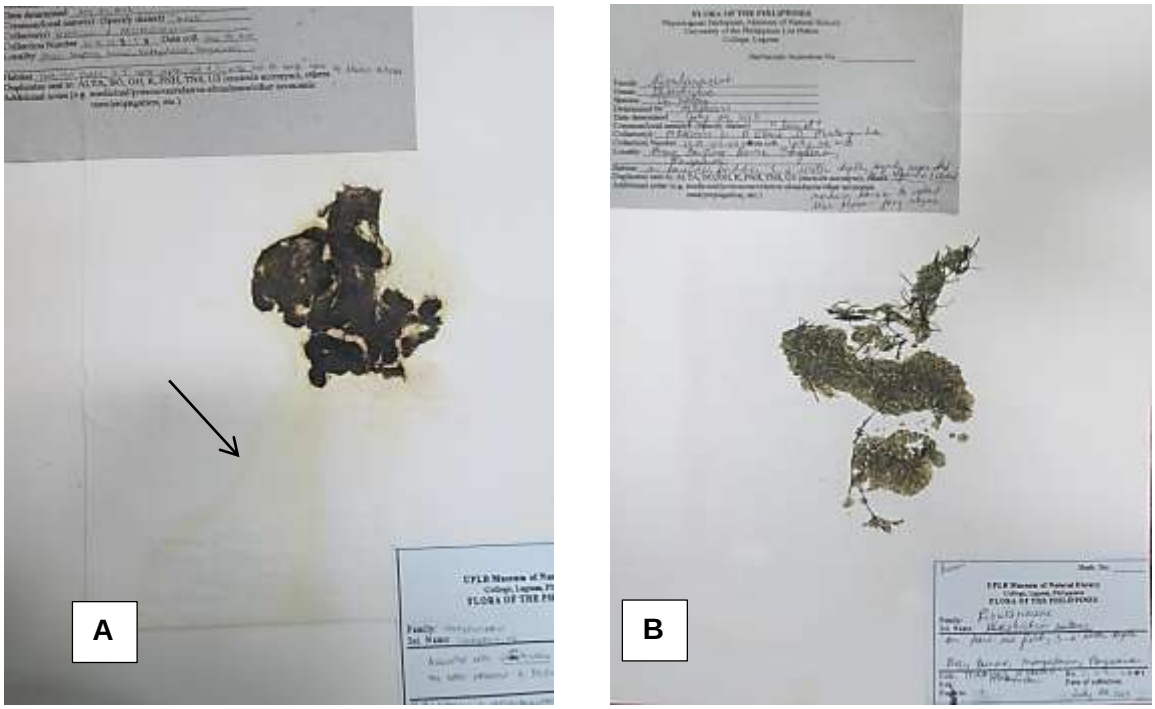

Figure 10.2: Herbarium specimens. A. With water marks. ↓ B. Drip-dried and cleaner herbarium sheet.

Prior to mounting of samples on the paper as described above, prepare the herbarium press by laying down one corrugated board on a flat surface or table (Fig. 10.3). On top of the board, place several pieces of newspaper and other absorbent materials. Remove the wet sample mounted on the paper from the slate and transfer this carefully on top of the pile of newspapers or absorbent papers. At this point, you may insert a piece of paper with code number and/or collecting data beside the sample, if this same information has not been written on the mounting paper earlier. Place a piece of absorbent cloth like muslin cloth, "katsa" or flour sack remnant directly on top of the wet sample and give it a light pressure with one's palm. There is no better substitute for absorbent cloth because algae will stick to other materials like paper. Place several pieces of newspaper or absorbent paper on top of the cloth and then capped by another corrugated board. The layer comprising a corrugated board with several newspapers, mounting paper, wet sample and absorbent cloth (in that specific sequence) is herein called one pressing unit. The process of pressing wet specimens unto absorbent materials and covered with absorbent cloth is repeated many times following the same sequence as in one pressing unit in order to come up with what is referred to as one completed pressing job. In effect, one **completed pressing job** is composed of 10-15 repeating pressing units (or about 10-15 cm thick).

The **completed pressing job** is then enclosed (sandwiched) between the two wooden herbarium pressers or frames termed top and bottom boards in Fig. 10.3, tied up securely on both shorter ends by cordage or belt or friction straps while pressure is applied unto the center of the pressers. It follows that the thinner (less bulky) the pressing job is, the faster is the drying process. The tied-up herbarium presser looks similar to the one illustrated in Fig. 10.3.

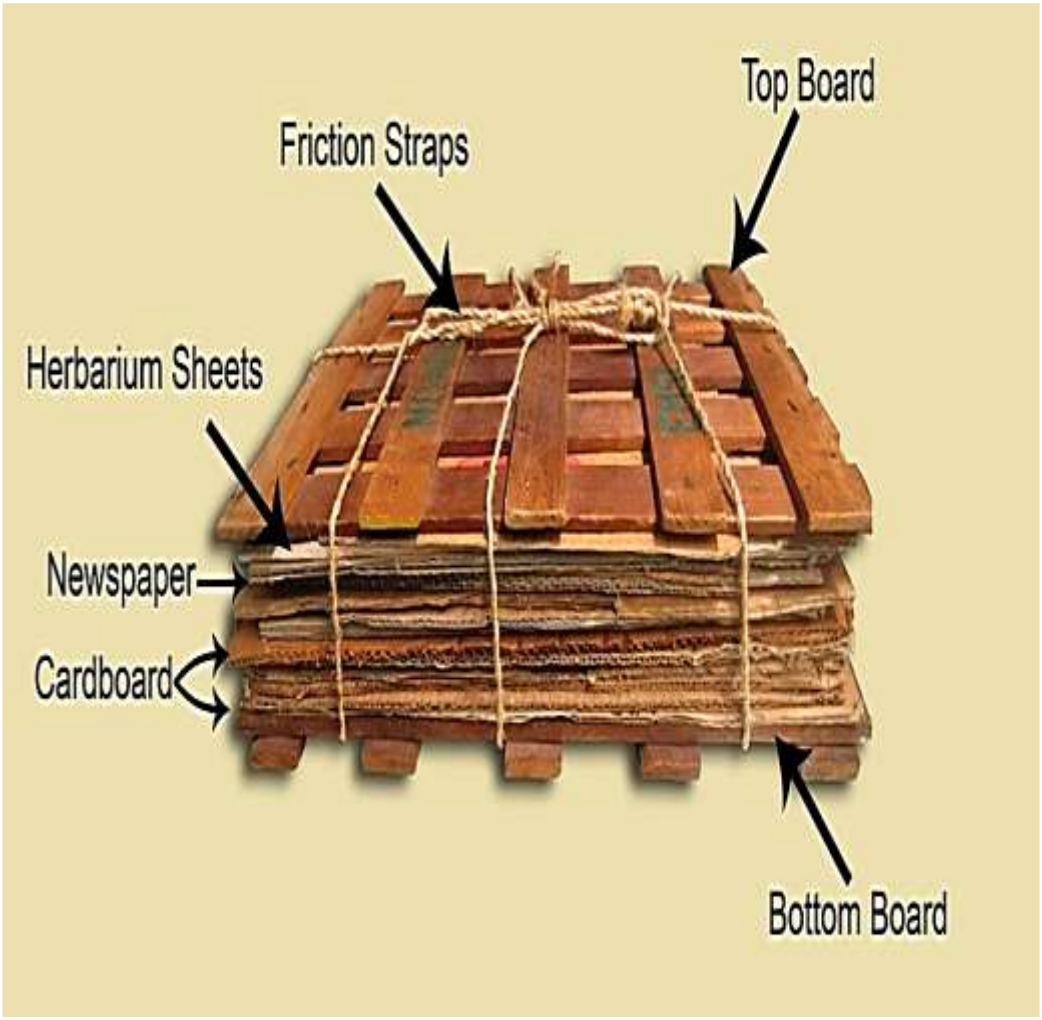

Figure 10.3: The plant presser or herbarium press.

The **bound plant press (one completed pressing job)** is usually air-dried in the shade for gradual drying or dried inside an incubator makeshift oven during inclement weather. The humidity buildup between the sheets and the availability of organic substrates (specimens, papers) are very conducive for fungal and bacterial growth, which can adversely affect the specimens that need to be preserved. If left unchecked, microbial growth can render the target specimens useless for taxonomic purposes. It is therefore important to promptly replace wet absorbent materials with dried ones to keep humidity and microbial growth in check. During sunny and dry days, replacing wet materials with dry ones once a day is deemed satisfactory; it should be more frequent during wet and humid days. Providing dry absorbent papers frequently also hastens the drying process of the target samples. Should samples be moderately overrun by fungal and bacterial growth as evidenced by moldy surfaces, one can usually rub off these unwanted growths using a cotton swab dipped in formalin or ethanol or ethanol in phenol solution acting as “poisoning” preparation. This process should be done periodically as necessary.

Direct solar drying of a bound herbarium press is also possible especially during the sunny days and during the course of on-going field work. The only real risks involved are some cases of rapid and over drying specially for smaller and fragile samples. When putting bound herbarium presses under direct sunlight, it is advisable to allow equal solar exposure of the two faces of the press by alternately turning over the herbarium press. Drying during rainy or cloudy days may also be possible with the use of incubator ovens, but this may constitute a fire hazard as some ovens can incinerate flammable paper and wood materials in the herbarium press. Besides, incubator ovens are usually energy-inefficient. Other heat sources may be used such as the heat-generating back section of most refrigerators and the heat exhaust of air conditioners. Pressers can also be made to “stand” beside wood stoves and other direct heat sources, but fire hazards should always be

minimized. Electric fans and blowers can also be used to hasten the drying process especially on wet and hazy days.

The timely replacement of absorbent materials represents an important chore in assuring the best dried herbarium specimens. This is akin to the replacement of baby diapers lest rashes develop. Saturated absorbent materials are allowed to dry under the sun or in the shade for re-use in the next cycle of replacement. When the samples are sufficiently dry, many of them adhere to the mounting sheets using natural adhesives from their tissues. Others may not adhere to the sheets readily and so they need to be attached by spreading glue on their back surfaces, or reinforced by small straps of paper to secure their places. Those not adhering to paper readily can be placed inside improvised brown paper packets which are then attached to the actual mounting paper using glue.

Proper labeling is an essential component of good research documentation. Incomplete collection data renders the sample less useful or of lesser scientific value. Such information should always accompany the samples throughout the specimens' processing steps from preservation to mounting and finally to curation. Upon mounting the pressed samples on the standard herbarium sheets, such collecting data should be entered into the permanent specimen label (Fig. 10.2B, Fig. 10.4A-B). Information should include but not be limited to scientific and local names, collecting date, locality including coordinates, collector information, field number, and some ecological information. Photographs of live samples may be attached to the standard sheets alongside the actual preserved samples.

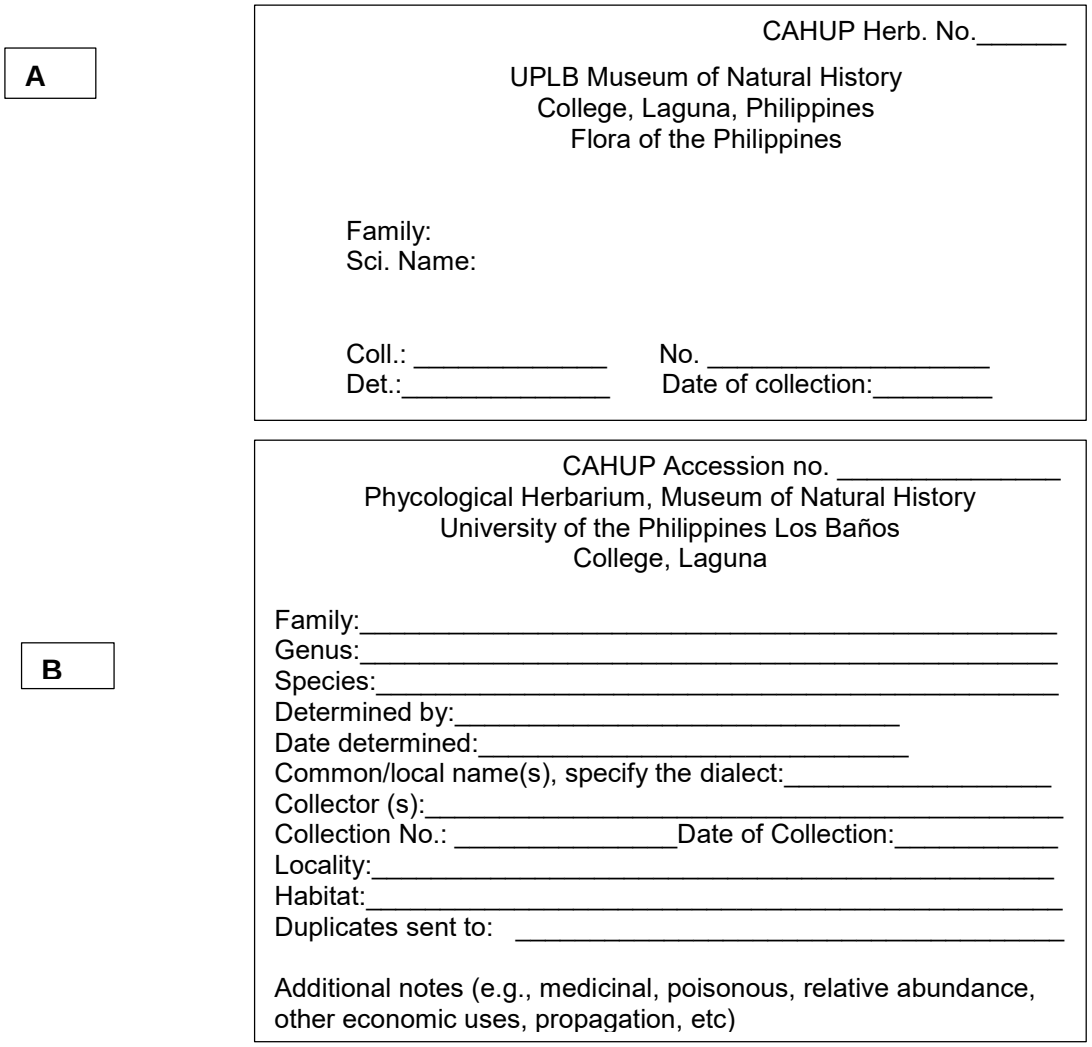

Figure 10.4: Herbarium labels. A. General label, stuck at the lower right hand corner of the herbarium sheet; B. Specific label, stuck at the upper left hand corner of the herbarium sheet.

Properly labeled mounted specimens of a single species obtained from various localities can now be stored in a folder made of Manila brown paper called the **species cover** while specimens of different species and belonging to the same genus are placed in another folder usually made of stronger Bristol board called the **genus cover.** Depending upon individual herbarium policies, mounted specimens can be arranged following a particular classification system, whether alphabetical or even by phylogenetic sequence.

Standard herbarium sheets are available pre-cut into standard sizes from outlets of scientific supplies, mainly abroad. The ideal quality of 100% rag and acid-free paper may be costly and unavailable locally. Suitable substitutes available locally from paper and stationery shops are Bristol board and illustration board. The former are available in large sheets which can be cut into a number of desired sizes at the local printing press shops using large industrial cutters. This can significantly bring down the cost of individual standard mounting sheets.

III. Summary

The importance of algal herbarium specimens have been well documented. Hence, it is important to have a reliable and well documented and curated algal herbarium specimens in order that they can be fully utilized. The preparation of herbarium specimens is an uncomplicated, straightforward task. However, detailed instructions on the proper procedures have been seldom spelled out in writing. This chapter detailed the step by step procedure in preparing algal herbarium specimens, especially for larger specimens of microalgae. May this simple protocol in preparing algal herbarium specimens, be useful in preparing voucher specimens for future studies, especially in taxonomy.

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Chapter 11. Note: Direct Phytoplankton Counting Techniques Using the Haemocytometer[1]

Milagrosa R. Martinez-Goss[2]*, R. Paul Chakroff[3], and Julia B. Pantastico[2,4]

I. Introduction

Direct enumeration of natural plankton populations poses a problem to most researchers. Laboratories with more accurate and faster quantifying equipment are more fortunate. Some of these devices used are the **inverted microscope** (Guillard 1973; Standard Methods 1965) with various glass chambers, **coulter counter** (Cushing and Nicholson 1966; Kubitschek 1969 and Parsons 1973), and the **millipore filters** (Frobischer 1968; Mulvany 1969).

Some less expensive counting tools are the various glass chambers, like the **Petroff-Hauser counting chamber** (Burnham et al. 1973), the **Sedgwick-Rafter** counting chamber (Standard Methods 1965), and the haemocytometer (Guillard 1973). Another method is the **drop transect method** that was adopted by Lackey (1938), a method improved with the use of the **Whipple disc** in counting filamentous algae like *Oscillatoria* (Olson 1950). Of these glass counting chambers, the **haemocytometer**, which can be used with a standard compound microscope, is one of the most popularly used because it is easier and more convenient to use. Besides, most of the unicellular microalgae being observed are within 2-30µm in diameter, and these dimensions are within the limits that the haemocytometer can handle (Guillard 1973). Moreover, the haemocytometer is easily accessible world-wide in supply houses because it is also being used in hospitals for counting human blood cells.

A **haemocytometer** is a thick glass slide in which the central region has parallel rectangular indentations intersected by a single indentation perpendicular to them forming an "H", that define two reflective rectangles under the cover slip known as counting chambers (Fig. 11.1). Each counting chamber has rulings or grids covering 9 mm^2. The boundary lines in an **Improved Double Neubauer ruling** are the center lines of groups of three (Fig. 11.2).

This chapter presents a simplified procedure using the haemocytometer for enumerating microalgal cells in unialgal or axenic culture and estimating the standing crop of phytoplankton based on the experience of the authors in counting such populations using Laguna Lake samples.

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Because this instrument is generally used for counting human blood cells, many phycologists working with microalgae may not be very familiar with its use, and this chapter hopes to be of help. Population densities in this lake have been observed to be within the millionth cells per milliliter (10^6 cells/ml), except in the case of *Microcystis* "blooms" where the highest recorded was 50.6 x 10^6 cells/ml.**

II. Procedure

Storage and preparation of sample.
It is always desirable to enumerate the components of the plankton community soon after collection prior to a change in population.

Water samples stored at 10ºC for 24 hours do not show an appreciable decrease in their counts compared to fresh materials. Lugol's iodine and formalin-acetic acid-alcohol (FAA) have also been used as chemical fixatives. However, plankton preserved in Lugol's iodine (1-2 drops per 10 ml sample) showed higher counts compared to those preserved in FAA: water sample (1:1).

Prior to counting, it is necessary to concentrate or dilute the water sample. If the population is sparse, it may be concentrated by **sedimentation, filtration, or centrifugation**. Sedimentation has the advantage of minimizing damage to cells but is time consuming. Filtration may be a faster method, but there are the problems of separating the cells from the paper and the possibility of losing minute cells into the filtrate. Centrifugation has been adopted for this purpose because it minimizes loss of organisms and it is a faster method. Water samples are usually centrifuged for at least 6 minutes at 1200 x g. Buoyant forms that do not settle down, like *Microcystis aeruginosa*, are pipetted and counted in a known volume of pre-centrifuged sample.

If the sample is too dense, it is usually diluted with filtered and sterilized lake water. In both cases, i.e., whether the water sample was diluted or concentrated, the remaining suspension is uniformly distributed. The concentration factor (cf) or dilution factor (df) were always noted, where,
cf = initial volume/final volume
df = final volume/initial volume

Filling the counting chamber.
First of all, the counting slide and the cover glass should be properly cleaned before using in order to decrease the viscosity of the fluid. A drop of the water sample is pipetted on top of each counting chamber. Immediately, the cover glass is placed on top with the latter's edges resting on the polished supports (Fig.11.1). The drop should not be too large to cause overflow of the water into the indentations or troughs nor too small to have an incompletely filled chamber. Just enough water is placed so that when the cover glass is applied, the water reaches only the two opposite edges of the cover glass. The haemocytometer can also be filled with the use of a wire loop, but counts are lower compared to the pipette-filled chambers (Guillard, 1973).

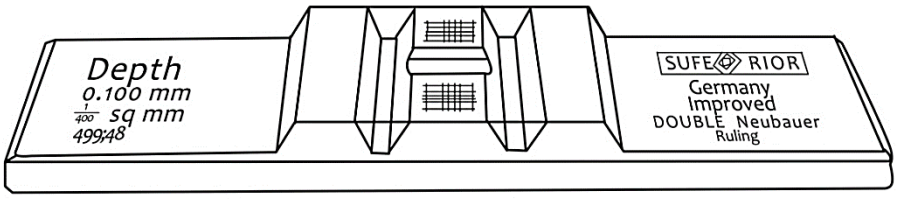

Figure 11.1: The haemocytometer.

Counting procedure.
The filled chambers are allowed to stand for 1-2 minutes prior to observation to give enough time for cells to settle down. Observation follows under low magnification to inspect the evenness of distribution of the cells. Ordinarily, the cells that are 6 µm or greater in diameter or length are

**M. Delmendo. Unpublished lecture delivered at the Phycological Society of the Philippines meeting, September, 1974.

counted under low power objectives, in the four corner blocks, A- D, and in the center block, E (Fig.11.2). Each of these blocks has an area of 1mm^2. or every dense suspension filled with minute organisms (5 µm or lesser in dimensions), it is best to count the cells in the smaller squares of 0.04 mm^2.

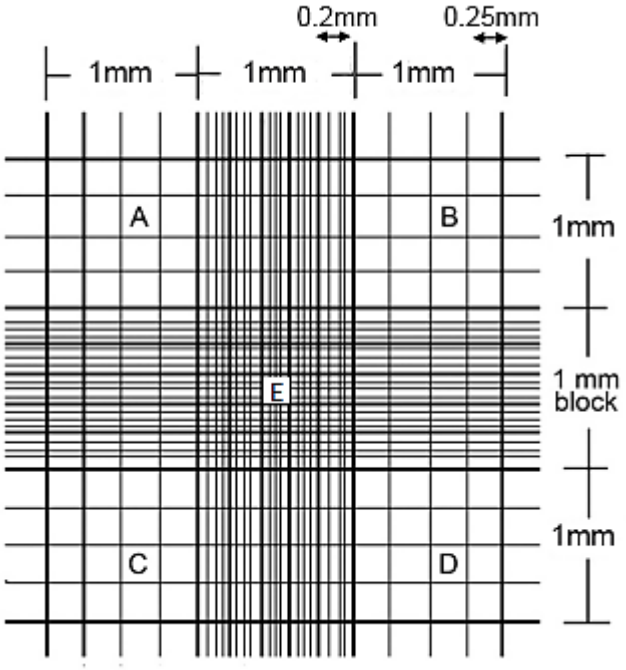

Figure 11.2: The improved double Neubauer ruling within one chamber of the haemocytometer.

Algal counts are recorded in individual 1mm^2 blocks, and the number of algae within a block is considered a separate estimate of the population. A unicellular organism is considered counted if all its parts are within the boundary lines or a portion of it is touching the upper and/or left hand boundary lines. The boundary line is the center line of three closely adjacent lines that separate each 1mm^2 block or 0.04 mm^2 area (Fig. 11.2). In Fig. 11.3, for example, there are 18 *Navicula* cells included in block C.

On the other hand, filamentous or colonial forms that have a tendency to cross more than one block are counted only once within a particular block. Therefore, aside from the above consideration, each organism is counted within a particular block if a major portion of the unit, i.e., a filament or colony, is found in that area. In Fig.11.3, the number of units included in block C are: 7 *Aulacoseira* (*Melosira*) *granulata,* 5 *Oscillatoria* sp., 3 *Melosira* sp. and 1 *Stigeoclonium tenue.*

It is preferable to make at least two fillings of each counting chamber to minimize the errors due to lack of random sampling and of specimen dispersion.

Units of expressing plankton counts.
There are various ways of expressing plankton counts. Three of these possible methods of computing for population densities are presented with sample problems. The methods are evaluated and discussed thoroughly based on actual experience of the authors.

1. Units/mL
This method considers each cell as one; and chains and colonies of organisms are also counted as one. In this manner, the **"direct count"** is also a **"clump count"** and bears a close relationship to the probable **viable count**, since each clump or chain would theoretically give rise to only one colony (Postgate 1969).

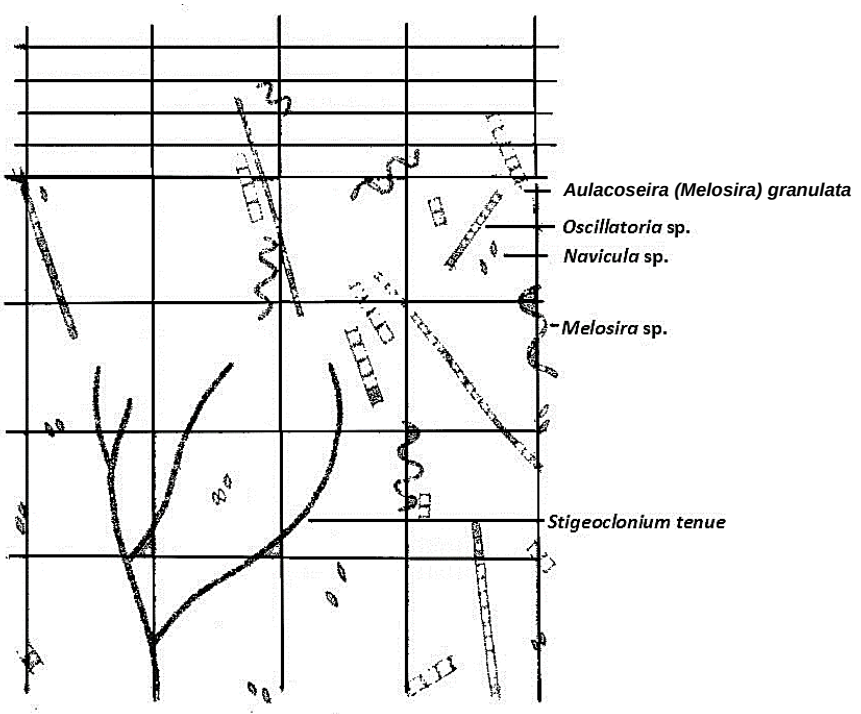

Figure 11.3: A representative distribution of the phytoplankton within block C of the haemocytometer (400x).

Assuming that the organisms in the plankton are 6 µm or greater in dimension, count the total units on each of the five ($1mm^2$) blocks (A-E) of a chamber. Since the chamber is 0.1 mm deep, then the total volume of the liquid scanned is 0.1 mm^3 or 0.001 cm^3 (1 $x10^{-4}$mL). The population density of the original water sample is computed by dividing the total units counted by the concentration factor (cf) or multiplying the total units enumerated by dilution factor (df).

For computation use the following equation:

$$\text{units/mL} = \frac{\text{total number of units x k}}{\text{total volume scanned (ml)}}$$

where, k = dilution or concentration factor, e.g.,

a) for concentrated water sample,

k = 1/cf

b) for diluted water sample,

k = df

As an illustration, two sample problems are presented below. The volume of the liquid within a 1 mm^2 block is equivalent to 1 x 10^{-4} ml. Counts in each block (A-E) are considered one trial. It is assumed that there are no organisms in the supernatant.

Problem 1.

In a 15 ml water sample concentrated by centrifugation to 1 ml, 30 units of organisms were counted in 10 blocks.

$$\text{units/ml} = \frac{\text{total units counted x 1/cf}}{\text{total volume scanned (ml)}}$$

$$= \frac{30 \times 1/15}{10 \times 1 \times 10^{-4}}$$

$$= 2{,}000.$$

Problem 2.

In a 1 ml water sample diluted with 14 ml filtered and sterilized bay water, 30 units of organisms were counted in a total of 10 blocks.

$$\text{units/ml} = \frac{\text{total units counted x df}}{\text{total volume scanned (ml)}}$$

$$= 30 \times \frac{(14+1)}{\frac{1}{10 \times 1 \times 10^{-4}}}$$

$$= 450{,}000.$$

This is a fast method of expressing counts and at the same time it immediately gives an approximate percentage occurrence of individual species in the population.

2. Cells/mL

This method of expressing plankton density is similar to the former except that filamentous, colonial, coenobial, or flocculent masses of organisms are recorded according to their number of units. To save time it is recommended that the number of cells of at least 10 units be obtained. The equation used for obtaining cell counts is basically the same as in unit counts.

If a situation arises such that the population is filled with minute unicellular species, then the cells are counted with the smaller squares in block E. At least 10 squares are scanned. The area of each 25 smaller squares is 0.04mm^2. The volume of the liquid over such area is equivalent to 0.004 mm^3 or 4 x 10^{-6} cm^3 (mL).

For computation use the following equation:

$$\text{cells/ml} = \frac{\text{total number of cells counted x k}}{10 \times 4 \times 10^{-6}}$$

This is a tedious and time-consuming method. Sometimes it may give inaccurate data for masses of organisms whose cells are not evenly distributed, e.g., *Nostoc commune.* There is also a greater possibility of increasing the errors in counting due to errors in enumerating the units and in determining the number of cells per unit. However, this method treats each cell of different species with equal significance. It also avoids the variability caused when a unit of species breaks apart during the process of shaking or centrifuging. Under the units/ml method, the segments are counted separately even though the number and dimensions of the cells are unchanged.

3. Biomass/mL

One of the methods that has been proposed for the estimation of algal cell volume is the **Whipple's Cubic Standard Method**. Using this method, one measures the length, width, and depth of the organism in micrometer units and the product of the three is recorded as the volume represented by the organism in "Cubic Standard Units" (Welch 1948). However, there are two drawbacks to this method.

a) the "cubic standard unit" changes in true size under different magnifications of the microscope, and
b) the volume recorded is usually an over-estimate size of the true volume of an organism because of great variability in shapes of different organisms.

The first drawback can be corrected by recording the measurement in microns and the volume in cubic microns or cubic millimeters (1mm^3 = 1 x 10^{9}µm^3). To convert the data into biomass/ml one must assume that the specific gravity of all the species is 1. Then a cell with a volume of 1µ^3 is equivalent to 1 picogram.

The second drawback can be solved by using different equations for obtaining volumes of different shapes of cells. Thus, the formula for computing the volume of a sphere is used for organisms that are nearly spherical (Kutkuhn 1958), like *Volvox*, *Nostoc*, etc.

$$\text{volume} = 4/3\pi r^3$$

`And, the equation for finding the volume of a cone-shaped species like *Dinobryon*, is

volume $=1/3\pi^2L$

While the volume of cylindrical or disc-shaped plankton that are either unicellular or filamentous as *Cyclotella, Coscinodiscus, Melosira,* etc., are obtained using the formula of a cylinder.

volume $= \pi r^2 L$

Finally, the volume of double cones and elliptical spheroids, like *Gomphonema*, *Schroederia, Scenedesmus, Euglena,* etc., can be computed using a modified formula intended for spherical forms. This is due to the fact that the length, width, and depth of the organisms are different from one another unlike in a sphere which has the same diameter across all planes,

volume $=1/6$ Lwd

In the above notations:

r-radius	L-length
w-width	d- depth

To save time, it is recommended that a mean volume be calculated for the common species that show little variation in dimension. When enough individuals have been measured to give a mean fiducial limits (usually not less than 25 individuals), count the number of species per mLof water sample and multiply the volumetric constant for that particular species. However, it must be realized that the mean volume of a particular species may change under different ecological conditions.

This is one of the most accurate plankton counting techniques in estimating percent composition of the population and total biomass of a given species in a community. It provides information needed for calculating plankton community diversity. On the basis of the time spent in computation, this method is the most time consuming. There is also the problem of obtaining the dimensions of some of the species, like the depth of the double cones or elliptical spheroids.

Table 11.1 shows the relationship of the values obtained from the three types of plankton count expression. Obviously, they are not comparable with one another. The number of units/ml will have the same value as cells/ml if all the organisms observed are discrete single cells. The counts for the former will always be lower compared to the aggregate species and the gap between the two values will vary depending upon their proportional rations. The non-relationship of the values is further supported by obtaining different relatively dominant species under the three methods of plankton expression, e.g. *Cyclotella meneghiniana* is relatively dominant in units/ml count, while *Microcystis aeruginosa* and *Oscillatoria proboscidea* are the relatively dominant species in the cell and biomass counts, respectively (Table 11.1). The interconvertibility of the three values is facilitated if the data on the units/ml, average cells/units, and the dimensions of the cells in microns are known.

Table 11.1: Comparison of the values obtained in using different ways of expressing phytoplankton counts from a surface water sample in a fishpen of Mayondon, Los Baños, Laguna.[a]

Species	Units per ml	Cells per ml	Picogram per ml[b] ($x10^3$)
Ankistrodesmus falcatus (Corda) Ralfs	333	333	4.029
Chlorella vulgaris Beijerinck	1,666	1,666	594.157
Coelastrum microporum Nägeli	266	2,121	416.967
Cyclotella meneghiniana Kützing	19,400	19,400	7,614.50
Cyclotella sp.	133	133	352.889

Fragilaria crotonensis Kitt.	2,800	2,800	298.480
Aulacoseira (Melosira) granulata (Ehrenberg) Simonsen	133	665	668.192
Merismopedia punctata Meyen	800	12,800	53.632
Microcystis aeruginosa Kützing	200	757,600	3,174,344
Oscillatoria amphibia Agardh	5,266	263,300	4,133.810
O. proboscidea Gomont	266	11,172	7,893.241
Palmelococcus protothecoides (Krüger) Chodat	333	333	3.863
Pediastrum duplex var. *clathratum* (A. Braun) Lagerheim	266	4,788	213.497
Scenedesmus armatus (Chodat) G.M. Smith	333	1,332	39.960
S. quadricauda var. *quadrispina* (Chodat) G.M. Smith		2,932	7.887
Schroederia setigera (Schröder) Lemmermann	200	200	0.833
Selenastrum bibraianum Reinsch	333	333	19.341

[a]Data taken from a portion of the doctoral dissertation of the senior author that was submitted to the Graduate School, U.P. at Los Baños,1976, entitled “Taxonomy and Ecology of Algae in Fishponds and Fishpens of Laguna and some Physiological Studies of *Navicula accomoda* Hust.”

[b]Parts per billion (ppb).

III. Summary

The **haemocytometer** is suggested to be used in direct plankton enumeration in conjunction with a standard compound microscope. A simplified procedure for its use was described. The possibility of using different units of expressing plankton counts were presented. Equations were proposed for the estimation of volume and biomass of phytoplankton with different shapes.

IV. Acknowledgment

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Chapter 12. Chlorophyll Analysis[1]

Milagrosa R. Martinez-Goss[2]* and Maribel L. Dionisio-Sese[3]

I. Introduction

Chlorophyll (chl) has always been associated with green vegetation. Green is a color that first earned its importance way back in 1776 when Ingen-Housz associated this color to the regeneration of foul air, as he wrote "this office is not performed by the whole plant, but only by the leaves and the green stalks." Since then the green chlorophyll pigments of leaves have become the "philosopher's stone" of organic synthesis, a veritable "elixir of life."

Chlorophylls are indeed the basic biological pigments involved in light absorption and photochemistry in plants, algae and photosynthetic bacteria.

Chlorophyll pigments, particularly **chlorophyll *a* (chl *a*)**, is widely accepted as a component in measuring biomass and the physiological condition of algae and plants in general. It is also a useful indicator of water quality wherein the ratio of the algal biomass to chl *a* levels is determined.

Our interest, however, is in the degree of the greenness of the chlorophyll pigment. This, in fact, has great value in the food industry. This color stimulates the appetite and renders palatability to dishes, as well as provides algal foods with that enhanced attractiveness. Hence, this chapter presents an overview of the chlorophyll composition of algae and assesses the different methods of analyzing their chlorophyll content.

A. Chlorophyll Composition in Algae

Chlorophyll *a* is the universal primary pigment present in all algae and plants, which are oxygenically photosynthesizing organisms. Table 12.1 shows that in addition to chl *a*, there are other pigments variably present in the different algal groups, viz., **chl *b*** which is common among the green-pigmented algae, **chl *c*** which is predominantly found in brown-pigmented types, and **chl *d*** which is the major photosynthetic pigment in a cyanobacterium, *Acaryochloris marina* (Graham et al. 2009).

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The concentration of chlorophyll in algae constitutes about 0.1 to 2% of the dry matter (DM) of algae (Table 12.2). However, the concentration of chlorophyll in laboratory-cultured algae could be as high as 4 to 5% of the total DM, as was observed in *Chlorella* (Haskin 1942). Field-cultured algae, like the marine benthic green alga *Ulva,* usually have a lower chlorophyll concentration (Haskin 1942).

Table 12.1: Chlorophyll pigment types in various algal groups (adapted from Graham et al. 2009).

Algal Group	Chlorophyll			
	a	*b*	*c*	*d*
I. Green-pigmented algae				
Prochlorophyta	+[1/]	+	-[2/]	-
Euglenophyta	+	+	-	-
Chlorophyta	+	+	-	-
Charophyta	+	+	-	-
II. Brown-pigmented algae				
Pyrrhophyta	+	-	+	-
Cryptophyta	+	-	+	-
Chrysophyta	+	-	+	-
Bacillariophyta	+	-	+	-
Phaeophyta	+	-	+	-
III. Red-pigmented algae				
Cyanobacteria	+	-	-	+
Rhodophyta	+	-	-	-

[1/] + present.
[2/] - has not been recorded in any member.

Table 12.2: Chlorophyll content of various groups of algae.

Algal Group	Chlorophyll					Chl ratio (*a*:*b*)	Condition	Reference
	Total	*a*	*b*	*c*	*d*			
I. Green Pigmented Algae								
A. Eukaryotic								
1. Freshwater								
Ankistrodesmus convolutus (mg·g^{-1}, dw)		22.5					laboratory	Chu et al. 1994
Chlorella pyrenoidosa (mg.g^{-1}, dw)	10.85	8.460	2.394			3.53	laboratory	Haskin 1942
Chlorococcum (mg·g^{-1}, dw)	2.2-8.67						laboratory (6-7 weeks)	McLean 1968
Haematococcus lacustris (mg·mL^{-1})	0.03	0.0189	0.0111			1.703	laboratory	Ding et al. 1994
2. Marine								
Codium tomentosum (mg·g^{-1}, dw)	0.32							Lubimenko 1928
Ulva lactuca (mg·g^{-1}, dw)	3.094	1.94	1.149			1.6		Haskin 1942
B. Prokaryotic								
Prochloron sp. (mg·g^{-1}, dw)	7.93	6.74	1.19			5.65		Withers et al. 1978
II. Brown Pigmented Algae								
Chroomonas sp. (mg·10^6 cells^{-1})		0.406		0.170		2.35	laboratory (7 days)	Faust and Gantt 1973
Chaetoceros gracilis (mg·L^{-1})		0.108-0.376					laboratory	Taguchi et al. 1987
Cyclotella sp. (mg·L^{-1})		0.142-0.524					laboratory	Taguchi et al. 1987
Ditylum brightwellii (mg·10^6 cells^{-1})	0.0156						laboratory	Stauber and Jeffrey 1988
Nitzschia closterium (mg.10^6 cells^{-1})	0.075						laboratory	Stauber et al. 1988
Dictyota dichotoma (% of dry wt)	0.78							Seybold and Egle 1938
Laminaria (% of dry wt)	0.23							Seybold and Egle 1938

III. Red Pigmented Algae								
A. Eukaryotic								
Gelidium (% of dw)		0.11						Lubimenko 1928
Porphyra laciniata (% of dw)		0.44						Seybold and Egle 1938
B. Prokaryotic								
Gloeocapsa montana (% of dw)		0.3						Sargent 1940
Gloeotrichia natans (mg·g^{-1}, dw)		10.0						Martinez-Goss (unpubl.)
Nostoc commune (mg·g^{-1}, dw)		9.2					tubular helical bioreactor (10 days)	Briones and Martinez-Goss 1994
Spirulina (mg·g^{-1}, dw)		11.0					laboratory (30 days)	Belay et al. 1994
Plant Species*								
*Spinacia oleracea** (spinach) (% of total)	35.04					3.87	treated with Triton X-100	Hladik and Sofrova 1980

* For comparison

Compared to higher land plants, like spinach, the chlorophyll concentration of the algae is only about 1/10 (Table 12.2). However their chl *a:b* ratio is approximately the same except in an instance where the prokaryotic alga, *Prochloron,* recorded a chl *a:b* of 5.65 (Withers et al. 1978), higher than that in spinach, which was 3.87 (Hladik and Sofrova 1980).

B. Chlorophyll *a* Content in Various Aquatic Ecosystems

Chlorophyll *a* content in various aquatic ecosystems was reported mainly due to phytoplankton and picophytoplankton. Table 12.3 shows that freshwater ecosystems where phytoplankton dominates yielded a higher chlorophyll *a* (18-2000 mg.m^{-3}) concentration than marine ecosystems (0.02-40 mg.m^{-3}). The chl *a* analysis done on the phytoplankton in an unfertilized concrete pond in the Philippines, in the vicinity of the University of the Philippines Los Baños (UPLB) Administration building on January 20, 1995, revealed that the chl *a* level was as much as 37 mg.m^{-3}, comprising about 0.15% of their total dry matter (Martinez-Goss and Guevarra unpubl.; Table 12.3). On the other hand, the chlorophyll *a* concentration in different trophic levels of lakes in Japan due to picophytoplankton ranged from 0.16 mg.m^{-3} (oligotrophic lakes) up to 1.73 mg.m^{-3} (hypertrophic lakes) (Takamura and Nojiri 1994; Table 12.3). These data are still lower than the data obtained from inland waters due to phytoplankton (Table 12.3).

Table 12.3: Chlorophyll *a* concentration of phytoplankton/picophytoplankton from various aquatic ecosystems.

Location	Chlorophyll *a* concentration (mg·m^{-3})	Reference
A. Phytoplankton		
1. Marine		
Antarctica	0.05-0.91	Tominaga 1971
Indian Ocean	0.02-0.60	Tominaga 1971
Oligotrophic ocean (ave.)	0.05	Strickland 1960
Fertile inshore blooms (ave.)	10-40	Strickland 1960
2. Freshwater		
England, Abbot's Pool	18-55	Hickman 1973
Ethiopia, Ethiopian Soda Lakes	2000	Talling et al.1973
Philippines, Laguna, College, UPLB[1/], Administration Pond	36.56	Martinez-Goss and Guevarra (unpubl.)
B. Picophytoplankton		
(Japan lakes)		Takamura and Nojiri 1994
oligotrophic	0.16	
mesotrophic	1.96	
eutrophic	1.52	
hypertrophic	1.73	

[1]UPLB=University of the Philippine Los Baños, phytoplankton sample taken on January 29, 1995.

II. Analysis

Algal chlorophyll analysis involves generally five steps, viz.: **1) concentration of sample; 2) cell disruption; 3) extraction of pigments; 4 identification of pigments, and 5) estimation.** Figure 12.1 presents a flow chart for these enumerated steps.

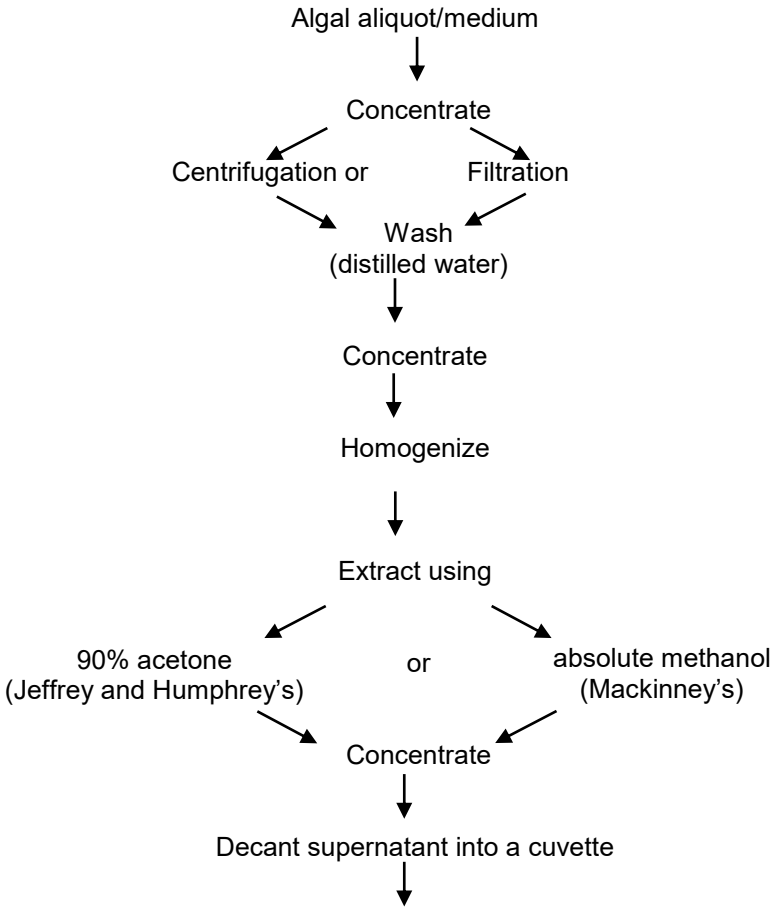

Figure 12.1: Flowchart for determination of chlorophyll *a* levels in algae.

Concentration of sample

This process is necessary only if analysis will be made on plankton samples or microalgal cultures which are sparsely populated. Concentration may be accomplished either by centrifugation or filtration. Filters could either be of the membrane type (0.45 µm porosity, 47 mm diameter) or of the glass fiber type (GF/C or GF/A, 4.5 cm diameter). A vacuum pump is needed to suck the filtrate through.

Cell disruption

The breaking up algal cells, together with their chloroplast and thylakoid membranes, could be accomplished mechanically or by the application of thermal treatment. Larger seaweeds, e.g., *Laminaria* and *Sargassum,* are initially cut into smaller pieces and boiled in water for 1 to 2 minutes to soften the tissues and allow faster breakup of the cells. This process, however, may result in the alteration of some products (Strain et al. 1971). Freezing and thawing of phytoplankton have been found to facilitate extraction (Jeffrey 1972).

Cell disruption is usually carried out together with extraction when dealing with unicellular and filamentous algae. A **mortar and pestle**, preferably one with smooth surfaces, such as one made of marble, is recommended to macerate the algal tissues. A glass tissue homogenizer may also

be useful for smaller quantities. Grinders made of glass seem to be more efficient in homogenizing algal samples than the teflon types.

Extraction of pigments

The chlorophyll pigments attached to the photosynthetic lamellae are extracted with ease when using organic solvents, e.g., 80 to 90% acetone or 90% to absolute methanol. These two different solvents are based on the two common methods for chlorophyll analyses. **Acetone** was first used in the method reported by Jeffrey and Humphrey (1975), while methanol is used in the method reported by Mackinney (1941). Most frequently, **methanol** gives a more quantitative extraction for samples, without involving any grinding techniques (Marker et al. 1980), due to its greater polarity over acetone. The data taken when the two extracting solvents were compared using unialgal culture of *Spirulina* and field sample of phytoplankton supports the finding that more chl *a* was extracted using methanol as compared to acetone but did not always work for chl *b* extraction (Martinez-Goss and Guevarra unpubl; Table 12.4). **Methanol extraction (Mackinney's method)** also yielded higher values than **acetone extraction (Jeffrey and Humphrey)** regardless of whether the algal samples were from unialgal cultures or ponds, as well as on the duration or method of incubation. In some instances, however, several extractions with methanol were required to remove the chlorophyll in thick-walled green algae, e.g., *Scenedesmus* (Bishop 1971). The formation of acidic chlorophyllides by chlorophyllase (Owens and Falkowski 1982) is also associated with the use of methanol. This reaction may be minimized by keeping the 100% methanol sample in the dark at -20ºC (Otsuki et al. 1987). On the other hand, a longer period of extraction, usually overnight, is necessary when using acetone. Acetone does not induce formation of oxidation products of chlorophyll which may interfere with analysis. For some algae, however, like the unicellular eukaryotic alga, *Cyanidium,* the extraction solvent used is N-N-dimethyl formamide (Volk and Bishop 1968).

Table 12.4: A comparison of the chlorophyll (chl) content of a unialgal culture of *Spirulina* sp. and phytoplankton sample from a concrete pond [1/] following different estimation procedures (Jeffrey and Humphrey and Mackinney methods) and under various methods of incubation (Martinez-Goss and Guevarra unpubl.)

Method of Incubation	Chlorophyll content (µg·mL^{-1})						
	Spirulina sp. (laboratory culture)		Phytoplankton sample (field sample)[1/]				
	Jeffrey and Humphrey	Mackinney	Jeffrey and Humphrey			Mackinney	
	Chl *a*	Chl *a*	Chl *a*	*b*	*c*	Chl *a*	Chl *a*
2-3 h at 4ºC	1.45 a[2/]	1.48 c	0.81 b*	0.68 b	1.18 a	1.16 a*	0.74 a
12 h at 4ºC	1.38 b*[3/]	1.78 ab*	0.79 c*	0.74 b	0.14 a	1.23 a*	0.71 a
2 min at 70ºC	0.55 c*	1.63 b*	0.86 a	1.02 a	0.15 a	1.15 a	0.72 a
2 min at 70ºC (12 h at 4ºC)	1.52 a*	1.92 a*	0.86 a*	0.83 ab	0.20 a	1.26 a*	0.72 a

[1/] Sample taken from the concrete pond near the Administration building, University of the Philippines Los Baños (UPLB), College, Laguna, Philippines on January 20, 1995.

[2/] Means followed by a common letter are not significantly different at 5% level following DMRT.

[3/] * Means between the Jeffrey and Humphrey's and Mackinney's procedures are significantly different at 5% level following DMRT.

Freshly collected algal materials should be extracted in alkaline conditions as soon as possible. Higher chlorophyll concentration was usually noted when the algal samples were kept at 4ºC than when maintained in a 70ºC water bath for 12 h (Martinez-Goss and Guevarra unpubl; Table 12.4). It is recommended that extraction be conducted in an ice bath in the dark to prevent volatilization of solvents, pigment breakdown, or bleaching of pigments. A pinch of $MgCO_3$ added into the acetone solution had been found to remove traces of acids coming either from the algae or from the glassware (APHA 1976). These acids tend to convert chl *a* to phaeophytin, which subsequently interferes with the colorimetric measurement data because phaeophytin happens to also absorb light and to fluoresce in the same region as chl *a* (Marker 1972; APHA 1976).

After extraction, the samples are transferred to a small capped centrifuge tube, and its volume brought up to at least 5 mL with the specific solvent, i.e., either acetone or methanol.

Identification

Extracted pigments are identified into their individual component pigments either **chromatographically** or s**pectrophotometrically** and read against a set of standard pigments (from Sigma Chemical Company). **Chromatographic separation** of pigments may be done directly from the solvent extracts or by initially transferring to petroleum ether prior to separation. Paper and thin layer chromatography have been used, but their results have been known to be difficult to interpret (Jeffrey 1981), due to the instability of the chlorophyll pigments. A more efficient and reliable separation technique applying a chemically bonded stationary phase is the **high performance liquid chromatography (HPLC).**

The **spectrophotometer** measures the absorbance of the extracted pigment over a range of visible light wavelengths. Each pigment is known to absorb a specific wavelength at which a characteristic absorbance peak will register for a particular sample. The absorbance peak for chl *a* in methanol is known to be at 665 nm, while in acetone, it is at 664 nm (Mackinney 1941; Jeffrey and Humphrey 1975). An instrument with a narrow band (i.e., 0.5 to 2 mm) is recommended because it can better resolve the narrow chl *a* peak.

Estimation

The concentration of chlorophyll is determined using the specific absorption (extinction) coefficient of a particular chlorophyll pigment in the specific solvent. The specific **absorption coefficient (α)** is defined as **α = D/dc;** where, **D** is the optical density or absorbency of the extract; **d,** the inside path length of the spectrophotometer cuvette in cm; and **c,** the concentration of the pigment in grams per liter. Table 12.5 shows a set of equations adapted to calculate, in micrograms per mL, the varying amounts of chlorophyll levels in different groups of algae where **A** is the absorbance or optical density reading at a given wavelength.

Based on the foregoing spectrophotometric analysis of algal chlorophylls, there are two methods that are commonly used, viz. the **I) Jeffrey and Humphrey method (1975)** and **2) Mackinney method (1941).** An example of estimating the amount of chl *a* in a cyanobacterium, *Spirulina*, following acetone extraction of Equation V in Table 12.5 is shown in Table 12.6. The two spectrophotometric methods of estimating algal chlorophylls, i.e., Jeffrey and Humphrey's (JH) and Mackinney's (Mac) methods, are compared in Table 12.7. It shows that the Mac method is more efficient and takes a shorter period of time to perform than JH, while the JH method has more chlorophyll conversion values than Mac.

Table 12.5: Proposed equations for estimation of chlorophyll pigments (µg chl·mL$^{-1)}$.

I. Higher plants and green algae with chl *a* and *b* (in 100% methanol extract)[1/]
chl *a* = 33.8 A^*_{650} - 12.5 A_{665}
chl *b* = 16.5 A_{665} - 8.3 A_{650}
Total chl = 25.5 A_{650} + 4.0 A_{665}
(in 90% acetone extract)[2/]
chl *a* = 11.93 A_{664} - 1.93 A_{647}
chl *b* = 20.36 A_{647} - 5.50 A_{664}
II. Diatoms, chrysomonads and brown algae containing chl *a, c_1, c_2* in equal proportions (in 90% acetone)[2/]
chl *a* = 11.47 A_{664} - 0.40 A_{630}
chl c_1 + c_2 = 24.36 A_{630} - 3.37 A_{664}

III. Dinoflagellates and cryptomonads containing chl *a and* c_2 (in 100% acetone)[2/]
chl *a* = 11.43 A_{663} - 0.64 A_{630}
chl c_2 = 27.09 A_{630} - 3.63 A_{663}
IV. Mixed phytoplankton populations containing chl *a* and *b,* and equal amounts of chl c_1 and c_2 (in 90% acetone)[2/]
chl *a* = 11.85 A^*_{664} - 1.54 A_{647} – 0.08 A_{630}
chl *b* = -5.43 A_{664} + 21.03 A_{647} – 2.66 A_{630}
chl c_1 + c_2 = -1.67 A_{664} - 7.60 A_{647} – 24.52 A_{630}
V. Algae with chl *a* only (in 90% acetone)-[3]/
chl *a* = 11.93 A_{664} - (cyanobacteria)
chl *a* = 11.41 A_{664} - (red algae)
chl *a* = 13.90 A_{665} - (cyanobacteria, in absolute methanol)[4/]

[1]/ Mackinney 1941.
[2]/ Jeffrey and Humphrey 1975
[3]/ Vonshak and Borowitzka 1991
[4]/ Boussiba 1988
*Absorbance at a given wavelength.

Table 12.6: An estimation of the amount of chlorophyll *a* (µg·mL^{-1}) in *Gloeotrichia natans,* a cyanobacterium, using the Jeffrey and Humphrey's method (Martinez-Goss and Guevarra unpubl.).

No. of sample	V* Volume or Algal medium (mL)	E Volume of extract (mL)	O.D. Absorbance at 664 nm	A O.D. x 11.93 = µg chl·cm^{-1} sol.	B AxE = µg chl·vol^{-1}	C B/V = µg chl·mL^{-1}	Ave. µg·mL^{-1}
1	5	3.78	0.603	7.194	27.1	5.43	
2	5	4.28	0.567	6.764	28.95	5.79	5.46
3	5	3.83	0.564	6.729	25.77	5.15	

*V = volume of algal medium;
E = volume of extract;
O.D. = optical density or absorbance at 664 nm;
A = O.D. x 11.93
B = A x E
C = B / V

Table 12.7. Comparative estimation of chlorophyll pigments in algae using two extraction methods.

	Methods	
	Jeffrey and Humphrey (1975)	Mackinney (1941)
Extracting solvent	acetone	methanol
Duration/efficiency of extraction	longer/less efficient	shorter/more efficient
Available conversion values	chl *a, b*, and c_1, c_2	chl *a* and *b*

The **fluorometer** is another instrument used for measuring chlorophyll concentration by emitting short-wavelength light that is absorbed by the chlorophyll molecule and re-emitted, i.e., fluoresced, at a longer wavelength. This instrument measures the intensity of fluorescence and the reading is used to calculate the pigment concentration. The method has several advantages over the spectrophotometric analysis, in having better sensitivity, requiring less concentrated samples (requires only at least 6 mL extract as compared to at least 10 mL for spectrophotometry), and providing fast and direct results. Further, the instrument is portable and easy to use but is expensive and measures only chl *a*.

III. Summary

The importance of algal chlorophyll in physiological and nutritional studies necessitated the review of the two common methods of estimating chlorophyll content in algae by spectrophotometry, i.e.,

the Jeffrey and Humphrey (acetone) method and the Mackinney (methanol) method. A detailed procedure in the chlorophyll analysis was presented and divided into five steps as: 1) concentration of samples (only if the sample has sparsely populated algae), 2) cell disruption, 3) extraction of pigments, 4) identification of pigments, and 5) estimation of pigment composition.

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Chapter 13. The Microscopes and Basic Microscopy Techniques

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I. Introduction

A. Historical Background and Definition of Terms

The **microscope** is an indispensable piece of equipment in the field of biology and its allied sciences, for the main purpose of observing objects not visible to the naked eye. It consists of a lens or a combination of lenses for enlarging or magnifying objects and for resolving finer details of specimens. There are different types of microscopes depending on the combination of lenses, light sources and their capability to magnify and resolve objects.

Among the widely used research microscopes are the **light microscopes** that use light, natural or artificial, as the power source. This type of microscope has gone a long way since its discovery in the early 16th century by the Dutch spectacle makers, the father and son, Hans Jansen and Zacharias Jansen, respectively (Ockenga 2015). Moreover, because it is a common piece of equipment in laboratory classrooms and research laboratories, its parts, uses, and careful operation are usually neglected. Hence, this chapter serves as a refresher note on light optical microscopy.

B. The Light Microscopes

Light microscopes can be classified into **one lens type** or **simple** and **two or more lenses type or compound** type depending on the combination of lenses. A **simple microscope** essentially has one lens or has one objective lens with a short focal length and a total magnification (magnification of the eyepiece lens x magnification of the objective lens) of up to about 300x. It is mainly used to enlarge image of an object within its focal length. Some examples are magnifying glasses and reading glasses. Anton van Leeuwenhoek (1632-1723) invented the first simple microscope (Barron 1965). On the other hand, there are microscopes that have two or more lenses that magnify features of an object. One of this type is the **dissecting microscope or simple**

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stereo-microscope, that may consist of one lens or a combination of lenses mounted together that function as one lens. Its total magnification (magnification of the eyepiece lens x magnification of the objective lens) is usually no more than 120x. A **dissecting microscope** gives a right side, three-dimensional image that makes it convenient to use in dissecting specimens, and that is how it got its name. The other advantage of the dissecting (stereo) microscope is that larger specimens can be observed. However, it views surface features of specimens only.

Another microscope with two sets of lenses is the **compound microscope**. It is called compound because it compounds the light by passing it through two or more lenses in order to magnify the object being viewed. The two sets of lenses are the ocular or eyepiece lens (near the eyes) and the objective lenses (near the object). A compound microscope may have three or more objective lenses (typically, 4x, 10x, 40x, and 100x). Hence, this type of microscope can give as much as 1000x magnification of an object , when a 100x objective lens is compounded (multiplied) by the eyepiece lens (typically 10x), which is higher than what is usually the magnification of a stereomicroscope (120x, even with an eyepiece magnification of 20x). Another feature of the compound microscope is that it can view the inner structure of the specimen, although in two dimensions only.

C. Contrasting Enhancement Techniques in Light Microscopy

Three contrasting enhancement techniques in light microscopy are hereby discussed, i.e., **brightfield microscopy (BF), dark field microscopy (DF**), and **phase contrast microscopy (PC)** (Kim 2013)**.** These techniques basically depend on the interaction of the specimen with light or adjustment of image contrast with the background. **Brightfield microscopy** is the usual conventional technique, which is suitable for observing the natural colors of specimens, living specimens, and even stained samples. The specimen appears dark on a bright background. In this case, the sample illumination is transmitted, i.e., illuminated from below and observed from above. However, in **dark field microscopy**, the specimen appears to be bright against a generally dark background. This type of microscopy produces a light cone which reaches the objective only when it is scattered by the sample (Kim 2013). This reinforces the image contrast. A brightfield microscope can be converted into a dark field type if the former has a condenser with a filter holder, by placing a patch stop filter into the filter holder. This is generally used to observe thin and unstained specimens. However. DF microscopy needs an intense amount of light to work with and usually shows less detail than BF microscopy (Kim 2013). It is also very sensitive to dust, so that a small amount of dust will already light up in the dark background. **Phase contrast microscopy** requires a special phase contrast objective and a special phase contrast condenser (Kim 2013). The optics will convert the differences in refractive index of the specimen into brightness differences. This will cause transparent objects to appear brighter or darker than their background. This technique is useful in observing special structures of living and unstained specimens. However, the parts are expensive. There are certain microscopes that are equipped with all the special optics for brightfield, dark field and phase contrast microscopy. For example, at the Plant Molecular Biology Laboratory of the International Rice Research Institute (IRRI), all the special optics are found in their microscopes, namely, a Carl Zeiss Axioplan microscope, an Axiophot epi-fluorescence microscope, and an Olympus BX 53 microscope.

Figure 13.1 shows the light micrographs of a young colony of the cyanobacterium, *Nostoc commune* Vaucher, taken with a light microscope in three different contrasting enhancement techniques, as brightfield (A), dark field (B), and phase contrast (C). The colonial sheath is visible both in dark field and in phase contrast. The specimen observed under the phase contrast microscope (C) shows the cellular components in the short trichomes of the cyanobacterium, while in brightfield (A) and in dark field (B) the trichomes appear as thread-like structures without showing that they are made of group of cells in a linear fashion.

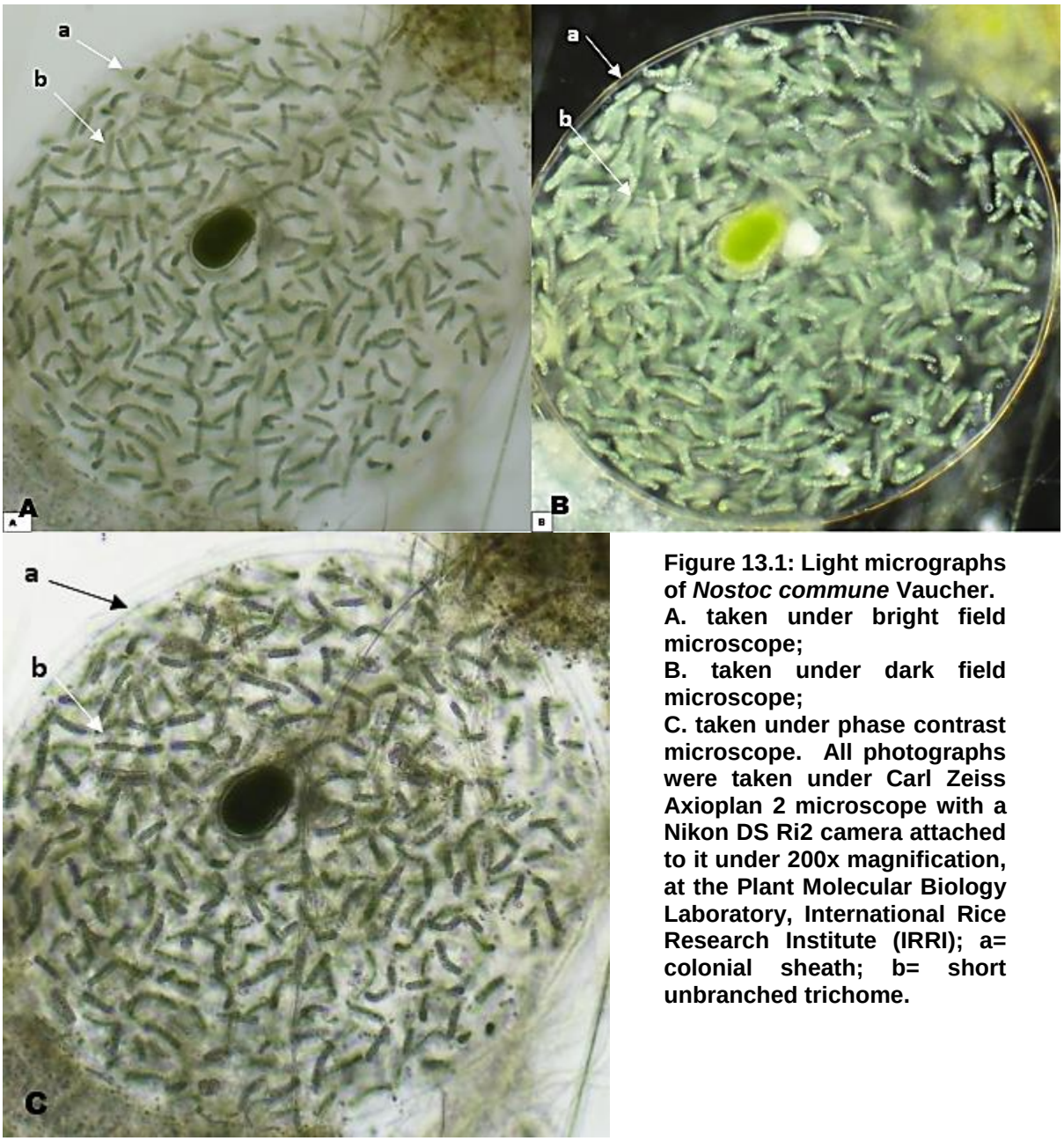


Figure 13.1: Light micrographs of *Nostoc commune* Vaucher.
A. taken under bright field microscope;
B. taken under dark field microscope;
C. taken under phase contrast microscope. All photographs were taken under Carl Zeiss Axioplan 2 microscope with a Nikon DS Ri2 camera attached to it under 200x magnification, at the Plant Molecular Biology Laboratory, International Rice Research Institute (IRRI); a= colonial sheath; b= short unbranched trichome.

II. Parts, Functions, and Operation

A. Parts and Functions

a. Compound microscope

A **basic compound light microscope*** has basically two parts, i.e., the **mechanical** and **optical parts** (Corrington 1941). The **mechanical parts** are secondary but necessary for support, operation and exactness of results, and may include the base, arm, stage (plus stage clips), dust shield, draw tubes, adjustments (coarse and fine), and revolving nosepiece. The **optical parts** are the primary main parts that give us the magnified and resolved image of the specimen or object.

Figure 13.2A shows the parts of a compound microscope, while Fig.13.2B shows the hypothetical optical parts that include the ocular eyepiece, objective lenses, the condenser, and the mirror. The **mirror** is an optical part that first receives and collects light. It has two reflecting surfaces, one on

*Descriptions and illustrations are based on the Euromex compound microscope and Euromex Model STD 9239916 for the dissecting scope. Another microscope model will have a light deviation from these models.

each side, one flat and the other concave. The flat surface should always be used when there is bright light and the microscope is equipped with a condenser to diffuse excessive light. Conversely, the concave mirror is used when there is not much available light. However, there are microscopes that have a light bulb as a light source. Above the mirror is the **condenser.** Based on its name, it focuses the beam of light on the specimen. This is equipped with an iris diaphragm, with a protruding lever, that controls the amount of light that will be delivered. The lever could move clockwise to open the iris diaphragm and counterclockwise to close it. The **optical lenses** are found above the stage. The first lens is the **objective (or object lens)**, so named because it is just above the object. The second lens is the **eyepiece or ocular**, at the top end of the draw tube, where the eye looks through. The objectives are long narrow tubes containing compound lenses for enlarging and resolving objects. They have different sizes that are directly related to their magnifying power. The scanner is the shortest, and allows a wider view of the object; next in length is the low power objective (LPO), while the longest tube is the high power objective (HPO) that has the greatest magnifying power and highest resolving power of the three. These are all dry objectives because they are made to be used in a medium of air. There are some microscopes that may be equipped with a special objective lens, called the oil immersion objective, which are used by immersing both the objective lens and the specimen in transparent oil of high refractive index, thereby increasing the numerical aperture of the objective lens or the resolving power of the microscope. All these objective lenses are mounted on a revolving nosepiece.

Parfocal objectives are objectives that are optically and mechanically designed so that the distance between the specimen and the aerial image is always constant. This means that in changing objectives from the LPO to HPO, one needs only slight refocusing with the fine adjustment to restore critical sharpness of the image, while the coarse adjustment does not need to be operated. This added feature saves time in microscopy work aside from avoiding glass slide breakages when the objective lens crashes onto it.

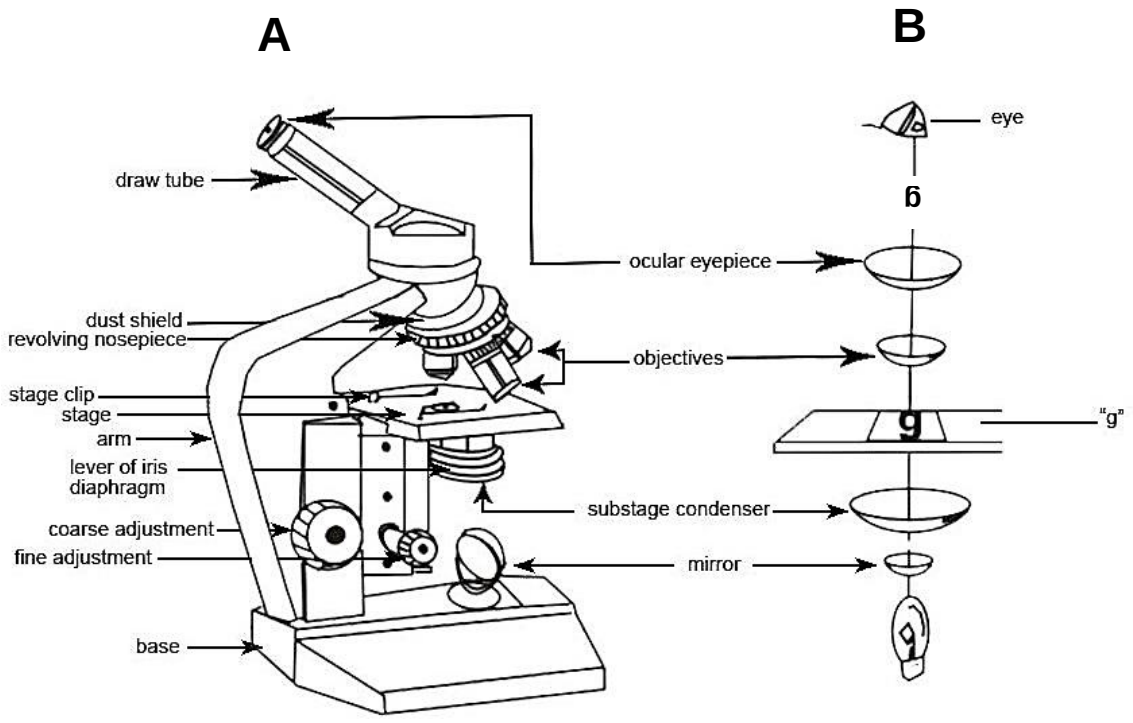


Figure 13. 2: The basic compound microscope (light microscope) (A) and its optical parts. (B) (Based on Euromex Model STD).

The **eyepiece or ocular** is so called because it is the part nearest the eye. It magnifies further the image formed by the objective and projects it. The magnification is engraved at the outer ring of the eyepiece.

Magnification and Resolving Power

The magnification and resolving power of the different lenses are summarized in Table 13. 1. **Magnification** is the extent to which the image of the specimen is enlarged as observed under the microscope**. Enlargement** occurs in two stages in a compound light microscope, at the objective lens and at the eyepiece or ocular lens. Therefore, the total **magnification** is the product of the magnifications of the objective and the ocular eyepiece lenses.

Resolving power or resolution, which is the ability of the objectives to break up an object into its component details, is mostly dependent upon its **numerical aperture (N.A.). Numerical aperture** is a measure of the microscope objective to gather light and resolve fine specimen detail at a fixed distance. The higher the N.A. of a lens, the better is the resolution of a specimen will be which can be obtained with that lens. Hence, an objective with a N.A. of 0.3 will have half of the resolving power of another whose N.A. is 0.6. Objectives with an N.A. of 1.0 or greater are achieved by placing **cedar oil (**with a refractive index greater than 1.0) between the lens and the cover slip. These are called **oil immersion objectives (OIO)** with 100x magnification. However, some compound microscopes may not be equipped with an OIO. Magnification and numerical aperture are engraved on the barrel of the objectives by a whole number followed by a decimal number, respectively.

Table 13. 1: Magnification and resolution features in a compound microscope.

Name of objective lens	Magnification	Numerical Aperture	Eyepiece lens magnification	Theoretical final magnification
Scanner	4	0.10	10	40
Low Power Objective (LPO)	10	0.25	10	100
High Power Objective (HPO)	40	0.65	10	400

b. Dissecting microscope

The **dissecting microscope** (Fig. 13.3) has basically the same mechanical and optical parts as the compound microscope but simpler than the compound microscope, both in terms of parts and functions. Fig. 13.3 shows the mechanical (A) and the optical parts (B). The basic **mechanical parts** include the 1) microscope base, 2) the stage and the attached stage clips, and 3) vertical limb or draw tube. The main function of these parts are to house, support and protect the optical parts. The **optical parts** include a single objective lens and two eyepiece lenses. These lenses are two separate light paths transmitting the image of the specimen to each of the eyepiece. The beam of light comes from the above the specimen. The usual maximum magnification is 120x.

B. Operation of the microscope

a. Compound microscope

Focusing

With the scanner in place, the observer looks through the eyepiece and adjusts the mirror, turning the coarse adjustment knob to ensure proper and uniform illumination. He/she begins by having this circle of light, the field of vision, as bright as possible using the scanner. If it is too bright, the brightness is reduced to a moderate amount by manipulating the iris diaphragm.

The observer then places a prepared slide with a letter “g” at the middle of the stage hole, securing it in place with the two stage clips holding both ends of the slide. He/she then moves the scanner all the way down by turning the coarse adjustment knob clockwise, and looks through the eyepiece, slowly moving the scanner upward by turning the coarse adjustment knob counterclockwise until the letter “g” comes into focus.

When the letter “g” is in focus, the observer has reached the right **working distance (WD)** between the specimen and the objective lens because the specimen is in focus. **Working distance** decreases as actual image magnification increases.

When the observer looks through the eyepiece again, he/she observes that the letter “g” is upside down in comparison to what can be seen with the naked eye on the stage. This is called a **virtual image**.

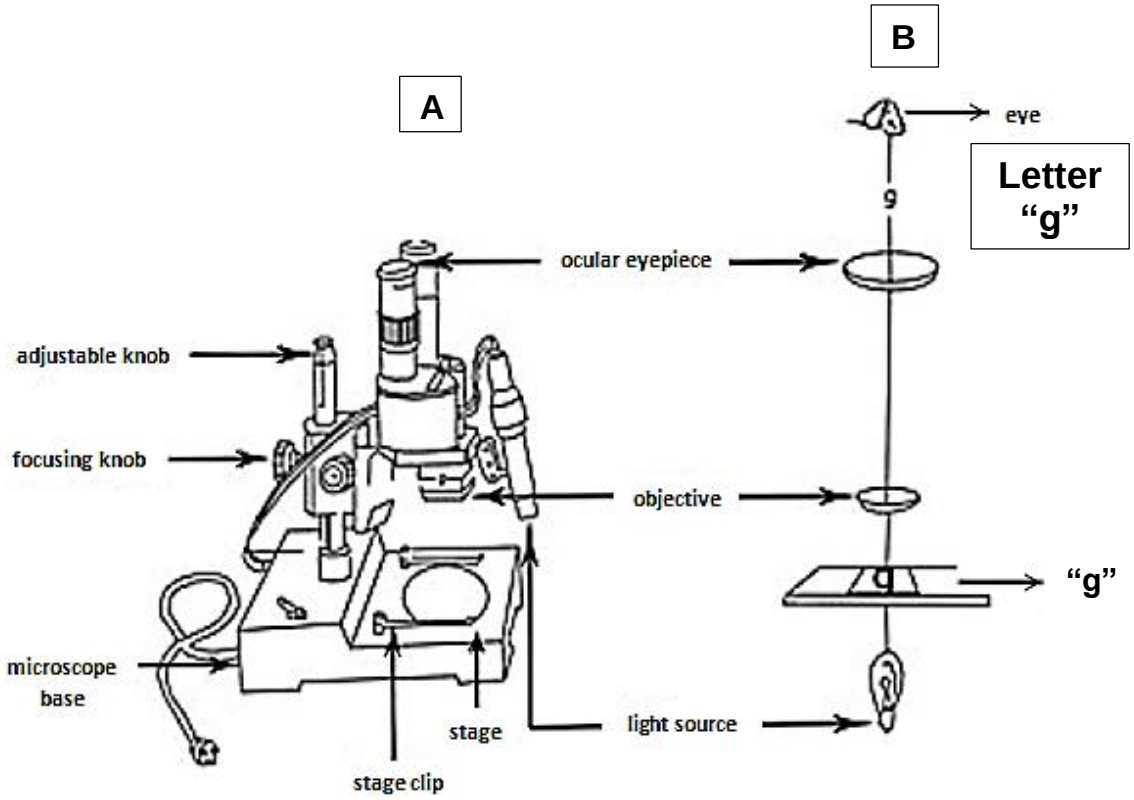


Figure 13.3: The dissecting microscope (A) and its optical parts (B) (Based on Euromex dissecting microscope, Model STD 9239916).

Orientation of images
Using the scanner of the compound microscope, the observer moves the slide “g” to the right, and sees the head. When he/she reverses the movement, and he/she will see the tail of the letter. If the slide is moved slightly upwards, more of the tail can be seen. Hence, if the observer wants to see more of the lower part of an object, he/she moves the slide upward, while if he/she wants to see the parts at the right end, he/she moves the object toward the left.

While looking through the ocular, the observer focuses and magnifies a particular point of the letter’s tail by turning the nosepiece until the LPO is in position. Then he/she puts the HPO in position.

In general, if the observer wants to see a particular point of a specimen, he/she places it at the center of the field of vision when focusing under LPO before shifting to the HPO. This minimizes moving the slide too much under HPO.

If the observer sees the image immediately, then the microscope being used is **parfocal** because the object is in approximate focus without further adjustment when switching from the LPO to the HPO and back again.

Depth of Focus
Depth of focus is a property of the microscope that indicates the thickness of a specimen that can be placed in focus under the microscope at one time. This can be demonstrated by observing

under the LPO a prepared slide of “threads” of three colors – green, red, and yellow. When the green thread is placed in focus, the red and yellow threads appear blurred. Try moving the coarse adjustment knob towards you and away from you until you will note that there is depth in the threads. The threads may appear from top to the bottom as green, yellow, and red, which is the actual arrangement of the threads on the slide.

b. Dissecting microscope
Observation of the specimens under the dissecting microscope gives the same orientation as what you see with your naked eye, except that the specimen is magnified. This is one reason that this equipment is a useful tool in dissecting specimens. The image that you see is called the **real image**.

The orientation of images observed under the compound light microscope is inverted and reversed mainly because of the convex lenses in the objectives and in the eyepiece, hence the light that passes through these lenses is refracted or bent. Therefore, the image is refracted through the objective lens, so an inverted image is produced in the objective lens. The eypepiece does a little more magnification of the inverted image, hence, producing an enlarged **virtual image**. On the other hand, in stereomicoscope or dissecting microscope despite having the same convex lenses as in the compound microscope, there is an additional **pair of erecting prisms** or **mirror system** that de-rotates and inverts the magnified image received from the objective lens and present it to the observer as it would appear as a real image, or as if it is observed without a microscope (Nothnagle et al. 2020).

III. Specimen measurements under the microscope

The linear measurements of specimens can be done under the microscope provided that there is a glass disk marked with scales, usually from 0 to 100, that is inserted to the eyepiece of the microscope This is called a **micrometer eyepiece disk** or **reticle** or **graticule.** However, the distance between the lines is not a known unit. It must be calibrated with a **stage micrometer** under different objectives of the microscope to give accurate measurements in micrometers. **Calibration** means comparing between a known measurement, which in this case is the stage micrometer, and the measuring instrument, or micrometer eyepiece or ocular micrometer scale. A **stage micrometer** is a simple microscope slide with a finely divided scale marked on the surface. Each distance between the two lines is of known true length, has a scale graduated in units of 1/100 mm, and is used to calibrate the **micrometer eyepiece** disk.

A. Calibration of the micrometer eyepiece with that of the stage micrometer.
The process of calibration is exemplified below.

The observer inserts the round micrometer glass disk or ocular micrometer into the eyepiece of the microscope, and focuses the eye lens so that the scale appears with maximum sharpness. Then, he/she takes the stage micrometer and places it on the stage of the microscope, and focuses on the scale of the stage micrometer. The stage micrometer has a scale that has been calibrated, i.e., each space in between the two vertical lines are of known unit of measurement. In this case each space is equivalent to 0.01mm.

Both the scales of the ocular micrometer (om) and those of the stage micrometer (sm) now appear sharply defined. Turning the eyepiece around puts the scales parallel to one another. The observer then moves the stage micrometer to allow its first line to coincide perfectly with the first line of the ocular micrometer scale, as in Fig.13. 4. At this point, he/she looks for another pair of lines (one from the ocular micrometer and the other one from the stage micrometer) that perfectly coincide with one another.

In between the intervals (coinciding lines), one can determine the number of smaller lines in both the om and the sm. For example, in Fig. 13.4 the number of smaller lines in the om, between nos. 2 and 3, is 10, while the number of lines in the stage micrometer is 5. Since each space in the

stage micrometer is equivalent to 0.01mm (or 1/100mm), therefore, the calibration factor (CF) of each space of the optical micrometer under this objective is computed based on the formula below:
CFo = (10/5) (0.01)
= 0.02 mm
= 20 µm
Hence, each space in the **om** under this objective is equivalent to **20µm**.

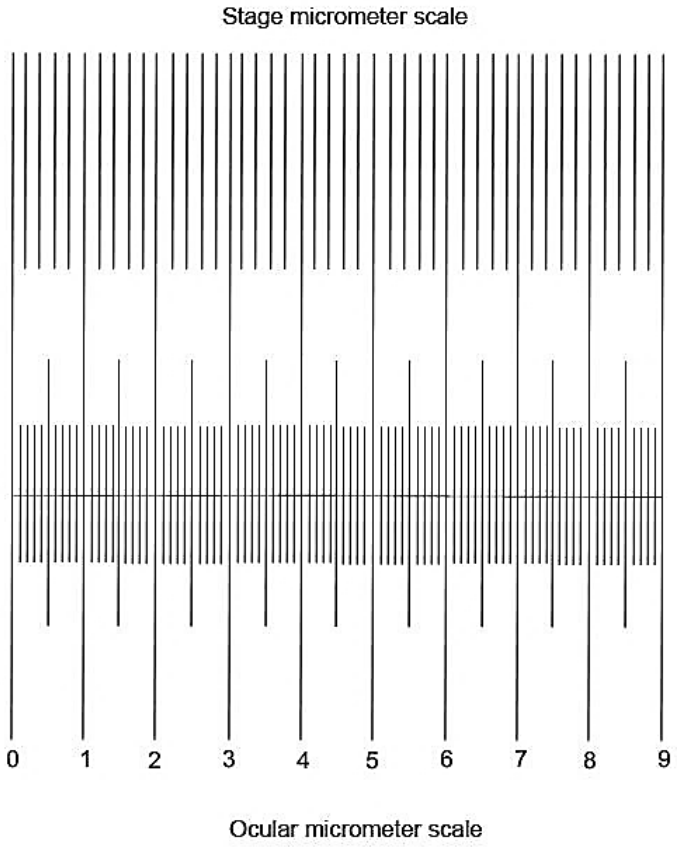
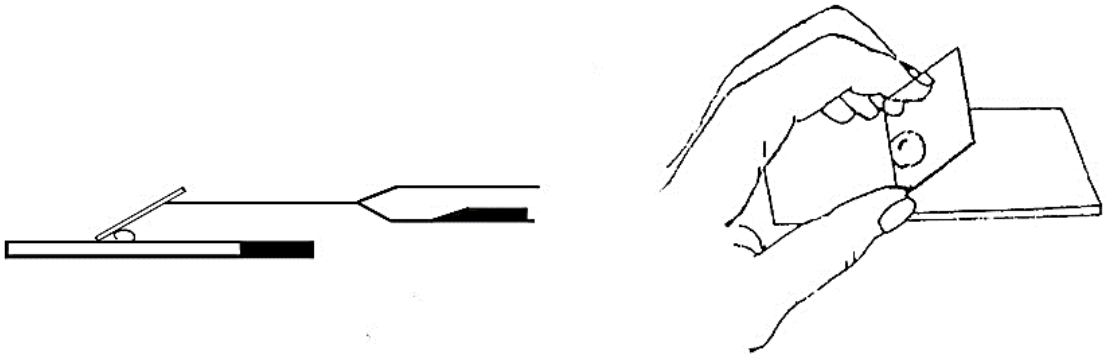


Figure 13.4: The ocular (eyepiece) and stage micrometer scale

IV. Preparation of Temporary Mounts

Temporary mounts are usually made for immediate observation and study of fresh materials (Corrington 1941). Water is used as the mounting medium and they are usually unsealed. If this is the case, the microscope should not be tilted when in use to avoid water spillage on the stage. Another problem with temporary water mounts is the drifting of cells due to water evaporating from the edge of the cover slip. This can be prevented by sealing the cover slip with clear nail polish or with petroleum jelly but preferably the latter is done when oil immersion objective lens is used. Also in most cases, the delicate specimens, like the flagellates should be observed first so that they are not destroyed over time due to intense illumination and/or warming up of the equipment.

The observer places a slide on a white sheet of paper, so that the observation can be clearer. Places a drop of pond water, taken from the bottom of the jar, at the center of the slide. He/she then picks up a cover slip by its edges between the thumb and forefinger, then lets one edge of the cover slip touch the outer ring of the water mount while the other end is held in a slanting position by a needle as shown in Fig 13.5 A-B.



A B

Figure 13.5 Preparation of temporary mounts involving careful placing of coverslips over the specimen (a and b).

Then the cover slip is then gently lowered to avoid trapping bubbles. A piece of filter paper is used to absorb excess water coming out of the edge of cover slip. The setup is mounted on the stage of the microscope and allowed to stand for 1-2 min before being studied.

V. Summary

The microscope is an important tool in biology. It allows us to observe minute organisms and detailed cellular structures that are not visible to the naked eye. There are three contrasting enhancement techniques presented in light compound microscopy, i.e., brightfield microscopy, dark field microscopy, and phase contrast microscopy. The basic parts of a compound microscope are divided into two major parts, the mechanical and the optical parts. These parts are described together with their functions. The compound microscope is differentiated from the dissecting microscope in this treatise. A detailed discussion in calibrating the ocular micrometer eyepiece is also presented. The preparation of temporary mounts is demonstrated together with the use of vital stains.

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Chapter 14. Fluorescence Microscopy: Instrumentation and Applications

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I. Introduction

A. History of Fluorescence Microscopy

Fluorescence has long been observed in organic compounds. The earliest record of this was made in the 1560s by Bernardino de Sahagun and Nicolas Monardes (Acuña et al. 2009). They reported that a particular white wood gave clear water a bluish hue when infused in it. The Aztec society called the infusion coatli which they used to remedy urinary problems. Monardes called the medicine lignum nephriticum indicating its wood origin and use. News about lignum nephriticum reached Europe by the 17th century, and wooden cups that exhibited the phenomenon when filled with water became popular in the continent. It was later identified that the plant sources for the original coatli and the wooden cups were different. The organic compound responsible for fluorescence in coatli is not present in the plant but an end product of oxidation of flavonoids found in the Mexican kidneywood tree source (Acuña 2009). The wooden cups, however, may have originated from narra wood exported from Philippines to Mexico, then to Spain (Safford 1916). In 1819, Edward D. Clarke observed the same phenomenon in fluorspar, a mineral made of calcium fluoride (Clarke 1819). Decades later in 1845, while observing a colorless quinine solution illuminated by a white light, John Frederick Herschel remarked that it exhibited a “beautiful celestial blue colour” (Herschel 1845). Motivated by Herschel's work, George Gabriel Stokes performed experiments on various substances and minerals in 1852. Stokes observed that certain materials emit light at a wavelength longer than the wavelength of light to which they were exposed

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(Stokes 1852). He called the phenomenon fluorescence, which is a term analogous to the term opalescence, another phenomenon derived from a mineral, in this case opal. Stokes suggested the use of fluorescence in chemical tests for compound separation and recognition. The use of ultraviolet light (UV) and fluorescence later became critical in the development of high resolution microscopic images.

In optical imaging instruments, resolution is limited by diffraction, as shown by Ernst Abbe in 1873. As a method to improve resolution, he proposed using ultraviolet light (Lipson et al. 1998). This was realized with the development of an ultraviolet microscope in 1904 by August Kohler and Moritz von Rohr (Land 1949). In 1908, Kohler, together with Henry F.W. Siedentopf, invented the fluorescence microscope (Young 1961). After a few years, fluorescence microscope became commercially available through the separate firms of Carl Reichert and Carl Zeiss. Since shorter wavelength light such as UV provided better image resolution and contrast, it became an essential light source for fluorescence microscopy. Developments in quantum mechanics, numerous investigations into fluorescent materials, and advances in optoelectronic and digital technologies paved the way for newer imaging techniques. For example, the use of laser and light-emitting diodes (LEDs) provided even better light sources. Optical filters made of engineered materials are now available in image formation. Higher temporal resolution of cameras and detectors enriched our understanding of various systems through dynamical and kinetic studies. Some of these exciting techniques pushed the spatial resolution beyond the diffraction limit, allowing objects at a few nanometers to be resolved even using only visible light (Schermelleh 2019).

B. Importance of Fluorescence Microscopy

Fluorescence microscopy is a powerful microscopy technique for structural and functional dynamics studies of biological specimens (Liu 2015). Research and development about fluorescence microscopy may be divided into three categories – development of fluorescent materials, enhancement of imaging techniques, and understanding of biological processes and functions. In the last decades, fluorescence microscopy has developed various techniques such as fluorescent wide-field microscopy, point scanning confocal microscopy, light sheet microscopy, total internal reflection microscopy, and super resolution microscopy, just to name a few. These techniques have since become common tools used by biologists in the study of prokaryotic and eukaryotic organisms including *Arabidopsis* and rice, among others. Choosing which technique to use depends on the nature of the sample. For example, for a dynamic (live or motile) sample, wide-field, confocal, and light sheet techniques are appropriate, while super resolution is best used for static samples. Sample thickness is also an important consideration when choosing which technique to use. Total internal reflection microscopy could only probe thin sections, about 15 μm or less, while light sheet microscopy and point scanning confocal microscopy could be used for samples thicker than 15 μm.

Fluorescence microscopy is an emerging tool in the phenotypic characterization of algal species. Phycologists use this technique to study the relationship of structures and functions in algae. For example, fluorescence microscopy is used in distinguishing photosynthetic flagellates from non-pigmented ones, and phytoplankton composition and abundance (Ansotegui et al. 2003). The fluorescence of green photosynthetic pigments, an indicator of photosystem II, can be used as an indicator of how algal species respond to environmental changes. This microscopy technique is also used in biofuels applications, specifically in the rapid and simple detection of **revertant** cells in an ethanol-producing cell population. Schulze et al. (2015) noted that in studying the cyanobacteria *Synechocystis* and *Synechococcus*, they were able to separate the non-ethanol producing cells (red) from ethanol producing cells (orange) by using a specific filter set in the microscope and using phycocyanin, the natural fluorophore of cyanobacteria. By considering the temporal behavior of fluorescence, kinetic studies could be performed and could elucidate some time-dependent behaviors of chlorophyll in photosystem-related processes (Suggett et al. 2010). The strength of fluorescence microscopy lies in its capability to probe higher spatial and temporal resolution of biological specimen with the use of fluorescent materials (Coling 2001). This chapter provides a basic introduction to fluorescence microscopy principles and instrumentation. Just like

any tool, the usefulness of fluorescence microscopy relies on how the user is skilled in microscopic techniques and knowledgeable of the principles of fluorescence. Familiarity with the basic optical principles allows the user to solve basic problems in image formation, or design a new experiment. This chapter also presents the applications of fluorescence microscopy in studies of algae and other plant species, and its advantages and challenges. The reaction of algae to light as in photosynthesis (Dionisio-Sese 1990), phototaxy (Choudhary 2019), attachment onto surfaces (Kreis 2017), photoprotection (Dionisio-Sese 2010; Fadeev 2012), and biofuel production (Arguelles 2020; Shih 2014) involves different photophysical processes. The high spatial and temporal resolution achieved in fluorescence microscopy allows deeper examination of these processes in the photobiology of algae.

II. Physical Basis of Fluorescence

Fluorescence is one form of a quantum mechanical process known as **photoluminescence**. Fluorescence happens when quanta of light known as photons excite a molecule to an energetic state. During **excitation**, electrons absorb photons with frequency v and their energy are raised to a higher state with an energy increase equivalent to E in the formula given below:

$$E = h\nu = hc/\lambda \quad [1]$$

where h is the Planck's constant (6.63×10^{-34} Js), ν is the frequency of light, c is the speed of light (2.998×10^{8} m/s) in free space, and λ is the wavelength of light.

Excited electrons go from the excited state (higher energy) to the ground state (lower energy) in a process known as **relaxation**. This process is usually accompanied by photon emission where the wavelength of light emitted (λ_E) is longer than the excitation light (λ_A) (Figure 14.1). The difference in wavelength ($\Delta\lambda = \lambda_E - \lambda_A$) is known as **Stokes shift**. In some molecules, another form of luminescence may occur, known as phosphorescence, wherein excited electrons undergo intersystem crossing to a triplet state through a non-radiative process as illustrated by the Jablonski diagram in Figure 14.2. From this state, electrons go down to the ground state with an even longer wavelength.

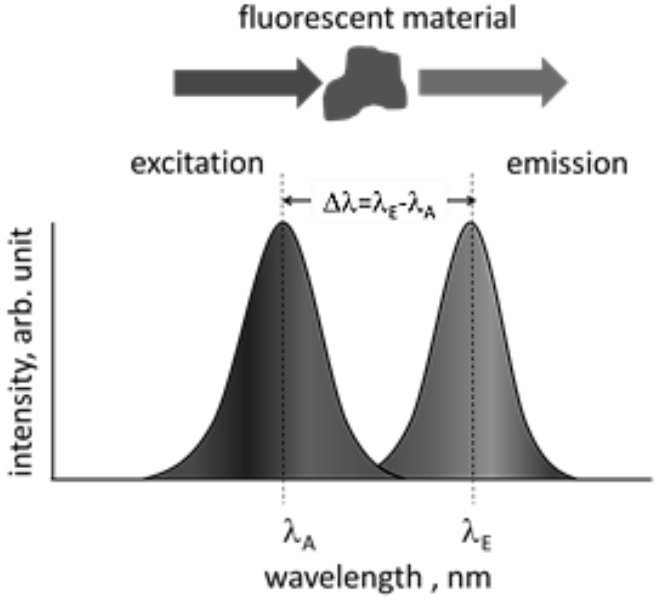

Figure 14.1: Resulting emission band with peak at a wavelength longer than the excitation light. The difference between two peak wavelengths is known as Stokes shift. From equation [1], a longer wavelength implies that the emitted photon has lower energy than the incident photon. The lost energy is involved in different non-radiative processes as shown in the Jablonski diagram in Figure 14.2.

As shown in Figure 14.2, the wide range of energies associated with excitation results in a broad spectral band for the emission. In an ideal situation, the spectrum of both the excitation source and emission have narrow bands. However, the actual spectrum of the excitation source shows a

band with a peak at a particular frequency. The excitation light is tuned so that it matches the resonance frequency of the fluorophore. The resulting emission also shows a band whose peak wavelength is longer than the excitation source. In order to make this process useful for microscopic applications, the bands are trimmed using filters.

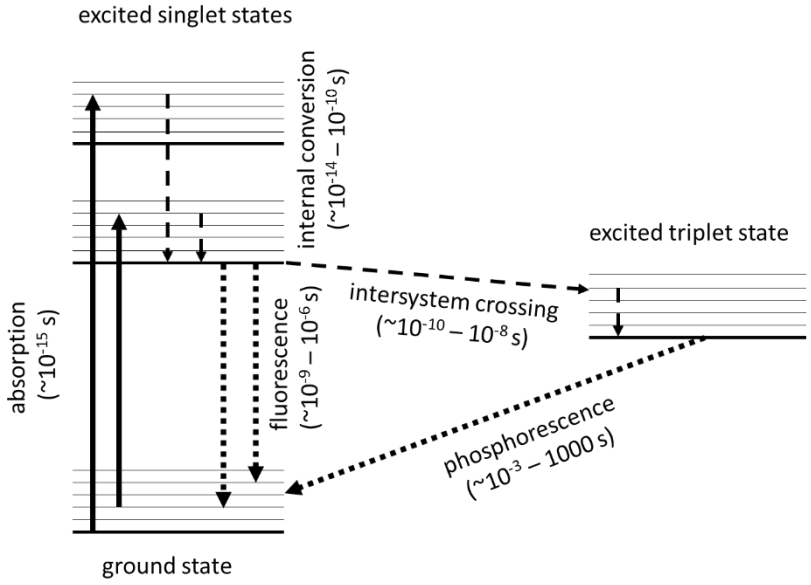

Figure 14.2: Jablonski diagram illustrating how photons are absorbed (solid line) and emitted (dotted lines), and the non-radiative processes (dashed lines) involved and their approximate time scales. The horizontal lines represent the allowed energy states for electrons.

Both **fluorescence** and **phosphorescence** emit at wavelengths longer than the excitation light. However, the difference lies in the rate at which photons are emitted. **Fluorescence** is a fast process, ranging from 1 nanosecond to 100 nanoseconds. On the other hand, **phosphorescence** is a slow process, ranging from 1 millisecond to 1 second (or up to an hour). To visualize the difference between fluorescence and phosphorescence, consider the light emission by a material when a pulse of ultraviolet light is incident as shown in Figure 14.3. Before the pulse hits the material, no emission could be observed. The material begins to emit light as the pulse of ultraviolet light reaches it. When the incident light is turned off, emission would almost instantaneously stop if the material is fluorescent. If on the hand the material is phosphorescent, it would continue to glow for 1 millisecond or longer after an incident light is turned off. Some objects take advantage of this phenomenon such as early warning devices, street signs, tools, and glow-in-the-dark toys.

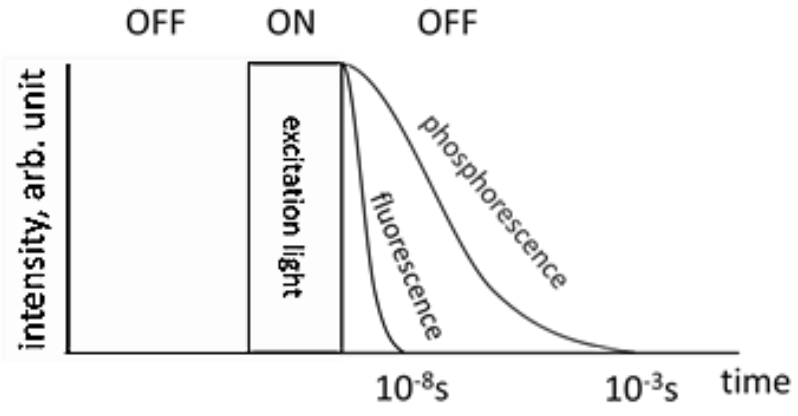

Figure 14.3: Both processes continue after turning off the excitation light. Light emission due to phosphorescence extinguishes slower than fluorescence.

III. Components of the Fluorescence Microscope

A. Fluorophores and Fluorochromes

A **fluorophore** is a fluorescent molecule or a functional group that absorbs light at a given wavelength and emits light at a longer wavelength. The light emitted from fluorophore could range from ultraviolet visible light to infrared. Fluorophores are usually characterized in terms of their extinction coefficient, quantum yield, and susceptibility to photobleaching. A good fluorophore would have a wide Stokes shift because a big difference in wavelength distinguishes the excitation light from the emission light.

Fluorophores can be broadly classified into two main classes - intrinsic and extrinsic (Lakowicz 2006). Intrinsic fluorophores are those that can be found naturally. Chlorophyll, a photosynthetic pigment found in plants, algae, and cyanobacteria, falls under this class. Intrinsic fluorophores also include aromatic amino acids, NADH, neurotransmitters, and flavins. One of the commonly used intrinsic fluorophore is green fluorescent protein (GFP). Its discovery in a jellyfish species in 1961 by Shimomura enabled labeling of single proteins in living systems (Shimomura 1962). It was later shown that GFP and other similar proteins could be engineered leading to other colors of fluorescent protein. Extrinsic fluorophores, on the other hand, are synthetically produced dyes or modified biochemicals that are added to the non-fluorescing samples. Addition of extrinsic fluorophores aims to change the spectral properties of the sample. These include quantum dots and organic dyes. Organic fluorophores typically contain aromatic groups or cyclic molecules (Rost 1996) as shown in Figure 14.4. The alternating single and double bonds, known as conjugated structures, act as antennas where electrons absorb photons as they resonate along the π-orbital.

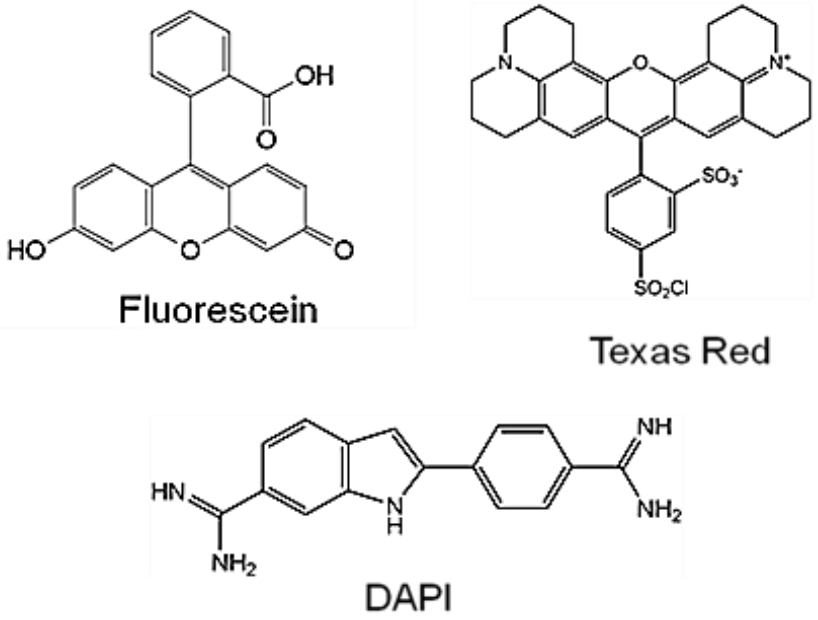

Figure 14.4: Chemical structure of common fluorophores used in wide field fluorescence microscopy. The presence of a conjugated structure or alternating single and double bonds provides a resonant system for absorbing light. Longer conjugated double bonds absorbs lower energy or longer wavelength light. DAPI= 4',6-diamidino-2-phenylindole

"Fluorochrome" is commonly used interchangeably with "fluorophore." However, fluorochrome refers to any fluorescing chemical or dye used in biological staining while fluorophore is any molecule or functional group that fluoresce. Depending on the chemical composition and staining characteristics, fluorochromes attach to specific areas of the specimen and leave others unstained. For example, DAPI or 4′,6-diamidino-2-phenylindole is a blue fluorescent stain that

intercalates in the AT region of double stranded DNA. Because of its high affinity to DNA this dye is often used as a nuclear stain. The standard nomenclature based on color index (CI) is published by the Society of Dyers and Colourists in collaboration with the American Association of Textile Chemists. The classification and naming of fluorochromes and other stains are discussed by Kiernan (2001). Some examples of commonly used fluorochromes are derivatives of or related to acridine, fluorescein, stilbene, or xanthene (Table 14.1).

Table 14.1: Some examples of fluorochromes used for fluorescence microscopy.

Name	Excitation Maximum/ Color Range	Emission Maximum/ Color of fluorescence	Application	Structures stained
Acridine orange	502 nm, cyan for DNA 460nm, blue for RNA	525nm, Green if double stranded DNA, 650nm, Red if single stranded RNA	DNA/RNA study	DNA RNA
Aniline blue	395 nm, UV	495 nm	Botany	(1 → 3)-β-D-glucan
4',6-diamidino-2-phenylindole {DAPI)	358 nm, UV	461 nm, blue	Cytology	Adenine-thymine rich regions of DNA
Calcoflour white	380 nm, UV	475 nm, Blue	Botany	Cellulose and chitin of cell walls of fungi and other organisms
Fluorescein isothiocyanate (FITC)	495 nm, blue	519 nm, yellow	Immunology	Detection of antigen-antibody reactions
Propidium iodide	493 nm, green	636 nm, orange red	Cytology	Cell viability assay, DNA content in cell cycle analysis

B. Fluorescence Microscope

Fluorescence microscopy makes use of fluorescence to achieve contrast in biological imaging. High contrast visualization enables the investigation of cellular or molecular structure, dynamics, and function at high spatial and temporal resolution. Tagging components of a sample with fluorophores emitting at different wavelengths further improves image formation. The simplified design and path of light in fluorescence microscopy are illustrated in Figure 14.5. There are six major components of fluorescence microscope – **light source**, **excitation filter**, **specimen**, **dichroic mirror**, **emission filter**, and **detector**. A high intensity excitation beam from a light source passes through an excitation filter. The transmitted light is then reflected by a dichroic mirror and directed to a specimen through an objective lens. Fluorescence emission from the

specimen is passed to the **dichroic mirror** and through a **barrier filter**. This filter blocks excitation light and only allows the emission light. The transmitted filtered light then passes through an emission filter for detection.

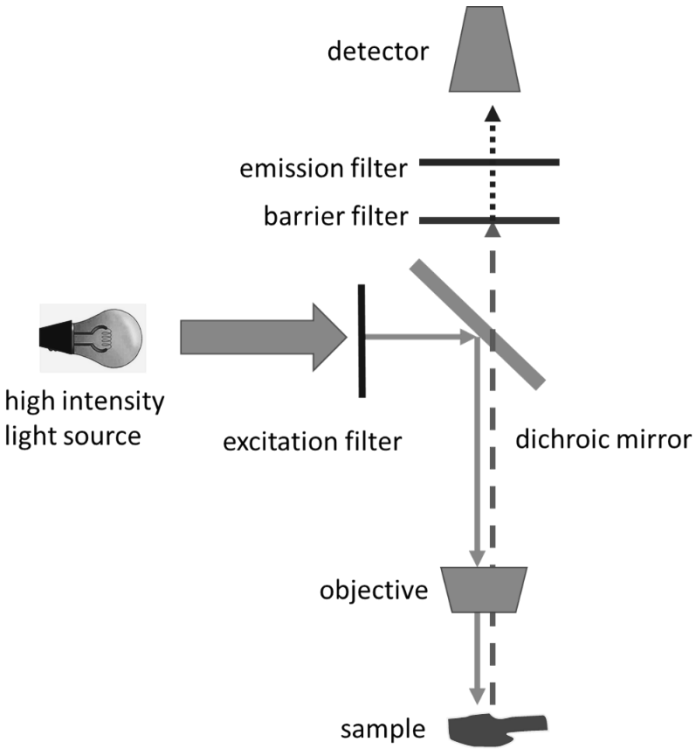

Figure 14.5: Beam path for fluorescence microscopy in an upright microscope. Different filters and mirrors are positioned to tune excitation light and emission light to produce a better image as described in Figure 14. 6

Light source

A light source is a crucial component of the microscope. The generation of stable high intensity light is required to excite the fluorophores. Traditionally, **mercury arc lamps** like **HBO** (H for Hg or mercury, B is the symbol for luminance, and O denotes unforced cooling) offer reliable broad spectrum illumination with a strong UV component for wide-field fluorescence microscopy. Another common source would be **metal halide (MH) lamps**. They have uniform power output across the spectrum compared to HBO and have longer bulb life time. Before any measurement, these lamps are given enough time to warm up to make sure that the emission spectrum has reached equilibrium and is stable. Both lamps contain mercury, posing environmental and health risks. In recent years, solid state **light-emitting diodes (LED)** are used as a superior alternative. LED as a light source has a narrow band with peak at a desired wavelength, is more stable than arc lamps, uses less electrical power, can be readily switched on and off, and has a longer lifetime.

Excitation filter

An ideal fluorophore would be one with a large Stokes shift as this would easily separate the excitation band from the emission band. Images formed are based on emitted light where the excitation light is filtered out. If both bands have a large overlap, it would be difficult to form an image. Further separation is needed using filters to ensure that image formation would result in high spatial resolution and contrast. Selection of filter properties should be made in consideration of the fluorescence emission spectrum as shown in Figure 14.6.

From the light source, the beam enters the excitation cube passing through the excitation filter. The function of the excitation filter is to isolate a narrow spectral band that excites the fluorophore. **Excitation filters** are made up of specially-coated optical glasses. The thin film coating could be

made as a long pass filter (which allows only longer wavelengths), a short pass filter (which allows only shorter wavelengths), or a band pass filter (which allows only a range of colors). Nowadays, most excitation filters operate as band pass filters, so that the excitation of unwanted fluorescence signals from the specimen and other sources is reduced.

Dichroic mirror or dichromatic beam splitters

Dichroic mirrors or **dichromatic beam splitters** are usually interference types that act as band pass filters. Using a dichroic mirror, only a narrow spectral band is transmitted, while the other portions of the spectrum are reflected. The band pass filtering capability of the splitters is achieved by using a specialized coating that allows the transmission of a certain spectral range. As a mirror, it is used to direct the excitation beam towards the specimen.

Emission or barrier filter

The **emission filter** functions similarly to the excitation filter. It allows only a certain spectral band from fluorescence emission, making a high contrast image with the darkest background possible. Instead of allowing a specific band, sometimes a whole range of spectra from certain wavelengths is required, and a long-pass filter may be used. Much consideration must be given in selecting the combination of filters to achieve a high resolution and good contrast image. Figure 14.6 shows the relationship between the excitation and emission bands and the filter range.

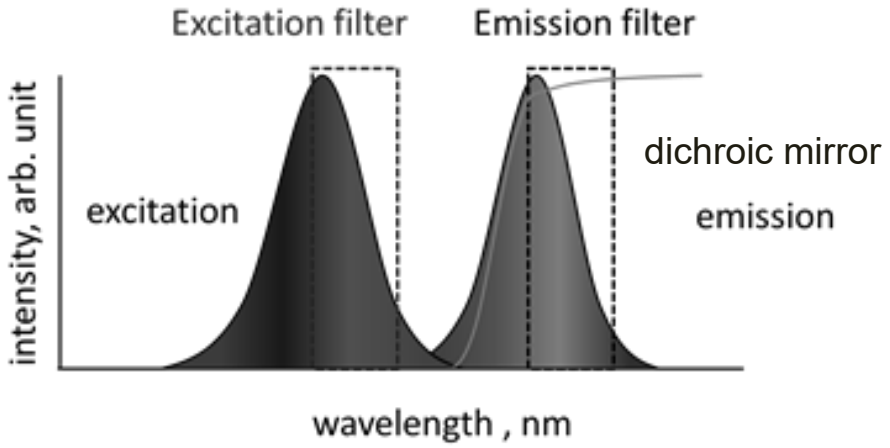

Figure 14.6: Excitation filter must allow transmission of light that contains the frequency (wavelength) of light for fluorophore excitation. In the same manner, the emission filter and the dichroic mirror must allow emission from sample while blocking excitation spectra.

IV. Advantages of Fluorescence Microscopy

Fluorescence microscopy has three important features that may be considered as its advantages over brightfield microscopy: image contrast, specificity of fluorescence labeling, and sensitivity of detection.

A. Image Contrast

Fluorescence microscopy is a common method for studying the dynamic behavior of cells, and this stems from its ability to isolate cells, individual organelles, and even proteins using multicolor fluorochromes (Kijani et al. 2015). This technique offers several advantages over other imaging techniques like brightfield microscopy, electron microscopy, and scanning probe microscopy for both *in vitro* and *in vivo* imaging. Figure 14.7 shows an example of the advantage fluorescence microscopy has over normal brightfield microscopy. Using propidium iodide and a certain filter set, one can detect live cells over dead algal cells.

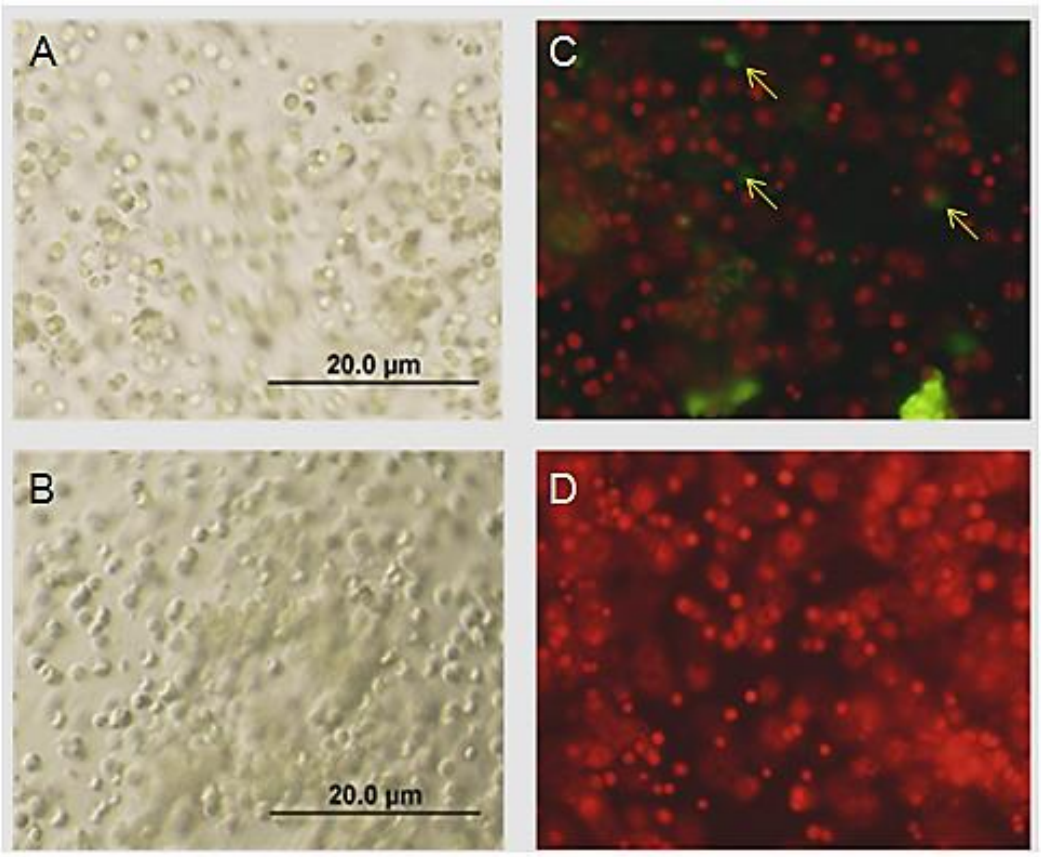

Figure 14.7: A sample of a cyanobacterium, *Chroococcus min*or (Kützing) Nägeli viewed using normal brightfield (A), phase contrast (B) and using fluorescence microscope (C&D). With the aid of propidium iodide (PI) and its filter (D), *Chroococcus min*or can be distinguished from debris within the samples. On the other hand, live algae (green colored cells with yellow arrow) can be distinguished from the dead (red) algal cells using the FITC filter set (C).

B. Selectivity** of Fluorescence Labeling

Molecules could be selectively labelled, and any change in emission intensity could be interpreted as certain processes or functions that the target molecule responds to or interacts with. However, this feature should be treated with care. Higher concentrations of dye may alter the system and cause it to emit light in a way that could be erroneously interpreted as the natural response of a system. Also, at higher concentrations, some binding sites of the dye become available for non-target molecules. Thus, dye should be strategically used in order to improve selectivity. A commonly used dye in fluorescence microscopy is acridine orange. Acridine orange is a nucleic acid- selective fluorescent dye which interacts with DNA and RNA. The monomeric binding of acridine to DNA results in green fluorescence, as seen in the vegetative cells in *Hapalosiphon welwitschii*, a cyanobacterium, (yellow arrows) but absent in the heterocysts of the same organism (red arrows) under the fluorescent microscope (Figure 14.8B). However, the nucleic acids could not be detected in unstained H. welwitschii under the brightfield microscope, although the vegetative cells (yellow arrows) can be differentiated from the heterocysts (red arrows) (Fig. 14.8A).

**Many authors use specificity interchangeably with selectivity. However, IUPAC discouraged the use of specificity and recommended the use of selectivity to refer to the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behavior. (Vessman 2001).

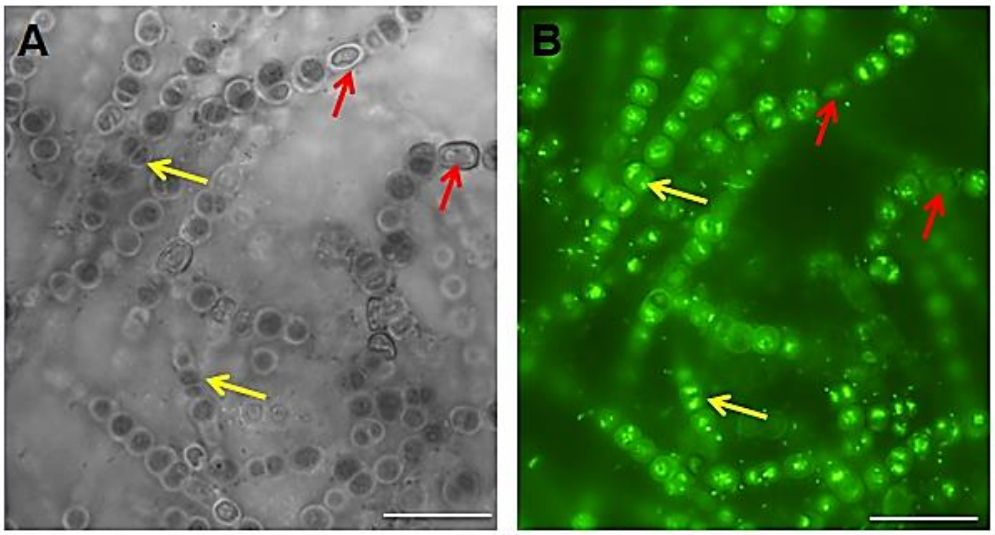

Figure 14.8: Images of *Hapalosiphon welwitschii* West & GS West viewed under brightfield mode, unstained (A) and under fluorescence mode, stained with 0.33% acridine orange (B). The yellow arrows point to the vegetative cells, and the red arrows point to the heterocysts (that look empty) in A; in B, the specificity of acridine orange for nucleic acid is attested to by its presence in the vegetative cells , by their green fluorescence, (yellow arrows) but nucleic acid is absent in the heterocysts (red arrows). Bar=50 µm, 100x.

C. Sensitivity of Detection

Unlike in brightfield microscopy which uses a broad range of visible spectrum (400-700nm) to view objects, fluorescence microscopy makes use of a desired and specific band of wavelengths to get the fluorescence signal. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are generally governed by the darkness of the background, and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence. The development of a sensitive light detector and camera made it possible to detect faint fluorescence signals. Time dependent emissions allow kinetics studies to be performed. It can also be used to differentiate microscopic techniques such as in differential interference contrast, where fluorescence and brightfield techniques are combined. An example of which is a study on the heterogeneity of fluorescence response of individual algal species (Suggett et al. 2010)

V. Tips in Using Fluorescence Microscope

Below are some guidelines on how to use the fluorescence microscope:

1. Use eyepieces with low initial magnification. For instance, use 8x magnification instead of 10x or 12.5x because fluorescence intensity decreases exponentially with an increase in total magnification.
2. Do not work with the microscope against a window. Operate it in a dark or screened corner of the room (adaptation of the eye). One can use a curtain to surround the area where fluorescence microscope is located. If it is not possible, cover the objective lens and luminous field diaphragm.
3. Use microscopes with short light paths. A long distance between the light source and the specimen means light losses at all free lens surfaces and reflecting mirrors.
4. When taking photos using a digital camera, set it at a high ISO (International Standards Organization) setting (ideally >200). Save images in either .tif, .png or .bmp file format. If necessary, analyze the image using Image J or any applicable image analysis software.

VI. Common Abbreviations of Optical Elements in Fluorescence Microscopy

Table 14.2. Common abbreviations of optical elements in fluorescence microscopy

Excitation filter	UV glass filter	UG
	Blue glass filter	BG
	Short pass filter (kurz is short in German)	SP or KP
	Exciter filter	EF
	Interference filter	IF
Beam splitters	Chromatic beam splitter	CBS
	Dichroic mirror	DM
	Teiler kante (edge splitter)	TK
	Farb teiler (color splitter)	FT
	Reflection short pass	RKP
Barrier filters	Long pass filter	L or LP
	Yellow or gelb glass	Y or GG
	Red glass	R or RG
	Orange glass	O or OG
	Kante	K
	Barrier filter	BA
When the filter type is also associated with a number, e.g. BA475, that designation refers to the wavelength (in nanometers) at 50% of its maximum transmission.		

VII. Summary

The fluorescence microscope provides high **image contrast**, allows the detection of live cells over dead cells, and **provides selectivity**. The selectivity feature is important in highlighting certain algal structures, such as those with nucleic acids, which then allows the differentiation of heterocysts from vegetative cells in nitrogen-fixing cyanobacteria. Fluorescence microscopy can also be used to perform **dynamical** and **kinetic studies** of biological functions such as lipid metabolism (for biofuel production) and photophysical processes such as photosynthesis, phototaxy, and photoprotection. It is therefore a vital piece of equipment which enhances teaching and research in biology and allied sciences. In view of the many advantages that this type of microscope provides in the academe and in research, this chapter presented a short historical perspective of this microscope and the physical principles of its operation. The physical component and its uses were also discussed to better appreciate the fluorescence microscope.

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Chapter 15. Classical Taxonomy

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I. Introduction

The diversity of plant and animal organisms, as evidenced from fossil records (3.5 billion years ago) predates the time of Aristotle (384-322 B.C.) and the diversity of fossils and living organisms has been ably and systematically catalogued. The classification of living organisms is accredited to the Swedish naturalist and physician Carl von Linné (also known as Carolus Linnaeus). Linnaeus classified organisms based on "appearances". This included classifying plants based on structures of their reproductive parts, too. For example, his system was based on *nuptiae plantarum*… the marriages of plants … which involved plants functioning as "husbands" and "wives" (Uno et al. 2001). Therefore, **classical or orthodox or traditional taxonomy,** which is also known as the **Linnean system** involves the use of morpho-anatomical features of organisms in classifying and identifying organisms. This is an artificial system of classification because it does not reflect natural or evolutionary relationships but it has been the bedrock of taxonomy for more than two centuries now. The Linnean system's strong features that are still surviving up to the present time are its hierarchical system of classification and the binomial system of nomenclature (Uno et al. 2001).

Classical taxonomy is very important because it is still the most widely used method of classifying, identifying, and naming organisms, which is based on morphological structures. The method is simple and easy to use and not expensive, although it is prone to subjective errors. It helps us categorize organisms so that we can easily communicate biological information. In contrast, the use of molecular techniques using computer technology and genetic or biochemical methods, which are more accurate, have not yet been widely used, because the supplies and materials are expensive under the present Philippine conditions (2020). Taxonomy uses hierarchical classification as a way of understanding and organizing the diversity of organisms. It creates these categories by looking at how organisms are related to each other not only morphologically but also genetically. It means, looking at the morphology, the form and structure of organisms, and see how many of these features do the organisms in a group share. The more

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shared traits they have the more related they are presumably in evolutionary history. **Molecular taxonomy** has succeeded in constructing phylogenetic trees using amino acid differences in the respiratory pigment cytochrome c as well as other protein or DNA itself (Hopsons and Wessels 1990). Indeed taxonomy using **molecular technique** and other techniques are useful in confirming the traditional taxonomic findings. Therefore, this chapter discusses the various traditional way of classifying, identifying, and naming algae.

Taxonomy is an integral part of systematics and it involves **classification, identification, and nomenclature**. The scientists involve in this branch of study are called **taxonomists** or particularly **phycologists or algologists. Classification** is grouping of organisms that have similarities in morpho-cytological features (**Linnaean approach**). **Identification** is the recognition of the essential and unique characters of an organism that are needed in finding similar characteristics in a known similar species, usually based on published literature, examination of type specimens, and consultation with the scientists who are experts in a particular group of organisms. While **nomenclature** is giving a new name to an organism**,** if the features of the organism do not fit any of those that have been previously published and studied. A new name is given based on the **International Code of Nomenclature (ICN)** for algae, fungi, and plants**,** formerly known as **International Code of Botanical Nomenclature (ICBN).**

II. Classification

Classification, based on the Linnean approach, lies mainly in making a judgment which similarities or differences between species are most important. On the basis of this, there is a degree of subjectivity. Therefore, in the 1950s, some taxonomists began to apply numerical methods for the evaluation of similarities and differences between species, which is called **numerical taxonomy** or **phenetics** (Raven and Johnson 1999). This method made a clearer delineation in the classification of organisms. On the other hand, classification based on Darwinian approach, considered evolutionary relatedness in assigning taxonomic affinity and not their degree of morphological similarity or differences. This is called **cladistics**, derived from the Greek word *clados*, meaning “branch”. It classifies organisms based on the historical order in which evolutionary branches arose during the history of the group, this will result into an **evolutionary tree** or a **cladogram.** A **clade** is a group of monophyletic organisms sharing one ancestral source**. Cladistic analysis** usually uses biochemical, structural and ultra-structural characteristics such as features of reproductive and vegetative structures and life history pattern as well as DNA sequence divergence, which is a subject matter in the modern type of taxonomy called **molecular taxonomy**.

A. Hierarchical Classification

Hierarchical classification is a system of grouping living things according to a hierarchy or level. The modern taxonomic classification system has eight main levels (or categories), from the most inclusive (broadest) to the most exclusive (specific), as: Domain, Kingdom, Division, Class, Order, Family, Genus, Species (Raven and Johnson 1999). Each grouping reflects perceived relationships. Historically, the early classification of eukaryotic algae and the prokaryotic algae (or the blue-green algae/cyanobacteria) were grouped in the algae, belonging to the kingdom **Plantae,** under the two-kingdom system of classification of living organisms according to Linnaeus, i.e., **Plantae or Animalia** (Raven and Johnson 1999). Later on, the algae were classified either in the kingdom Monera or kingdom Protista, based on the **five-kingdom system of classification of Whittaker** (Whittaker 1969; Hagen 2012). In this system of classification, the eukaryotic algae were grouped in the kingdom Protista (=Protoctista) while the prokaryotic blue-green algae or cyanobacteria were included in the kingdom Monera. However, in the latest **three domain system of classification** of biological organisms of Woese (Woese et al. 1990), the algae are grouped either in the domain Bacteria, where the cyanobacteria belong, or in the domain Eukarya (Eucarya), where the eukaryotic algae are included. On the basis of the latest phylogenetic and molecular studies, some of the green algae and all the red algae are included in

the kingdom Plantae, while the brown pigmented algae belong to the kingdom Chromista (Adl et al. 2005; Leliaert et al. 2012; Cavalier-Smith 2018). Table 15.1 shows the classification of the three microalgae, i.e., *Nostoc commune*, *Chlorella vulgaris*, and *Porphyridium purpureum* in eight hierarchical levels based on Adl et al. 2005; Leliaert et al. 2012; Champenois et al. 2015, and AlgaeBase.

Table 15.1. Sample classifications for a prokaryotic microalga (*Nostoc commune*), the eukaryotic freshwater microalga (*Chlorella vulgaris*), and the eukaryotic marine microalga (*Porphyridium purpureum*).

	Nostoc commune	*Chlorella vulgaris*	*Porphyridium purpureum*
Domain	Bacteria	Eukarya	Eukarya
Kingdom	Eubacteria	Protista	Plantae
Division	Cyanobacteria	Chlorophyta	Rhodophyta
Class	Cyanophyceae	Trebouxiophyceae	Porphyridiophyceae
Order	Nostocales	Chlorellales	Porphyridiales
Family	Nostocaceae	Chlorellaceae	Porphyridiaceae
Genus	*Nostoc*	*Chlorella*	*Porphyridium*
Species	*N. commune*	*C. vulgaris*	*P. purpureum*

B. The Species

In **biology**, the **species** are groups of organisms that remain relatively constant in their characteristics, and can be distinguished from other species, and do not normally interbreed with other species in nature. Therefore, this definition is true among sexually reproducing organisms because outcrossing is a barrier and serve as a criterion to a species. However, among bacteria and other asexually reproducing organisms, this criterion cannot serve for species recognition, instead the constancy of their morphological, biochemical, and molecular characteristics serves to differentiate species. This is known as the **phylogenetic species concept** that also differentiates eukaryotic organisms (Graham et al. 2009). On the other hand, the **morphological concept of a species** means the smallest groups that can be reliably defined by structural characters and are relatively easy to differentiate (Graham et al. 2009). An example of a phycology book that relies on morphological characters is “The Freshwater Algal Flora of the British Isles” (John et al. 2002). In **taxonomy, a species** is also the binomial name of an organism or a two-part name of any organism, that was developed by Carolus Linnaeus and published in his two-volume set called “*Species Plantarum*” (Species of Plants) in 1753 (Simpson, 2006; Uno et al. 2001). It is also known as the **scientific name** of an organism. It includes the generic name that starts with an upper-case letter, followed by the specific epithet, with all letters in lower cases. All scientific names should be italicized because they are in Latin. For example, the cyanobacterium that is locally called in Ilokano as “tab-taba” is *Nostoc commune,* the sea grapes or locally called in Bisaya (Waraynon) as “lato,” or in Ilokano as “ar-arusep” is an edible species of *Caulerpa racemosa,* while “gamet” in Ilokano is any of the edible species of *Porphyra* spp. (Cordero, 2008).

Guiry in 2012, estimated that there are about 72,500 algal species, of which 44,000 have probably been published, and 33,248 names have been processed by AlgaeBase.

III. Identification

Identification is a process that involves recognition of unique and essential characters of an organism needed to compare these features with the features of an identified organism that are available in published scientific monographs, journals, or books. This is called comparison of an unknown specimen with **written descriptions** (Simpson 2006). Another source of reference is comparing the unknown specimen with a **known living or preserved known specimen.** Living algal specimens are available in various algal culture collections worldwide and even locally in the Philippines, e.g., at the Microbial Culture Collection of the Museum of Natural History, University of the Philippines Los Baños (Martinez-Goss et al. 2014) and the Philippine National Collection of Microorganisms in National Institute of Molecular Biology and Biotechnology (BIOTECH),

University of the Philippines Los Baños, College, Laguna. A comparison of the dissected and microscopically examined samples may sometimes be necessary between the unknown and known samples.

Preserved algal samples are also found in various local and international herbaria. It is usually preferred to compare with the type specimens. See chapter 10 for some international and local herbaria. A **type specimen** or a **holotype** is the specimen designated by the original author at the time the species name and description were published. Types of diatom specimens in slides are preserved in various herbaria, in particular in their diatom section of the cryptogamic herbaria. Some herbaria that have wide collection of diatom slides are shown in Table 15.2.

Table 15.2. Some herbaria with large slide collection of diatom types.

Type of collection	Section of herbarium	Name of Museum/Herbarium	Location of the Museum/Herbarium
12,000 type specimens	Diatom Section	British Museum of Natural History	Cromwell Rd., South Kensington, London SW7 5BD, United Kingdom
Some collections: Boyer, CS Cleve & Möller Foged, N Peragallo Rabenhorst Smith, HL Smith, W Tempere & Peragallo	Diatom Section	Academy of Natural Sciences of Philadelphia (ANSP)	1900 Benjamin Franklin Parkway, Philadelphia, Pa.,19103, USA
Grunow	Cryptogamic Section	Naturhistorisches Museum Wien	Burgring 7, Vienna 1010, Austria
Mann, A		Smithsonian National Museum of Natural History	16th St. & Constitution Ave., NW, Washington, DC, 20560, USA

Image comparison is also one way of identifying an unknown specimen, especially nowadays, when photographs and illustrations are easily available on the Internet (webpage images), books, journal articles, and other useful resources (Simpson 2006). This is a fast way of identification before one can narrow down into a more specific taxon but this method must be accompanied by some more technical descriptions, such as morphological descriptions, physiological characterization and habitat description of the organism. Another method of identification is asking for **expert opinion** on the matter. This may be true for some special groups of organisms that someone is an expert on. This depends upon the accessibility and willingness of the expert. However, in most cases, the experts are so willing to help because the information that the inquirer is giving may be useful in their study of that particular organism. For example, The Phycology group at the Institute of Biological Sciences, University of the Philippines Los Baños (UPLB), College, Laguna, Philippines has been fortunate in having the identification of a specific Trentepholiaceae verified by one of the world's experts in this group of algae, Dr. Fabio Rindi of the Universita Politecnica delle Marche in Italy. Dr. Rindi was referred to the authors by Dr. Michael Guiry, of Algaebase, who works in this group of algae, too.

Another useful method in identification is the use of a **taxonomic key**. A **taxonomic key** is a device that is constructed in such a way that the user is presented with a series of choices about the characteristics or features of the unknown organism, by making a correct choice at each step of the key, the user is led to the identity of the organism. The most common type is a **dichotomous key.** This consists of two contrasting adjectives or phrases, in which each is known as a **lead**. The two leads together comprise a couplet. The leads of a couplet may be indented and/or numbered.

The lead that best fits the organism to be identified is selected, then all the couplets hierarchically beneath that lead (either by indentation or numbering) are sequentially examined until an identification is obtained. For example, Fig. 15.1 shows the differentiation among the eight genera in the family Euglenophyceae (Arguelles et al. 2014), while Fig. 15.2 differentiates four species of *Trachelomonas* (Arguelles et al. 2014). However, in most cases, the keys are either of the **natural,** or **artificial** or **practical** type, which means that they do not reflect natural groups or are not concerned about classification. Seldom are keys of the natural or phylogenetic type. Identification should not rely solely on the keys but this must be followed up by checking the written morpho/anatomical descriptions, illustrations/photographs, or specimen comparisons.

Key to the eight Genera in the family Euglenophyceae

1a. Large in length (>6 µm), discs, stellate, or reticulate chloroplast with pyrenoid …… 2
1b. Small in length (<6 µm), lens-shaped chloroplast without pyrenoid …… 7

2a. Protoplast without a lorica …….. 3
2b. Protoplast surrounded by a rigid brownish lorica …… 5

3a. Cells metabolic (shape changeable) …….. 4
3b. Cells rigid ……... 6

4a. Cells diverse in shape, chloroplasts with or without pyrenoids …… *Euglena*
4b. Cells are spindle-shaped, chloroplasts without pyrenoids …… *Euglenaformis*

5a. Cells with lorica, with a distinct collar, but no posterior tailpiece …….. *Trachelomonas*
5b. Cells with lorica, but no distinct collar, but with posterior tailpiece …….. *Strombomonas*

6a. Cells usually coffee-bean shaped with a cleavage furrow …… *Cryptoglena*
6b. Cells ovoid to pyriform with a long pointed spine at the posterior end …… *Monomorphina*

7a. Cells usually flattened and leaf-like *Phacus*
7b. Cells usually radially symmetrical *Lepocinclis*

Figure 15.1: An example of a numbered dichotomous key to the eight genera in the family Euglenophyceae (adapted from: Arguelles et al. 2014).

Key to the four species in the genus *Trachelomonas*

1a. Lorica without a collar …… *T. volvocina*

1b. Lorica with a collar …… 2

2a. Lorica smooth …… *T. armata*

2b. Lorica rough, maybe spiny …… 3

3a. Collar is slightly bent and irregularly toothed with spines …… *T. similis*

3b. Collar is short with a ring of spines …… *T. hispida* var. *coronata*

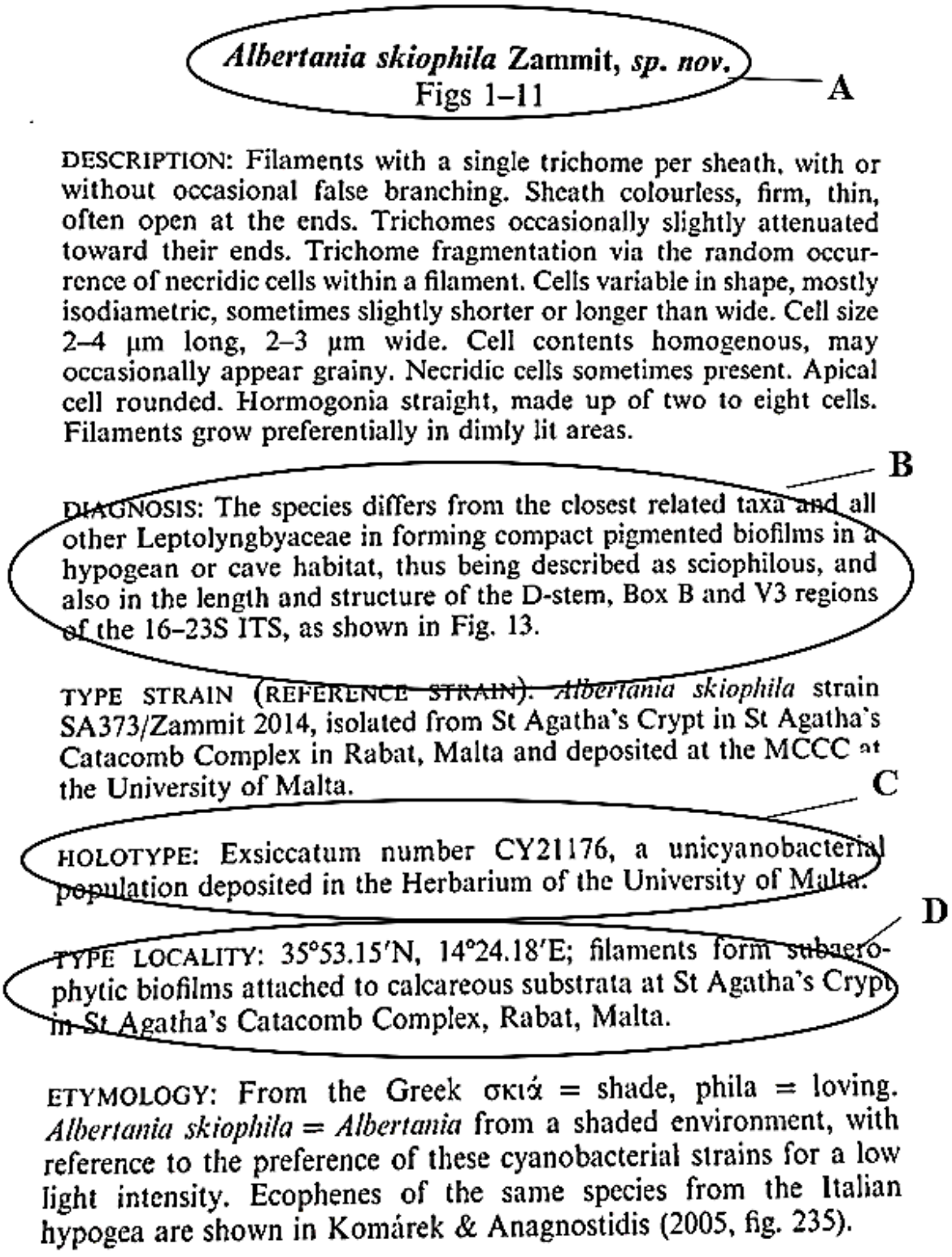
Figure 15.2: An example of a numbered dichotomous key to the four species of *Trachelomonas* (adapted from: Arguelles et al. 2014).

Current names of the identified organisms should be based on AlgaeBase (algaebase.org/search/species/Google Search).

IV. Nomenclature

Nomenclature is assigning a name to an organism, when all attempts to identify it have not been successful, following the formal system of naming organisms based on the **International Code of Nomenclature for algae, fungi, and plants (ICN)**, that embodies decisions of the Nomenclature Section of the XIX International Botanical Congress in Shenzhen, China in July, 2017. This Shenzhen Code supersedes the Melbourne Code (McNeill et al. 2012). This code was known as the International Code of Botanical Nomenclature (ICBN) before 2011. The principles of the ICN are the same as those embodied in the 2000 St. Louis Code (Simpson 2006), such as, 1) every taxon has only one correct name. The names assigned to a species is known as the **scientific name,** and it is composed of two names, hence, it is also called a **binomial name** and was introduced by Carolus Linnaeus and was widely practiced after the publication of his Species Plantarum in 1753 (Raven et al. 1992); all scientific names and below the rank of family should have an **author,** the name of the person who first validly published the name. The author's name is part of the scientific name but in practice the author's name may not always be cited. 2) all scientific names must be associated with some physical entities, known as nomenclatural types. Some of the type specimens are the holotypes, lectotypes, syntypes, and paratypes (Simpson, 2006). A **holotype** or simply the type is the specimen or illustration upon which a name is based, originally used or designated at the time of publication by the author. It is recommended that a holotype is deposited in an internationally recognized herbarium. 3) The third principle of the ICN is priority of publication, which generally states that the one validly published first, is the correct name. However, priority of publication only applies to taxa at the rank of family and below. This principle applies only for plants and algae after the publication of the Species Plantarum on May 1, 1753. Furthermore, a valid publication must follow four criteria, such as, the new name must be published in a scientific journal commonly available to the scientific community. The name must be a proper Latinized name, with the rank indicated, e.g., as *species novum* or *genus novum.* A diagnostic description of the species is needed to differentiate it from a similar or related taxon. One major change from the Melbourne Code of ICN is that the diagnostic description of the new species may now be written in English and no longer in Latin. 4) The fourth criterion is that the new name must not only be based on a nomenclatural type but must also include the location of the type.

A valid publication of a new species of a cyanobacterium, *Albertania skiophila* Zammit, illustrating all of the above criteria is shown in Fig. 15.3. of a cyanobacterium, *Albertania skiophila* Zammit, illustrating these criteria (Zammit 2018). In this case, the full citation of the scientific name includes not only the binomial names but also the authorship and the rank indicated (A). The scientific name is in Latin, meaning shade-loving (A). The diagnostic characteristics are presented. This time it is written in English (B). The holotype is indicated and deposited in the Herbarium of the University of Malta (C), while the type locality was identified in St. Agatha's catacomb complex in Rabat, Malta (D).



Albertania skiophila* Zammit, *sp. nov.
Figs 1–11

A

DESCRIPTION: Filaments with a single trichome per sheath, with or without occasional false branching. Sheath colourless, firm, thin, often open at the ends. Trichomes occasionally slightly attenuated toward their ends. Trichome fragmentation via the random occurrence of necridic cells within a filament. Cells variable in shape, mostly isodiametric, sometimes slightly shorter or longer than wide. Cell size 2–4 µm long, 2–3 µm wide. Cell contents homogenous, may occasionally appear grainy. Necridic cells sometimes present. Apical cell rounded. Hormogonia straight, made up of two to eight cells. Filaments grow preferentially in dimly lit areas.

B

DIAGNOSIS: The species differs from the closest related taxa and all other Leptolyngbyaceae in forming compact pigmented biofilms in a hypogean or cave habitat, thus being described as sciophilous, and also in the length and structure of the D-stem, Box B and V3 regions of the 16–23S ITS, as shown in Fig. 13.

TYPE STRAIN (REFERENCE STRAIN). *Albertania skiophila* strain SA373/Zammit 2014, isolated from St Agatha's Crypt in St Agatha's Catacomb Complex in Rabat, Malta and deposited at the MCCC at the University of Malta.

C

HOLOTYPE: Exsiccatum number CY21176, a unicyanobacterial population deposited in the Herbarium of the University of Malta.

D

TYPE LOCALITY: 35°53.15′N, 14°24.18′E; filaments form subaerophytic biofilms attached to calcareous substrata at St Agatha's Crypt in St Agatha's Catacomb Complex, Rabat, Malta.

ETYMOLOGY: From the Greek σκιά = shade, phila = loving. *Albertania skiophila* = *Albertania* from a shaded environment, with reference to the preference of these cyanobacterial strains for a low light intensity. Ecophenes of the same species from the Italian hypogea are shown in Komárek & Anagnostidis (2005, fig. 235).

Figure 15.3: Example of a valid publication of a new species description and its other components A= shows full citation of the binomial scientific name in Latin, the author's name, and the rank of the taxon. B=diagnostic characteristics are presented in English. C= holotype is designated and indicates where it is deposited. D= type locality is identified. (With permission from the author and Phycologia) (adapted from: Zammit, 2018).

V. Summary

Classical taxonomy or also known as traditional or orthodox taxonomy is still a very important field of study. This is also called the Linnean system that was developed by Carolus Linnaeus, a Swedish physician and naturalist. The system creates taxonomic rankings by looking at how organisms are related to each other by their shared morphological structures or appearances. In effect, it utilizes similarities in morphology, the form and structure of organisms, implying shared evolutionary history, to classify living organisms, to identify species, and give new names. The Linnean system is artificial in the sense that it is just based on morphological structures and not on natural or evolutionary relationships; nevertheless it became the foundation of biology. It has contributed to the hierarchical system of classification and the binomial system of nomenclature that are still popular and are still being used even at present. Molecular techniques in taxonomy are useful in confirming the classical or traditional findings.

VI. Acknowledgments

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Chapter 16. Molecular Techniques for Taxonomic Studies of Philippine Microalgae

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I. Introduction

Algae are an indispensable component of various ecosystems and have played a pivotal role in the evolution of life on earth. They occupy a significant position in the global mineral cycles and in the mitigation of global warming by sequestering CO_2 from the environment. Being photoautotrophic, they are the primary producers at the base of many aquatic food webs. Not only do they provide food for other organisms, they also provide man with nutraceuticals, and are commercially important in the cosmetics industries, drug delivery, biomolecule separation and computer chip manufacturing. In recent years, algae have been tapped in energy production, such as in the production of biofuels like biodiesel and ethanol. They are also important sources of livelihood especially for people living in coastal areas.

Among the different kinds of algae, **microalgae** are the most poorly identified, relying mostly on their ultra-morphological characteristics. Morphological identification of actively growing and developing algae is quite difficult and requires in-depth knowledge of the taxonomy of microalgal species. The Philippines, being in the tropics, has a rich flora which includes microalgal species that may offer potentials that are yet undiscovered and unidentified. This is where molecular techniques can help in identifying and classifying microalgae. **Molecular phylogenetics** is the branch of phylogeny that analyzes hereditary molecular differences to gain information on an organism's evolutionary relationship. Even though molecular-based identification is simple and reliable, only a few of the microalgal strains have been identified based on molecular markers, and a large number of strains are yet to be recognized. Identification of these species is important

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for phylogenetic studies, biodiversity analysis, screening genes for manipulation studies, biotechnology research, biofuels industry, and other commercial applications.

This chapter contains procedures on how quality DNA can be successfully extracted from microalgae at minimum expense and with minimum chemical and equipment use. Microalgae have been problematic to researchers whose task is to break their cell walls in order to extract nuclear material for molecular work and other cellular materials for commercial purposes. The cell walls of microalgae, such as *Chlorella*, contain microfibrillar and trilaminar components that contain **sporopollenin** (He et al. 2016), a substance found in the extremely resistant walls of spores and pollens.

The target genes used for identification of microalgae are molecular markers from the nuclear and chloroplast genome (Rasoul-Amini et al. 2009; Hamaji et al. 2013), such as gene specific markers for ribulose 1,5-biphosphate carboxylase/oxygenase (*rbc*L) from the chloroplast genome, and internal spacer 2 region (ITS-2) (Hall et al. 2010; Hadi et al. 2016), and 18s *r*RNA (*r*DNA in bioinformatics terms) region (Matsumoto et al. 2010) from the nuclear genome. Nuclear-encoded 18S *r*DNA sequences have been, until recently, the primary source of data for inferring phylogenetic relationships among green algae (Proschold and Leliaert 2007). The ITS-2 region of the nuclear ribosomal operon has been proposed as a DNA bar code for algae and land plants (Moniz and Kaczmarska 2009), and has been widely used in species level phylogenetics of green algae (Suliman et al. 2008; O'Kelly et al. 2010). The *rbc*L from the chloroplast genome is used instead of mitochondrial DNA for the obvious reason that the chloroplast is more specific to algae and green plant evolution than the mitochondrion.

II. Preparation of Materials and Detailed Procedures

A. Preparation of Unialgal Clonal Cultures of Microalgae from Water Samples

Double-layered Whatman (No.1) filter paper was used to remove debris and solid particles from the water sample, then the filtrate was centrifuged. The pellet was resuspended in 50mL of BG-11 liquid medium, and incubated at 25±1°C, light intensity of 100 µmol photons $m^{-2}s^{-1}$ and 16:8 light/dark cycle until visible growth is seen. A serial dilution of the broth culture was done and was plated on BG-11 agar medium to obtain individual colonies. Individual colonies were then plated onto BG-11 agar medium to obtain unialgal colonies. Lastly, the number of cells was counted using a haemocytometer (Improved double ruling Neubauer counting chamber).

B. DNA Extraction (Freeze-Thaw/Glass Powder Method; see Figure 16.1)

About 20 mL of microalgal sample was collected and centrifuged at 5,000 rpm for 5 minutes. The supernatant was pipetted out, being extra careful to take out as much medium as possible without disturbing the pellet. A 30 µL Tris-EDTA (TE) buffer (pH 8.0) with 0.5% sodium dodecyl sulfate (SDS) was added to the pellet, then vortexed for 5 seconds and left at room temperature for more than 1 hour. It was then transferred to a microcentrifuge tube and was left to freeze. Making sure the microcentrifuge tubes were uncapped, they were thawed immediately in boiling water. This freeze-thawing process was repeated three times. The sample was then ground with 20 mg glass powder using a micropestle (micropipette yellow tip with melted end). It was then centrifuged at 13,000 rpm for 1 minute, after which the supernatant was taken out and an equal volume of cold isopropanol was added. The mixture was left at room temperature for 2 minutes and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was then taken out carefully so as not to disturb the precipitated DNA. The pellet was washed with 1 mL cold 70% ethanol and centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet left to air-dry to get rid of the ethanol. The pellet was resuspended in 30 µL TE buffer or until completely dissolved. Lastly, it was stored in -20°C freezer until further use.

Tris-EDTA (TE) Buffer Preparation

Ethylenediaminetetraacetic acid (EDTA), (0.5M stock solution, pH 8.0)

An 18.6 g quantity of EDTA (disodium salt dihydrate, MW 372.24) was added to 80 mL of double distilled water (ddH_2O). Using a pH meter or strips of pH paper, the pH was slowly adjusted to 8.0,

the required pH to dissolve EDTA, by adding about 2.2 g of sodium hydroxide (NaOH) pellets (MW 40.00) while vigorously mixing with a magnetic stirrer or by hand. Distilled water (dH_2O) was added to make a total volume of 100 mL solution. Lastly, the solution was autoclaved for 15 minutes at 121°C, left to cool to room temperature and the lid was tightened for storage at room temperature.

Tris-HCl (1M stock solution, pH 8.0)
A 12.1 g quantity of Tris base (MW 121.10) was dissolved in 70 mL of ddH_2O. Using a pH meter or strips of pH paper, the pH was adjusted to 8.0 by the slow addition of about 5.0 mL concentrated hydrochloric acid (HCl), after which. ddH_2O was added to make a total volume of 100 mL solution. Lastly, the solution was autoclaved for 15 minutes at 121°C, left to cool to room temperature and the lid was tightened for storage at room temperature. As a precautionary measure, a yellow-colored solution must be replaced by a different source of Tris because it indicates poor quality, and the pH must be measured at room temperature.

To make 1X Tris-EDTA buffer (pH 8.0)

Volume	Stock Solution	Final Concentration
1.0 mL	1.0 M Tris-HCl	10 mM
200 µL	0.5 M EDTA	1 mM

+ Double distilled water (ddH_2O) to make 100 mL solution.

Glass Powder Preparation (Radha et al. 2013)
Broken test tubes were ground with a sterile mortar and pestle inside a fume hood using protective goggles and gloves. The correct particle size, approximately 100 µm, was checked under the microscope and the bigger particles were sieved out using a nylon or silk mesh. In a 1-L glass beaker, 400 g glass powder was resuspended in 800 mL ddH_2O and stirred for 1 hour. The slurry was then allowed to settle for 90 minutes and the supernatant, which contains the fine particles used for glass milk, was transferred into a large 250-mL centrifuge bottle. The glass particles were pelletized by spinning at 6,000 rpm for 10 minutes and resuspended in 250 mL ddH_2O, after which concentrated nitric acid was added while in the fume hood. The glass particles were continuously washed and spun up to five times until the pH of the slurry became neutral. It was then sterilized at 250°C for 2-3 hours and dried. Aliquots were made in microcentrifuge tubes to prevent contamination. For DNA extraction, 20 mg glass powder was added to freeze-thawed DNA samples in section B. Lastly, the aliquots were stored indefinitely at room temperature.

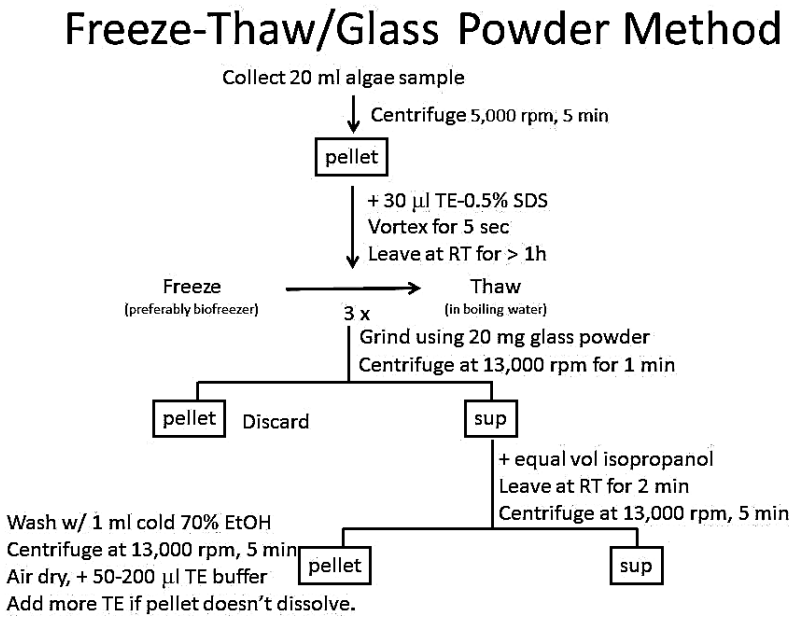

Figure 16.1: Flow diagram of the freeze-thaw/glass powder method of DNA extraction
sup =supernatant.

C. DNA Quantification Using the Spectrophotometer

A spectrophotometer was used to measure the amount of UV irradiation absorbed by the nucleotide bases and to ascertain whether the sample is pure (i.e., without significant amounts of contaminants such as proteins, phenol, agarose or other nucleic acids). For quantifying DNA and RNA, absorbance readings were taken at 260 nm wavelength. The reading at 260 nm allows calculation of the concentration of nucleic acids as follows:

1 OD at 260 nm for double-stranded (ds) DNA = 50 ng/µL of dsDNA
1 OD at 260 nm for single-stranded (ss) DNA = 20-33 ng/µL of ssDNA
1 OD at 260 nm for RNA molecules = 40 ng/µL of RNA

Readings were also taken at 280 nm and 320 nm. The absorbance reading at 280 nm gives the amount of protein in the sample, while the reading at 320 nm indicates non-nucleic absorbance. It is worth mentioning that pure preparations of DNA and RNA have A_{260} and A_{280} values of 1.6-1.9, respectively. If there is contamination with protein, this ratio is less than 1.6, and accurate quantification of the amount of nucleic acid is not possible. Values greater than 1.9 indicate contamination with RNA. The ratio was then calculated after subtracting the non-nucleic acid absorbance at A_{320} using the following formula:

$$\text{DNA Purity } (A_{260}/A_{280}) = \frac{(A_{260} \text{ reading} - A_{320} \text{ reading})}{(A_{280} \text{ reading} - A_{320} \text{ reading})}$$

Yield (µg) = OD (A_{260}) × dilution factor (e.g., 200, 3 µL sample in 597 µL TE buffer) × total vol in mL × 50 (ng per OD for dsDNA)

Concentration = yield in µg/vol in µL

D. DNA Visualization and Analysis by Agarose Gel Electrophoresis

Agarose gels allow separation of nucleic acids based on charge migration and mass. Because of the net negative charge of the phosphate backbone of the DNA, it has a negative charge and will migrate toward the positive pole. During electrophoresis, nucleic acids are forced to migrate through the pores of the gel. Smaller fragments migrate faster than longer fragments, thereby separating nucleic acids with different sizes.

10X Tris/Borate/EDTA (TBE) Electrophoresis Buffer

The following, 1 g NaOH (MW 40.00), 108 g Tris base (MW 121.10), 55 g boric acid (MW 61.83) and 7.4 g EDTA (disodium salt dihydrate, MW 372.24), were weighed and added to 700 mL deionized or distilled water in a 2-L flask. The mixture was stirred using a magnetic stir bar and deionized water was added to bring the total solution to 1 L, then stored at room temperature.

1X TBE Electrophoresis Buffer

About 9 L deionized or distilled water was added to 1 L of 10X TBE and stirred before storing at room temperature (indefinitely).

1% Agarose

A 2.0 g quantity of agarose (electrophoresis grade) was added to 200 mL 1X TBE electrophoresis buffer in a 600-mL beaker or Erlenmeyer flask and stirred to suspend agarose. The beaker was covered with aluminum foil, and heated in a boiling-water bath (double boiler) or on a hot plate until all agarose was dissolved (approximately 10 minutes). To ensure that all agarose had dissolved, it was reheated for several minutes until it appeared translucent, then it was covered with aluminum foil and kept at about 60°C until ready to use. It is worth mentioning that samples of agarose powder can be pre-weighed and stored in capped test tubes until ready for use. Alternatively, solidified agarose can be stored at room temperature and then re-melted over a boiling-water bath (15-20 minutes) or in a microwave oven (3-5 minutes per beaker) prior to use. When re-melting agarose, evaporation will cause the concentration to increase. If necessary, one could compensate by adding back a small volume of water.

Ethidium Bromide (1 µg/mL) Staining Solution
To 500 mL deionized or distilled water, 100 µL of 5 mg/mL ethidium bromide was added. It was then stored at room temperature in unbreakable bottles, preferably opaque-colored (or aluminum foil-wrapped white bottles). The bottles must be labeled "CAUTION" since ethidium bromide is a mutagen and a suspected carcinogen. It is necessary to always wear rubber gloves when handling the chemical.

Loading Dye
The following, 0.25 g bromophenol blue (MW 669.96), 0.25 g xylene cyanol (MW 538.60), and 1 mL 1M Tris (pH 8.0), were dissolved in 60 mL deionized or distilled water, then 50 g sucrose (MW 342.30), or 50 mL glycerol, was added to make 100 mL total solution, which was stored at room temperature.

0.2% Methylene Blue Stock Solution
A 0.2 g quantity of methylene blue-trihydrate (MW 373.9) was added to 100 mL of distilled water then stirred until completely dissolved and stored at room temperature.

0.025% Methylene Blue Staining Solution
About 62.5 mL of 0.2% methylene blue stock solution was added to 437.5 mL of distilled water, then stirred until completely dissolved and stored at room temperature.

pBR322/BstN I Size Markers (0.1 µg/µL)
To 75 µL distilled and autoclaved water, 10 µL of a solution of 10 µg/µL pBR322 and 10 µL of the appropriate 10^X buffer were added, after which 5 µL BstN I was added and incubated at 60°C for 60 minutes. In order to check for complete digestion 5 µL (plus 1 µL loading dye) was electrophoresed in a 1-2% agarose gel. Exactly five bands were visible, corresponding to 1,857 bp, 1,058 bp, 929 bp, 383 bp and 121 bp. If in any case additional bands appeared, it indicates incomplete digestion, thus additional enzyme must be added and incubated again at 60°C, and then stored at -20° C for until one year.

Casting an Agarose Gel (Wide Mini-Sub® Cell GT Horizontal Electrophoresis System)
The end dams were snapped onto each end of the gel casting tray. The end dams seal the ends of the tray, eliminating the need to tape the ends. The gel comb was inserted into the slot of the tray. Approximately 25 mL melted agarose was poured into the tray, until it reached a depth of about 3 mm. Enough space was ensured between the bottom of the comb and the glass plate (0.5-1.0 mm) to allow proper formation of the wells and to avoid sample leakage. There should be no air bubbles in the gel and the gel casting tray should not be tilted or slanted in order to obtain a uniform gel thickness. The gel was allowed to solidify for approximately 20 minutes or until the agarose gel turned opaque. After the gel solidified, the comb was carefully removed from the gel, ensuring that the wells are properly formed or have not been damaged during the comb removal. The end dams were then removed from the tray by carefully sliding them out.

Loading and Running a Gel (Figure 16.2)
A micropipette with a fresh tip was used to add 15 µL PCR sample-loading dye (1 volume of loading dye was mixed to 6 volume DNA sample, ensuring that no ethanol is present in the sample as this will cause the sample to float out of the well upon loading). The mixture was loaded into the assigned well of a 1% agarose gel. It was made certain that the tip of the pipette would not push through the bottom of the sample well and not overload the well. In addition, air bubbles had to be removed in the gel trapped between the wells by rinsing them with electrophoresis buffer. Ensuring that the entire gel is submerged in the electrophoresis buffer, 5 µL of the pBR322-*BstN*I size marker was then loaded into one lane of gel. The loaded gel was placed into the center of the chamber and the casted gel in parallel to the electrode wires to ensure uniform migration of the DNA during electrophoresis. Approximately 350 mL of 1X TBE running buffer was carefully poured into one side of the chamber until the level of buffer was approximately 2 mm above the top of the gel. The chamber must not be overfilled. Any spills should be wiped on the apparatus before proceeding to the next step, ensuring that the patch cords are attached to the cover, and that the

female jacks and the banana plugs on the chamber, are completely dry before sliding the cover onto the electrophoresis chamber. The electrophoresis apparatus should always be covered to protect against electric shock. The electrodes were then connected in order for the DNA to migrate towards the anode (positive electrode). The red patch cord was connected to the red electrode terminal and the black patch cord to the black electrode terminal on the power supply. The power was set to 130 volts for 20-30 minutes. Adequate separation should occur when the cresol red dye front has moved at least 50 mm from the wells. The gel may be stained either with 1 µg/mL ethidium bromide for 10 minutes or 0.05% methylene blue (or proprietary) stain for 30 minutes, followed by 20-30 minutes de-staining with water to remove excess stain. The staining buffer can be saved and reused. It is important to note that when bromophenol blue loading dye was added to the sample, 10 µL of sample should be left without loading dye in order to send the sample for sequencing. The temperature of the buffer was monitored periodically during the run and when the buffer became overheated, the voltage was reduced. Melting of an agarose gel during electrophoresis was also monitored since it is a sign that the buffer may have been incorrectly prepared or it could be due to high voltage resulting in high temperature.

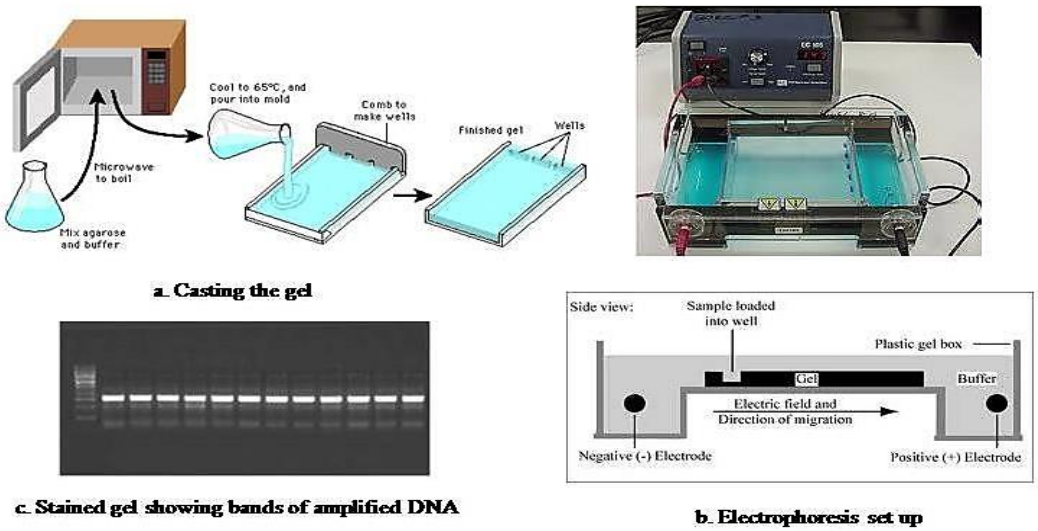

Figure 16.2: Agarose gel electrophoresis. (a) casting gel, (b) electrophoresis set-up, and (c) stained get showing bands of amplified DNA.

Visualization

Ethidium bromide–DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of DNA to be visualized against a background of unbound dye. The gel image can be recorded using a gel documentation system. Suitable eye and skin protection were worn during visualization since UV light can damage the eyes and skin. Since UV light also damages DNA, if DNA fragments are to be extracted from the gel, a lower intensity UV source should be used or exposure of the DNA to UV light should be minimized.

E. Polymerase Chain Reaction (PCR)

How to Avoid Contamination

During PCR, usually more than 10 million copies of a template DNA can be generated. Therefore, care must be taken to avoid contamination with other templates and amplicons that may be present in the laboratory environment. General recommendations to lower the risk of contamination were followed, such as: (a) procedures, like preparation of the DNA sample, setting up of the PCR mixture, thermal cycling and analysis of PCR products, were performed in separate areas; (b) the PCR mixture was set up in a laminar flow cabinet equipped with UV lamp; (c) fresh gloves were worn for DNA purification and reaction set up; (d) dedicated containers were used for PCR and positive displacement pipettes or pipette tips with aerosol filters were used to prepare DNA samples and PCR reaction set-up; (e) PCR-certified reagents, including high quality,

nuclease-free water were used; and (f) control reactions were performed without template DNA to check for the absence of contamination.

Reaction Set Up

To prepare several parallel reactions and to minimize the possibility of pipetting errors, a PCR master mix was prepared in a single tube by adding water, buffer, dNTPs, primers and *Taq* DNA Polymerase. The master mix was aliquoted into individual PCR tubes and all solutions were vortexed and centrifuged after thawing, keeping all components on ice. The algal lysate was then added using a fresh tip and mixed. The components of the PCR mix are as follows:

Cycle Sequencing Mix

Components	*µL*
Forward Primer (5 pmol) *	1
Reverse Primer (5 pmol) *	1
Taq DNA Polymerase	0.2(1U)
dNTP mix (10mM each nucleotide)	2.5
Distilled Water	15.8
10x PCR Buffer w/ Mg^{2+}	2.5
Algal lysate	2
Total volume	*25*

S*The primer pair for *rbc*L gene (Hadi et al. 2016) included sequences forward primer 5'–ATGTCACCACAAACAGAGACTAAAGC–3' and reverse primer 5'–GAAACGGTCTCTCCAACGCAT– 3'.

*The primer pair for ITS-2 region (Hadi et al. 2016) included sequences forward primer 5'–AGGAGAAGTCGTAACAAGGT–3' and reverse primer 5'TCCTCCGCTTATTGATATGC–3'.

*The primer pair for 18s *r*DNA sequences (Hall et al. 2010) included sequences forward primer 5'–GGGTGATCCTGCCAGTAGTCATATGCTTG–3' and reverse primer 5'–GATCCTTCCGCAGGTTCACCTACGGAAACC–3'.

When using a thermal cycler without a heated lid, 25 µL of mineral oil should overlay the reaction mixture. All samples were stored on ice until ready to amplify according to the prescribed conditions as follows:

Reaction Condition (rbcL and ITS-2)

	Temperature	*Time*	*Cycle*
Denaturation	95°C	5 min	35
Annealing	55°C	30 sec	
Extension	72°C	1 min	

Reaction Condition (18 rDNA)

	Temperature	*Time*	*Cycle*
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing	55°C	30 sec	
Extension	72°C	1.5 min	
Final Extension	72°C	10 min	1

*Each profile may be linked to a 4°C hold after completing the cycle profile, while amplified DNA holds well at room temperature.

DNA Purification

The protocol for the QIAquick PCR purification kit (http://www.qiagen.com) was followed. This kit is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (Figure 16.3). For cleanup of other enzymatic reactions, the protocol was also followed as described for PCR samples while the MinElute Reaction Cleanup Kit was used as an alternative. Fragments ranging from 100 bp to 10 kbp were purified from primers, nucleotides, polymerases, and salts using the accompanying QIAquick spin columns by a microcentrifuge.

Important Points Before Starting

Ethanol (96-100%) was added to Buffer PE before use. All centrifugation steps were carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature. A 1:250 volume pH indicator I was added to Buffer PB (i.e., add 120 µL pH indicator I to 30 mL Buffer PB or 600 µL pH indicator I to 150 mL Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of 7.5. The pH indicator I was added to entire buffer contents; it was not added to buffer aliquots. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Then five volumes of Buffer PB were added to one volume of the PCR sample and mixed. It is not necessary to remove mineral oil. For example, add 500 µL of Buffer PB to 100 µL PCR sample (not including oil). If pH indicator I has been added to Buffer PB, the color of the mixture must be yellow. If the color of the mixture is orange or violet, 10 µL of 3 M sodium acetate (pH 5.0) was added and mixed. The QIA quick spin column was placed in a 2-mL collection tube. To bind the DNA, the DNA sample was applied to the QIA quick column and centrifuged for 60 seconds. The flow-through was then discarded and the QIA quick column was placed back into the same tube. Collection tubes were re-used to reduce plastic waste. For washing, 0.75 mL Buffer PE was added to the QIA quick column and centrifuged for 60 seconds. The flow-through was discarded and the QIA quick column placed back in the same tube. The column was centrifuged for an additional one minute to remove any residual ethanol. The QIA quick column was placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50 µL Buffer EB (10 mM Tris Cl, pH 8.5) or water (pH 7.0 – 8.5) was added to the center of the QIA quick membrane and centrifuged for 1 minute. Alternatively, for increased DNA concentration, 30 µL elution buffer was further added to the center of the QIA quick membrane and left to stand for 1 minute, then centrifuged at high speed and stored at -20°C.

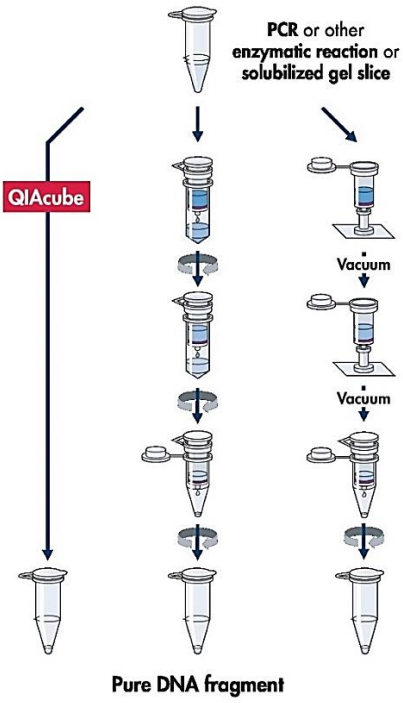

Figure 16.3: DNA purification using QIAquick (http://www.qiagen.com/).

F. Principles of DNA Sequencing (Source: National Center for Biotechnology Information)
The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. In 1977, twenty-four years after the discovery of the structure of DNA, two separate methods for sequencing DNA were developed: the chain termination method and the chemical degradation method. Both methods were equally popular to begin with, but for many reasons the chain termination method is the method more commonly used today. This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis.

The DNA to be sequenced, called the template DNA, is first prepared as a single-stranded DNA. Next, a short oligonucleotide is annealed, or joined, to the same position on each template strand. The oligonucleotide acts as a primer for the synthesis of a new DNA strand that will be complementary to the template DNA. This technique requires that four nucleotide-specific reactions – one each for G, A, C, and T – be performed on four identical samples of DNA. The four sequencing reactions require the addition of all the components necessary to synthesize and label the new DNA, including: (a) DNA template, (b) primer tagged with a mildly radioactive molecule or a light-emitting chemical, (c) DNA polymerase which is an enzyme that drives the synthesis of DNA, and (d) one dideoxynucleotide, either ddG, ddA, ddC, or ddT.

After the first deoxynucleotide is added to the growing complementary sequence, DNA polymerase moves along the template and continues to add base after base. The strand synthesis reaction continues until a dideoxynucleotide is added, blocking further elongation. This is because dideoxynucleotides are missing a special group of molecules, called a 3'- hydroxyl group, needed to form a connection with the next nucleotide. Only a small amount of a dideoxynucleotide is added to each reaction, allowing different reactions to proceed for various lengths of time, until, by chance, DNA polymerase inserts a dideoxynucleotide, terminating the reaction. Therefore, the result is a set of new chains, all of different lengths (Figure 16.4).

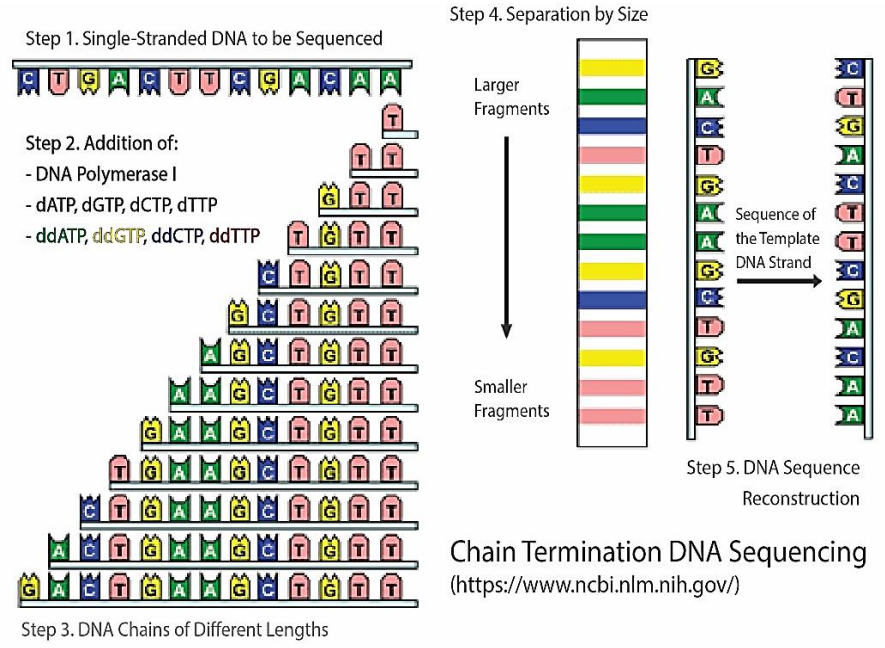

Figure 16.4: Chain termination sequencing involves the synthesis of new strands of DNA complementary to a single-stranded template (step 1). The template DNA is supplied with a mixture of all four deoxynucleotides, four dideoxynucleotides (ddATP, ddCTP, ddGTP and ddTTP) – each labeled with a different color fluorescent tag – and DNA polymerase (step 2). As all four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a dideoxynucleotide. The result is a new set of DNA chains all of different lengths (step 3). The fragments are then separated by size using gel electrophoresis (step 4). As each labeled DNA fragment passes a detector at the bottom of the gel, the color is recorded. The DNA sequence is then reconstructed from pattern of colors representing each nucleotide sequence (step 5) (https://www.ncbi.nlm.nih.gov/).

To read the newly generated sequence, the four reactions are run side-by-side on a polyacrylamide sequencing gel. The family of molecules generated in the presence of ddATP is loaded into one lane of the gel and the other three families, generated with ddCTP, ddGTP, and ddTTP, are loaded into three adjacent lanes. After electrophoresis, the DNA sequence can be read directly from the positions of the bands in the gel.

Variations of this method have been developed for automated sequencing machines. In one method, called cycle sequencing, the dideoxynucleotides, not the primers, are tagged with different colored fluorescent dyes, thus all four reactions occur in the same tube and are separated in the same lane on the gel. As each labeled DNA fragment passes a detector at the bottom of the gel, the color is recorded, and the sequence is reconstructed from the pattern of colors representing each nucleotide in the sequence.

III. Cladogram Construction Using Bioinformatics

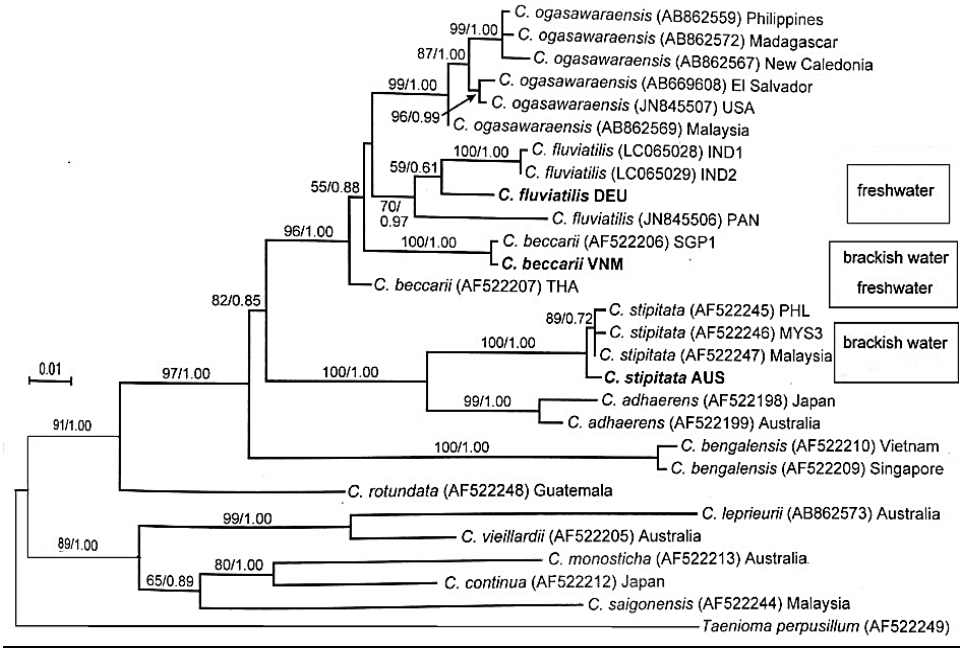

Figure 16.5: Maximum likelihood (ML) phylogeny of *Caloglossa* species inferred from the partial *rbc*L gene sequences (Kamiya et al. 2016).

Biological information is encoded in the nucleotide sequence of DNA. Bioinformatics is the field that identifies biological information in DNA using computer-based tools. Some bioinformatics algorithms aid the identification of genes, promoters, and other functional elements of DNA. Other algorithms help determine the evolutionary relationships between DNA sequences. Figure 16.5 illustrates the maximum likelihood (ML) phylogeny of *Caloglossa* species inferred from the partial *rbc*L gene sequences (Kamiya et al. 2016).

Because of the large number of tools and DNA sequences available on the Internet, experiments done *in silico* (“in silicon” or on the computer) now complement experiments done *in vitro* (in glass or test tubes). This movement between the molecular and computational sciences is a key feature of modern biological research.

Using BLAST to Find DNA Sequences in the Databases (electronic PCR or e-PCR)
The BLAST search in e-PCR explores for sequences that contain identical matches to the forward primer. It is different from *in vitro* PCR in that it does not seek sequences that are complimentary to the primer sequence. However, it resembles the annealing step of the forward primer in *in vitro* PCR in that it seeks out and delimits the 5’ end of target sequence.

In initiating a BLAST search, BLASTn tool was accessed at the National Center for Biotechnology Information (NCBI) website by opening www.ncbi.nlm.nih.gov/BLAST. The DNA sequences of the primers were entered in the “Enter your query sequence” window. These are called the query sequence(s). Any non-standard nucleotide characters were omitted from the window because they are not recognized by BLAST. The Nucleotide collection (nr/nt) database was selected from the drop-down menu under “Database” of the “Choose Search Set” section. Optimize for “somewhat similar sequences (BLASTn)” were selected in order to optimize under “Program Selection” section. “BLAST” at the bottom of the screen was clicked and the query sequences were sent to a server at the National Center for Biotechnology Information in Bethesda, Maryland. There, the BLAST algorithm was used to match the primer sequences to the millions of DNA sequences stored in its database. While searching, a page showing the status of the search is displayed until results are available.

The results of BLAST search were displayed in three sections. First, a graphical overview illustrates how many significant matches (=’hits’) in the database align with the query sequence and the relative location of the query sequence against the sequence in the database. Matches of differing lengths are coded by color. Second, a list of significant alignments or hits, is generated with the Accession information. Third, a detailed view of each primer sequence (query) aligned to the nucleotide sequence of the search hit (subject) is shown. It was noted that a match to the forward primer (nucleotides 1–25), and a match to the reverse primer (nucleotides 26–51) are within the same Accession.

The scores in the E-value column were considered on the list of significant alignments. The Expectation or E-value is the number of alignments of the query sequence that would be expected to truly occur in the database if the query sequence occurs by chance in the database. The lower the E-value, the higher the probability that the hit is related to the query. The list of significant identities is tabulated from the lowest E-value to the highest E-value. The names of any significant alignments that have E-values less than 0.1, and less than 0.001 was noted. The “Score” and “E-value” provide measurements for the significance of each hit. The primers which are relative to this subject sequence were checked at the “Alignments” section, taking note that the lowest and highest nucleotide positions in the subject sequence indicate the borders of the amplified sequence. Subtracting one from the other gives the difference between the two coordinates. However, the actual length of the fragment includes both ends, so one nucleotide was added to the result to determine the exact length of the PCR product amplified by the two primers. Then lastly, the + or the – allele were determined.

The sequence at the database hit was considered and copied for future use. The Accession link contains the sequence datasheet wherein basic information about the sequence, including its base pair length, accession number for the GenBank entry, source, and references among others can be accessed. The entire nucleotide sequence of the gene or DNA sequence that contains the PCR product can be found at the bottom section of the report lists. In order to get the amplicon, or amplified product, the nucleotides between the beginning of the forward primer and end of reverse primer were highlighted and pasted into a text document. Then, any extra nucleotides were trimmed from the ends, and all non-nucleotide characters and spaces deleted. Lastly, a phylogenetic tree was generated using ClustalW for multiple alignment of sequences.

IV. Summary

The chapter presented the procedures involved in molecular techniques for algal taxonomy. Following the preparation of unialgal clonal cultures of microalgae obtained from water samples, DNA was extracted by the freeze-thaw/glass powder method, and quantified using the spectrophotometer. The DNA was then visualized and analyzed by agarose gel electrophoresis. Samples of template DNA from the sample were amplified by Polymerase Chain Reaction (PCR), then purified using the QIAquick PCR purification kit. A BLAST search was then used to find relevant DNA sequences online in electronic PCR (e-PCR) databases. A phylogenetic tree of the

microalgal samples tested can be generated using BLASTn or ClustalW for multiple alignment of sequences following the procedures performed above.

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GLOSSARY

adsorption A form of immobilization of microalgal cells by adhesion of a thin layer of molecules to the surface of a support material.

affinity immobilization Immobilization of microalgal cells by enzyme immobilization techniques that make use of affinity tags that are present or added at a specific position far from the active site in the structure of the native proteins. It could be used to create strong affinity bonds between the enzyme structure and a solid support functionalized with the complementary affinity ligand.

agar slants Agarized media placed in test tubes, cooled at an appropriate angle (for the slant), and generally used for long storage of usually unialgal or axenic microalgae.

agarized media Nutrient media that have been hardened with agar for cultivating algae.

agarose gel electrophoresis Agarose gels allow separation of nucleic acids based on charge migration and mass. Because of the net negative charge of the phosphate backbone of the DNA, it will migrate toward the positive pole. During electrophoresis, nucleic acids are forced to migrate through the pores of the gel. Smaller fragments migrate faster than longer fragments, thereby separating nucleic acids with different sizes.

algal beads Beads containing immobilized microalgae incubated in an alginate carrier with nutrient medium.

algal paste (or microalgal paste) A microalgal concentrate in paste form.

alginate carrier A receptacle for immobilized algae used for industrial and biomedical purposes, usually in the form of sodium alginate, a polymer of D-mannuronic acid and L-glucuronic acid extracted from kelps.

algology The study of algae.

artificial media Nutrient media that are formulated from either inorganic and/or organic chemicals for culturing algae. They basically contain nitrogen, phosphorus, potassium, and other macro- and micronutrients for optimum growth of particular alga(e) as opposed to the natural media where algae naturally grow.

aseptic Free from contamination caused by harmful bacteria, viruses or other microorganisms.

autoclave A piece of equipment that uses steam to sterilize small equipment, glassware, solutions and other objects.

axenic culture A culture which only contains a single species/variety/strain of the organism and entirely free of any contaminating organisms.

barrier filter (or emission filter) A part of the fluorescence microscope that functions similarly to the excitation filter and allows a certain spectral band from fluorescence emission, making a high contrast image with the darkest background possible. Instead of allowing a specific band, sometimes a whole range of spectra from certain wavelengths is required, and a long-pass filter may be used.

beam splitter An optical component of the fluorescence microscope that divides a beam of light into two at different directions.

bioinformatics The field that identifies biological information in DNA using computer-based tools.

BLAST search In e-PCR, it searches for sequences that contain identical matches to the forward primer. It differs from in vitro PCR because it does not seek sequences that are complementary to the primer sequence. However, it resembles the annealing step of the forward primer in in vitro PCR in that it seeks out and delimits the 5'end of target sequences.

brightfield microscopy A contrasting enhancement technique in light microscopy, where the specimen appears dark on a bright background suitable for observing the natural colors of living specimens or even stained samples

calibration Determining the size of a unit in an unknown measuring instrument (like an ocular micrometer) to a known measuring instrument (e.g. a stage micrometer) whose scales are measured.

cell immobilization Induction of dormancy in microorganisms, such as microalgae, for medium-term storage of cells in receptacles such as alginate beads.

centrifugation A separation or concentrating process that depends on the action of centrifugal force to separate particles in a solid-liquid mixture into two, consisting of sediment and the supernatant liquid.

chemical sterilization The use of chemicals to destroy or remove all viable microorganisms, including spores of fungi and bacteria, and viruses.

chlorination of water The process of adding chlorine or chlorine compounds such as sodium hypochlorite to water. This method is used to kill certain bacteria and other microbes in tap water as chlorine is highly toxic to contaminants.

chlorophyll A general term for the green photosynthetic pigment in plants, algae and cyanobacteria.

chlorophyll a The universal primary photosynthetic pigment since it constitutes the reaction center of photosystems I and II in most algae and land plants.

chlorophyll b An accessory photosynthetic pigment of green algae and land plants.

chlorophyll c An accessory photosynthetic pigment of the chromists, like the diatoms, dinoflagellates and brown algae.

chlorophyll d An accessory photosynthetic pigment found in some red algae and cyanobacteria; a major photosynthetic pigment in the cyanobacterium, *Acaryochloris marina* .

chromatography A technique used in the separation of a mixture by passing it as a liquid solution or suspension (e.g., a mixture of chlorophyll pigments in liquid chromatography) or as a vapor (e.g., a mixture of volatile substances in gas chromatography) through a medium in which the unknown components (mobile phase) move into the stationary phase at different rates by which they are identified against a standard..

cladistics Classifying organisms based on the historical order in which evolutionary branches arose during the history of the group. A method of classifying organisms according to the proportion of measurable characteristics that they have in common. It is assumed that the higher the proportion of characteristics that two organisms share, the more recently they diverged from a common ancestor.

cladogram A branching diagrammatic tree used in cladistics to show phylogenetic relationships.

cladogram construction In e-PCR, the BLAST searches for sequences that contain identical matches to the forward primer. BLAST, housed at the National Center for Biotechnology Information (NCBI) website, is accessed, and the DNA sequences of the primers are entered. Significant matches and significant alignments, or hits, are given, and the number of alignment with the query sequence can be found. A phylogenetic tree using BLASTn or ClustalW for multiple alignment of sequences can be generated.

classical (or orthodox) taxonomy Scientific classification of organisms that is usually based on their form and structure (morphology).

classification Grouping together of organisms with similar morphological features (Linnaean approach) and /or those with evolutionary relationships (Darwinian approach). Establishment of a hierarchical system of categories from the most inclusive (broadest) to the most exclusive (specific).

compound microscope A microscope made up of two or more lenses (eyepiece lens and the objective lens) that magnify features of an object, giving it an inverted and laterally reversed image.

condenser The optical lens part of a compound microscope which collects and focuses the light from the illuminator on to the specimen.

coulter counter A piece of equipment for counting and sizing particles suspended in electrolytes.

cryopreservation The storage of a living organism, or a portion thereof, at an ultralow temperature, usually with a cryoprotectant, such that it remains capable of survival upon thawing.

cryoprotectants Substances or compounds used during the preservation of microorganisms and microalgae at ultralow temperatures (typically -130^{o}C or colder). These substances lower the temperature at which intracellular water freezes, decrease cell volume due to osmotic pressure, alter membrane properties such as solute permeability, and reduce cell membrane disruption caused by the formation of ice crystals in and around the cells. They promote the formation of smaller ice crystals.

cryptogamic herbarium A repository for dried spore-bearing plants.

culture medium (pl. culture media) A nutrient system, which can be solid, liquid or semisolid, designed to support the growth and propagation of microorganisms or cells.

cycle sequencing A variant of chain termination DNA sequencing developed for automated sequencing machines, where the dideoxynucleotides – not the primers – are tagged with different colored fluorescent dyes, so that all four reactions occur in the same tube and are separated in the same lane on the gel. As each labeled DNA fragment passes a detector at the bottom of the gel, the color is recorded, and the sequence is reconstructed from the pattern of colors representing each nucleotide in the sequence.

dark field microscopy A contrasting enhancement technique in light compound microscopes that shows the specimen to be bright against a generally dark background, which is generally useful in observing thin and unstained specimens.

defined media Nutrient media that have definite or quantifiable amounts of nutrients.

depth of focus A property of the microscope that indicates the thickness of the specimen that can be placed in sharp focus under the microscope at one time.

diatoms Photosynthetic protists/microalgae, particularly heterokonts or stramenopiles, that are usually unicellular to colonial forms with cell walls (frustules) mainly made up of silica and are usually golden brown due to the predominance of the carotenoid pigment called fucoxanthin.

dichotomous key A taxonomic key or reference tool consisting of two contrasting adjectives or phrases, in which each is known as a lead, in which the lead that best fits the organism to be identified is selected, and all the couplets hierarchically beneath are sequentially examined until an identification is obtained.

dichroic mirror (or dichromatic beam splitter/s) An optical component of the fluorescence microscope that allows the excitation light to pass through while reflecting emission light from the specimen.

diffraction A wave phenomenon where a wave redirects as it passes around an obstacle or a gap. The degree of redirection depends on the size of the obstacle or gap relative to the wavelength.

disinfection A process that kills or reduces the number of pathogenic microorganisms in an environment or on a surface.

dissecting microscope Also known as a stereoicroscope, it is a type of light microscope with two or more lenses, giving a right-side three dimensional image of an object making it convenient to use in dissecting specimens.

DNA purification Isolation of DNA from a sample using a combination of physical and chemical methods.

DNA quantification using a spectrophotometer A spectrophotometer (absorbance at 260 nm) used to measure the amount of UV irradiation absorbed by the nucleotide bases (DNA and RNA).

DNA visualization and analysis DNA fragments can be extracted and amplified through electrophoresis, and can be visualized under the fluorescence microscope since ethidium bromide–DNA complexes display increased fluorescence.

double Neubauer ruled haemocytometer An improved glass counting chamber that has rulings covering nine square millimeters. Boundary lines of the rulings are the center lines of the groups of three; triple boundary lines also separate the 25 groups of 16 small squares in the central square millimeter; these lines are permanently etched on the polished surface of the glass and appear brightly illuminated, providing sharp contrast to the bright lines and the cells.

dry heat cabinet A chamber used to sterilize items that may be damaged by moist heat or penetrated by moist heat like powders, petroleum products, sharp instruments, and other items.

dry heat sterilization The killing or removal of all microorganisms, including bacterial spores, by dry rather than wet heat. It requires longer exposure time (1.5 to 3 hours) and higher temperatures than moist heat sterilization.

e-PCR A computer program that is used to examine DNA sequences for sequence tagged sites (STSs), each of which is defined by a pair of primer sequences beginning an experimental PCR product.

emission filter (or barrier filter) A filter that blocks the excitation range of the fluorophore, allowing only its emission range to reach the detector.

enrichment medium A medium that contains nutrients to enhance the population density of a wide variety of microalgae sampled or collected in the wild.

entrapment The immobilization of microalgal cells by catching or confinement, usually in alginate beads.

evolutionary tree A visual representation of the branching pattern of evolution, showing evolutionary relationships among various biological species based on similarities and differences of their morphological and genetic characteristics

excitation filter A filter made up of specially coated optical glasses whose function is to limit the spectral band of excitation light that hits the fluorophore using a bandpass filter or a shortpass filter.

excitation light In microscopy, a light source with a short wavelength (or higher energy) used to excite a fluorophore. Ultraviolet light is commonly used as the light source. Excitation light is selected to have its peak wavelength match the resonance wavelength of a fluorophore.

exoskeleton (or cell wall, frustule) The cell wall of a diatom that is usually made of silica.

exsiccatae plantae A herbarium (plant/algal) specimen in dried form, which is mechanically pressed or air-dried to be kept in the herbarium.

eyepiece See ocular.

fixative A chemical that is usually used in “killing” tissues, which has a deep penetrating ability and an immediate action that tends to coagulate and stabilize proteins in specimens so that they do not distort or deteriorate during preservation, study, and storage. Fixatives usually raise the refractive index of the tissue, making it more susceptible to staining.

fixing fluid See fixative.

fluorescence microscope An optical microscope that examines materials that fluoresce under ultraviolet light based on the principle that fluorescent materials emit visible light when they are irradiated with light of shorter wavelength like UV rays or with violet-blue visible rays. It provides image contrast such as differentiation of live cells from dead cells and highlights certain structures such as those with nucleic acid materials. It can also perform kinetic studies in biological processes.

fluorochrome A dye that fluoresces, or a photoreactive chemical that can absorb energy via the interaction of an orbital electron in the molecule’s atomic structure with a photon of light. It is used in labelling biological materials in fluorescence microscopy.

fluorophore A molecule or functional group, usually with a conjugated structure, that is capable of fluorescing.

forced-air sterilizer A dry-heat sterilizer equipped with a motor-driven blower that circulates heated air throughout the chamber at a high velocity, permitting a more rapid transfer of energy from the air to the instruments.

formalin An aqueous mixture of formaldehyde gas in water, with a small amount of methyl alcohol. The latter is added to prevent the formaldehyde from forming a solid mass through polymerization. Commercial formalin is usually sold as 37% formaldehyde in the Philippines.

freeze-drying The elimination of water from a frozen algal suspension by sublimation under reduced pressure where water is evaporated under a vacuum from a frozen algal sample without passing through the liquid phase. Freeze-dried microalgae maintain their original shape and texture.

freeze-thaw method A technique which allows the extraction of quality DNA from microalgae by alternating freezing and thawing the material, at minimum expense and with minimum chemical and equipment use.

frustule Diatom cell wall made up mostly of silica. See exoskeleton.

gaseous sterilizer A sterilizer that makes use of chemicals that sterilize in a chambersimilar to an autoclave. It denatures proteins by replacing functional groups with alkyl groups. Ethylene oxide kills all microbes and endospores, but requires exposure of 4 to 18 hours.

genus cover A Bristol board folder containing a collection of algal herbarium specimens of different species and belonging to the same genus.

glass powder method A technique for extracting quality DNA from microalgae using finely ground glass done by either by grinding the algal materials in glass powder with a mortar and pestle or by simply shaking the algal material in fine glass powder in a test tube.

glutaraldehyde A colorless, viscous liquid that penetrates cellular structures slowly, causing less damage to flagellates and retains in vivo fluorescence.

ground state A lower energy state to which a molecule undergoing photoluminescence descends upon relaxation, from a previous excited state (higher energy).

haemocytometer Originally designed for counting human blood cells, it is also used for counting microalgae. It is a thick glass slide in which the central region has parallel rectangular indentations intersected by a single indentation perpendicular to them forming an “H,” that define two reflective rectangles under the cover slip known as counting chambers (0.1mm deep). Each chamber consists of nine squares (1mm^2 each). The central counting area contains 25 large squares (0.04 mm^2 each) which has 16 smaller squares.

halogens A group of elements that includes chlorine, iodine, bromine, fluorine and astatine, belonging to group VIIA of the periodic table. Halogens are fairly toxic but effective disinfectants alone or in compounds. Examples include iodine (like iodine tincture in alcohol solution), one of first antiseptics used, and chlorine (mixed in water forms hypochlorous acid) which is used to disinfect drinking water, pools, and sewage.

HBO An acronym for the mercury arc lamp: H is for Hg or mercury; B is the symbol for luminance; O is for unforced cooling.

heat sterilization Sterilization with the use of heat, whether through moist heat sterilizationor dry heat sterilization.

heavy metal A metal such as copper, selenium, mercury, silver, and zinc, which is used to kill or reduce the number of pathogenic microorganisms by its addition to a medium or use on a surface which comes into contact with the microorganisms. Such metals are veryeffective in tiny amounts.

hemocytometer See haemocytometer

herbarium A collection of dried, preserved plant/algal specimens together with some associated data used for scientific study.

herbarium label A label on a herbarium specimen containing the scientific and local names, collecting date, locality including coordinates, collector information, field number, and some ecological information.

herbarium presser See plant presser.

herbarium sheet A sheet of paper on which the plant specimen is temporarily or permanently mounted. Standard herbarium sheets are pre-cut in standard sizes.

hierarchical classification A system of grouping living organisms based on their shared traits. There are at present eight levels, the most inclusive being the domain, and the most exclusive, the species.

high efficiency particulate air filters (HEPA) Air filters that remove bacteria from air that are usually used in operating rooms in hospitals, or in laminar flow hoods, or in inoculating chambers.

holotype A specimen designated by the original author at the time the species name and description were published.

identification The process of giving a name to a particular unknown organism based on a known name of an organism from published taxonomic materials and other means of references.

image comparison One way of identifying an unknown specimen using scientifically published photographs and illustrations of a known or identified specimen.

immobilized algae Algae that have been induced to a state of dormancy by various means and for various purposes.

incident light Light illuminating a system. In fluorescence microscopy, the incident light on a specimen is the transmitted light through an excitation filter. The incident light on a detector or camera is the filtered emission from a specimen.

incineration See burning.

inverted microscope A type of light microscope in which the light source and condenser are placed above the stage pointing down, while the objectives and turret are below the stage pointing up. The image observed gives the same orientation as what can be seen with thenaked eye, except that the specimen is magnified, making it useful in dissecting specimens, aside from other micromanipulations.

ionizing radiation Refers to short-wavelength, high-intensity radiation, used to destroy microorganisms. The term includes gamma rays, X-rays, electron beams, or higher energy rays, which have short wavelengths (less than 1 nanometer). This radiation reacts with DNA to cause mutations, resulting in a damaged cell and the production of peroxides. It is commercially used for the sterilization of disposable items (cold sterilization).

isotype A plant specimen that is a duplicate or very similar to the type specimen of the holotype of a species which may be used as a reference specimen if the type specimen is lost.

ITS-2 region of the nuclear ribosomal operon. This has been proposed as a DNA bar code for algae and land plants, and has been widely used in species-level phylogenetics of green algae. RbcL from the chloroplast genome is used instead of mitochondrial DNA because the chloroplast is more specific to algae and green plant evolution than the mitochondria.

laminar flow hood A canopy or enclosure that provides an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures.

L-drying See liquid drying

liquid (L)-drying A preservation technique where the algae to be preserved are not frozen but maintained at temperatures just above freezing in the liquid state while moisture from the sample is removed quickly under vacuum.

light-emitting diode (LED) A specialized diode that emits radiation.

light microscope A microscope that uses light, whether from a natural or an artificial source, as a source of illumination.

macronutrient A nutrient needed in large amounts like sodium nitrate, usually in $g.L^{-1}$, in the preparation of media for culturing algae.

measuring eyepiece (or ocular micrometer eyepiece, reticle or graticule) A glass disk marked with scales, usually from 0 to 100, inserted into the ocular eyepiece. When calibrated with a stage micrometer, it can be used to take dimensions of cells/tissues under a particular objective of a light compound microscope.

mechanical convection sterilizer See forced air sterilizer.

mechanical parts The secondary parts of the light microscope that support the lenses.

membrane filter A porous article or mass used to filter out microbes. Membrane filters have uniform pore sizes (e.g., 0.22 and 0.45um) and are used to filter most bacteria but do not retain spirochetes, mycoplasmas and viruses. Filters with 0.01 µm pores retain all viruses and some large proteins and are used in industry and research.

mercury arc lamp A reliable broad spectrum source of illumination with a strong UV component for wide-field fluorescence microscopy.

metal halide (MH) lamp A source of illumination for fluorescence microscopy which hasuniform power output across the spectrum and has a longer bulb life time compared to HBO (mercury arc lamps).

microalgae A vast group of oxygenic photosynthetic, microscopic unicellular or multicellular algae which usually cannot be seen with the naked eye.

microalgal paste See algal paste.

micronutrient (or trace element) A nutrient needed in small quantities (as in mgm or $microgram.L^{-1}$) like cobalt nitrate, in preparing media for culturing algae.

microwave radiation A form of non-ionizing radiation ranging from 1 mm to 1 m which may kill vegetative cells but not bacterial endospores in media.

millipore filter A trade name of a porous device composed of nitrocellulose or polycarbonate with a controlled pore size (0.2- 20 µm) used to remove impurities or solid particles (liquid or gas) passed through it.

moist heat sterilization Sterilization usually done with steam under pressure as in an autoclave. The most dependable sterilization method where steam must come in direct contact with the material to be sterilized. Moist heat sterilization kills microorganisms by coagulating their proteins. Latent heat is liberated when steam condenses on a cooler surface. Later on, hydrolysis and breakdown of bacterial proteins occur.

molecular phylogenetics The branch of phylogeny that analyzes hereditary molecular differences to gain information on an organism's evolutionary relationship.

molecular taxonomy A type of taxonomy in which biochemical characteristics, such as the DNA sequence divergence, are used as the basis for the classification of organisms.

mountant (or mounting media) The solution in which the specimen is mounted, generally under a cover glass (slip), utilizing natural or synthetic resins that are easy to handle, with a high refractive index. These also act as sealants, adhesives, and preservatives. Examples include naphrax, styrax, and Canada balsam.

nomenclature Giving a new name to an organism, if the features of the organism do not fit any of those that have been previously published and studied.

numerical aperture (N.A.) A measure of the ability of the microscope objective to gather light and resolve fine specimen details at a fixed distance: the higher the N.A., the better the resolution of a specimen which can be obtained with that lens. The N.A. of the objective lens is etched on the barrel of the objectives.

numerical taxonomy Classification of organisms by numerical methods based on their characteristic states to create a taxonomy using numeric algorithms like cluster analysis rather than using subjective evaluation of their properties.

nutrient medium (pl. nutrient media) A formulation of organic and/or inorganic compounds, either in solid or liquid state, used for the cultivation, isolation, or storage of microorganisms, such as microalgae.

objective (or object lens) In microscopy, the lens(es) just above the object or specimen, mainly used to gather light and enhance the magnification of the specimen being observed.

ocular lens (or eyepiece) In microscopy, the optical lens part of the microscope nearest the eye which further enhances the magnification of the specimen being observed. The magnification (usually 10x) is etched on the barrel of the eyepiece.

oil immersion objective (OIO) lens In microscopy, the objective lens that increases the resolving power of a microscope by linking the objective lens and the specimen using transparent oil (like a cedar oil) of high refractive index, thereby increasing the numerical aperture of the objective lens. Its magnification is usually 100x.

optical lenses In microscopy, the lenses found above the stage of a compound microscope, including the objective or object lens and the eyepiece or ocular eyepiece.

optical parts of the microscope The parts of the microscope through which light passes, that magnify and resolve images.

organic chemical components Components of a nutrient medium that are derived from carbon-containing compounds.

oven-type sterilizer A dry heat sterilizer that has heating coils at the bottom of the unit that cause the hot air to rise inside the chamber via gravity convection. This type of sterilizer is slower in heating, requires longer time to reach sterilizing temperature, and is less uniform in temperature control throughout the chamber than the forced- air type.

oxidizing agent In chemistry, the chemical compound that has the ability to accept electrons in the chemical reaction. Examples include hydrogen peroxide and nitric acid.

parfocal objectives In microscopy, objectives that are optically and mechanically designed so that the distance between the specimen and the aerial image is always constant such that little or no adjustment is needed when shifting from the low power objective (LPO) to the high power objective (HPO).

periodic transfer (or subculturing) A method of maintaining microalgal cultures that involves the aseptic transfer of an inoculum from a log phase culture into a pre-sterilized, fresh culture medium at regular intervals.

phenetics See numerical taxonomy.

phenotype In genetics, phenotype is the term for the observable traits of an organism.

phosphorescence Emission of light resulting from electronic relaxation from the triplet state to the ground state. Unlike fluorescence, phosphorescence is a slow process, involving radiation of lower energy photons (longer wavelengths), which continues even after excitation stops.

photoluminescence A quantum mechanical process which requires light to excite a molecule to an energetic state. It includes fluorescence and phosphorescence.

photon A massless elementary particle at rest representing a quantum of light or other electromagnetic radiation; the smallest discrete amount or quantum of electromagnetic radiation. The energy of a photon is dependent on the radiation frequency. In free space, a photon travels at 2.998×10^8 m/s.

phycology (or algology) The scientific study of algae.

phylogenetic tree See evolutionary tree.

physical sterilization Destruction or removal of all viable microorganisms, including spores of fungi, bacteria and viruses through physical means, such as the use of heat (moist heat or dry heat).

pickle A colloquial term for the preservation of specimens in fluid or liquid state, from the fact that the usual preservative component in the solution has an acid component like glacial acetic acid.

plankton net A piece of equipment for collecting plankton consisting of a single net ring, as well as a towing line and bridles, a filtration nylon mesh net and a sample collecting small bucket.

plant presser (or herbarium presser) A setup constructed for the drying of plant/algal herbarium specimens made by laying down a corrugated board on a flat surface or table, on top of which are placed several pieces of newspaper and other absorbent materials or absorbent papers on top of the wet specimen.

polymerase chain reaction (PCR) A laboratory technique that allows the production of multiple copies of a segment of DNA very rapidly and accurately.

preservation in liquid or fluid Storage of specimens in liquid preservatives to protect them from possible deterioration.

preservative A solution that has a germicidal effect and prevents autolysis, providing an environment around the specimens free from bacteria, zooplankters, and other infestations so that the specimens are maintained close to their “normal” appearance for long periods of time.

quadrant streak plate method The isolation of microalgae by streaking a red-hot metal inoculating loop on the water sample containing the algae, streaking in parallel zigzags along four quadrants of an agarized nutrient medium in a Petri dish. After incubation, isolated colonies would be expected in the last quadrant.

quaternary ammonium compounds (Quats) Quaternary ammonium compounds or quatsare cationic (positively charged) detergents which are effective against Gram-positive bacteria and less effective against Gram-negative bacteria.

radiation The process of emitting radiant energy in the form of waves or particles. Exposure to radiation is one method of microbial control. It falls under two categories, ionizing and non-ionizing radiation. Non-ionizing radiation includes infrared and ultraviolet light. Ionizing radiation includes gamma rays, X-rays, electron beams, or higher energy rays that have short wavelengths (less than one nanometer).

real image An image formed when light rays, after reflection or refraction, converge at a point before a mirror or lens. An upside-down and magnified image of the microscopic specimen being viewed and also called a real image because the light rays actually pass through the place where the image lies.

relative humidity The ratio of the actual vapor pressure of water in the air to that in air saturated with water vapor. It is frequently expressed as a percentage.

relaxation In fluorescence and luminescence, it is the process that takes place as the molecule goes from the excited state (higher energy) to the ground state (lower energy).

resolution (or resolving power) In microscopy, the ability of the objectives to break up an object into its component details; the greater the resolving power, the smaller the minimum distance between the two lines or points that can be differentiated. Resolving power is dependent upon the numerical apertures of the optical lenses, including the substage condenser.

scientific name The name assigned to a species of an organism, composed of two names, a generic name (name of the genus), in which the first letter is capitalized, and a specific name, the name of a species, in which the first letter is not capitalized, plus the name of the author.

selected slides A type of permanent diatom slide that is made up of usually one type or one species of a diatom or some selected group of diatoms.

selective media Nutrient media that are suited for cultivating a specific microalga like Tañada's medium, which is meant to cultivate diatoms; or BG-11 minus nitrogen, which is used to culture nitrogen-fixing cyanobacteria.

simple microscope A microscope that essentially has one lens or has one objective lens, with a short focal length and a total magnification of up to about 300x. It is mainly used to magnify objects. Some examples are magnifying glasses and reading glasses.

single-cell isolation Isolation of a single cell of a microorganism, such as a microalga.

solidified media Media in liquid state that have been hardened with agar for cultivating algae.

species cover In the preparation of herbarium specimens, a sheet of Manila paper that is acid free and brown, with dimensions of 42 cm x 30 cm (length x width), when folded. It encloses a collection of algal herbarium specimens of one species, further enclosed in one genus cover.

spectrophotometer An instrument that measures the light absorption of a certain liquid like chlorophyll solution, as a function of wavelength (in UV and visible regions) and follows Beer Lambert's law of light absorption.

spirit collection A term for a collection of preserved biological specimens in an alcohol-based preservative based on the fact that ethyl alcohol is one of the components.

spread plate technique A method of isolating discrete microalgal colonies by inoculating a plate containing a solidified nutrient medium, and spreading the inoculum evenly over the surface using an L-shaped rod which are later incubated. Following incubation, isolated microalagal colonies would grow on the surface of the medium.

stage micrometer A simple microscope slide with a finely divided scale marked on the surface. Each distance between the two lines is of known true length, has a scale graduated in units of 1/100 mm and is used to calibrate the ocular micrometer eyepiece disk.

static-air type of sterilizer A kind of oven-type dry-heat sterilizer with heating coils at the bottom of the unit that cause the hot air to rise inside the chamber via gravity convection. It is slower in heating, requires longer time to reach sterilizing temperature, and is less uniform in temperature control throughout the chamber than the forced-air type of sterilizer.

stereomicroscope See dissecting microscope.

sterile Aseptic or totally clean of any contaminating living microorganisms.

sterilization A process that destroys or removes all viable microorganisms, including spores of fungi and bacteria. It is a process in establishing aseptic conditions and is important in maintaining isolated strains of microalgae.

Stokes shift In fluorescence microscopy, the difference between band peaks of the excitation spectrum and the emission spectrum.

streak plate method Isolating algal colonies by dipping an inoculating wire loop in a water sample and making parallel zigzag streaks on the surface of a solidified nutrient medium in a Petri dish.

strewn slides or strewn mounts A type of permanent diatom slide which contains a mixture of diatoms from a sample.

subculturing See periodic transfer.

taxonomic key An identification device that consists of sequentially choosing among a list of contrasting characteristics that best fit the unknown organism which will ultimately lead to the identity of the unknown organism.

temporary mount A mount usually made for the immediate observation and study of fresh materials. Water is used as the mounting medium, and it is usually unsealed.

trace metal A chemical element whose concentration is needed in low amounts in a culture medium (in $mgm.L^{-1)}$ for the optimum growth of microalgae. An example is boron (as H_3BO_3) which is needed at 2.86 $mgm.L^{-1}$ in BG-11 medium.

type specimen A specimen designated by the original author at the time the species name and description were published.

ultraviolet (UV) light A form of non-ionizing, electromagnetic radiation ranging from 10nm to 400nm, which is unable to penetrate substances, and can only be used for sterilizing surfaces. UV is commonly used as an illumination source for fluorescence applications.

unbuffered Without any solution or chemical to resist changes, especially in the pH of a medium.

undefined media Nutrient media that have nutrient compositions that are not quantifiable.

unialgal isolate An isolate of a single algal species.

virtual An image is formed by a convex mirror or a concave lens where light diverges from a point. No image is formed on a piece of paper placed on the virtual image plane. The image observed in front of an eyepiece of a microscope is a virtual image.

voucher specimen In taxonomy, these are parts of plant/algal/animal specimens, or wholly preserved specimens, that were collected and studied, and that serve as object references documenting the identity of the name used in scientific publications arising from the study.

wavelength In electromagnetic radiation, the distance between two successive crests or troughs of a wave; its unit of measurement is in meters. Wavelength is inversely proportional to frequency, which means that the longer the wavelength, the lower is the frequency, and vice versa.

working distance (WD) In light microscopy, the distance between the specimen and the objective lens when the specimen is in focus.

TAXONOMIC INDEX

SUBJECT INDEX

ABOUT THE EDITORS

Dr. Windell L. Rivera received his PhD degree in Medical Science (Molecular and Cellular Microbiology) from the Institute of Tropical Medicine, Nagasaki University School of Medicine, Japan in 1999. Currently, he is Professor 12 of the Institute of Biology, College of Science of UP Diliman. He was conferred the title, UP Scientist III by the UP System in 2015 and 2018. Credited to his name are more than 100 scientific articles published in Web of Science- and Scopus-indexed journals. His research interests include molecular diagnostics and epidemiology, molecular protozoology and medical biotechnology. He received awards from various agencies, among them are: 2006 TOYM in Medical Science and Microbiology, 2006 Outstanding Young Scientist in Medical Science, 2009 PSM Outstanding Microbiologist, 2010 NAST Outstanding Research in Tropical Medicine, 2010 NRCP Achievement Award in the Biological Sciences, 2013 UPAA Distinguished Alumnus in Science and Technology, 2014 CHED REPUBLICA (Outstanding Research Publication in the Natural Sciences), 2014 Eusebio Y. Garcia Award for Molecular Biology and Molecular Pathology, 2015 DOST-NAST Outstanding Research and Development Award for Basic Research, 2016 Thomson Reuters Promising Star Awards, and 2017 Top 100 Scientists of Asia. Recently, he was elected Academician of the National Academy of Science and Technology, Philippines.

Nerissa K. Torreta, is an Associate Professor of the Plant Biology Division, Institute of Biological Sciences, University of the Philippines Los Baños. She teaches both undergraduate and graduate courses in Botany notably Introductory Phycology (BOT 101) and Advances in Phycology (BOT 202). She completed her baccalaureate and Master of Science in Botany degrees at the University of the Philippines Los Baños and also did a Post-graduate Diploma in Science at the University of Queensland in Brisbane, Australia. A phycologist at heart, she has attended and presented papers on algae in many conferences both here and abroad. She has co-written papers on microalgae published in refereed journals, several of which are in ISI journals. A former officer and lifetime member of the Philippine Phycological Society, Inc. and the Philippine Society for Microbiology, Inc. and the current Secretary of the Biology Teachers Association of the Philippines, UPLB Chapter, she continues to encourage students, faculty and researchers to study microalgae.

ABOUT THE EDITORS

Dr. Milagrosa R. Martinez-Goss, Professor Emeritus at the Plant Biology Division, Institute of Biological Sciences, University of the Philippines Los Baños (UPLB) is a recognized expert in phycology, specifically on microalgae.

Dr. Martinez-Goss is a pioneering scientist in the field of taxonomy and diversity of diatoms. Until Dr. Martinez-Goss entered the field, researches in this study have been led by scientists from the USA, Japan and other European countries. One of her important contributions in this field of study was the discovery of three new species/varieties of *Nitzschia* and *Tryblionella* from Laguna de Bay. Her phycological studies in this lake helped determine its carrying capacity by looking at its phytoplankton productivity. Her studies on paleolimnology of the lake based on diatom studies and her re-discovery of the elusive freshwater red alga, *Compsopogon coeruleus* (Balb.) Mont. in the vicinity of Laguna de Bay also provided further insight into the lake's history. These significant research results on the lake's history and carrying capacity helped improve the management of the lake by research and academic institutions involved in this effort. With more than forty years of experience in teaching and research, she has implemented over 50 research projects, four (4) of which were foreign-funded.

A prolific writer of scientific papers, she has written a total of about 182 scientific papers, four of which are published as books. She was also a guest editor in three scientific journals, i.e., Philippine Agriculturist, 1986, vol. 69 (4b), The Philippine Scientist, 2014, vol. 51, including one ISI-international journal, Journal of Applied Phycology, 2007, vol. 19 (6). The high standards of Dr Martinez-Goss' scientific writings are attested to by the numerous awards and recognitions she has received. Among these are the ten (10) International Publication Awards from the University of the Philippines System for her publications in ISI journals, and the two (2) Outstanding Scientific Papers awarded by the National Academy of Science and Technology (on July 12, 2001 and on July 9, 2015).

Dr. Martinez-Goss was the chairperson of the Local Organizing Committee of the 6th Asia-Pacific Conference on Algal Biotechnology held on October 12-15, 2006 in Dusit Hotel Nikko, Makati City, Philippines. Dr. Martinez-Goss was also mainly responsible for having reinstituted the Philippine Phycological Society, Inc. in 2007 as its Charter Member and she became the Founding President of the Society from 2007 to 2009. She is also actively involved in the maintenance of algal cultures and the phycological herbarium as one of the curators of the Museum of Natural History, UPLB since 1977.

Methods in Microalgal Studies

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