STUDY ON THE EFFECTIVENESS OF DIFFERENT PRESERVATIVES ON THE PRESERVATION AND FIXATION OF PHYTOPLANKTON (*Tetraselmis sp.)* CELLS

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Roll No.: 0122/08

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JUNE 2023

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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LIST OF ABBREVIATIONS

ml	Mililiter
F	Factor
T	Treatment
Cell/ml	Cells per mile liter
mm	Millimetre
g	Gram
%	Percent
KI	potassium iodide
DHA	Docosahexaenoic acid

Abstract

A study was conducted on the effectiveness of different preservatives such as Formaldehyde, Ethyl alcohol and glycerin, Logul's solution (neutral), Logul's solution (acidic), Glutaraldehyde, and Transeu solutions on the preservation of phytoplankton (*Tetraselmis sp*.) cells. The main goal was to find appropriate chemical preservatives for preserving phytoplankton samples in laboratory conditions and determining the extent of damage caused by preservatives during long-term storage cells of phytoplankton. *Tetraselmis sp.* served as the experiment's target species. The Chlorodendrophyceae family includes it under the name marine phytoplankton. The Marine Fisheries and Technology Station, BFRI, Cox's Bazar provided the isolated Tetraselmis sp. sample (one liter of concentrated sample water). This sample was obtained by the BFRI in Bangladesh from the Bay of Bengal. The result showed among different preservatives Lugol's solution (acidic) was the most effective preservative, whereas Glutaraldehyde was determined to be the least effective and the highest mean intact cell was found at formaldehyde and the lowest mean intact cells was found at Glutaraldehyde. This study would help to provide knowledge for choosing appropriate preservation during laboratory experiments and help to find the more suitable preservatives.

Keywords: Phytoplankton, *Tetraselmis sp.*, Preservatives, Fixation, Preservation

Chapter One

Introduction

1.1 Background of Study

The word "plankton", used to characterize tiny plants (phytoplankton) and animals (zooplankton) that move passively in aquatic systems, derives from the Greek word "planktos," which means "drift" or "wander.". Plankton can modify their depth by actively swimming and changing their buoyancy, but they cannot move independently of ocean currents. The foundational elements of the entire aquatic chain of food worldwide are phytoplankton. They are minute plant components, ranging in size from a few millimeters to a few microns (Marshall, 2009). These microscopic plants must get sunlight in order to perform photosynthesis. They need basic, straightforward inorganic chemical nutrients like phosphate (PO_4) and nitrate (NO_3) in addition to light and oxygen (O_2). In the form of carbon dioxide (CO_2), they also need carbon. The "glass-like" shell of diatoms necessitates the use of a specific silicon compound (Silicate, SiO_4). Phytoplankton is responsible for more than 90% of the total marine primary output. Many published publications detail their unique functions in calcification, silicification, and nitrogen fixation. They are presented as primary consumers' direct food sources, including zooplankton and larvae of fin fish and shellfish. Energy from phytoplankton may be transferred to organisms at higher trophic levels, according to several studies (Rajkumar et al., 2009).

Microalgae are now being used more frequently in biotechnological applications, primarily for bioremediation, the production of nutraceuticals and medicines, and bioenergy. Climate science, geology, environmental management, and conservation are just a few of the many areas of marine science that depend on research on marine phytoplankton. For a complete image of the algal community to be presented, it is essential to comprehend the presence, range, and abundance of phytoplankton species. This can only be done if the samples being examined at the time are of high enough quality.

The most frequent uses of plankton samples are for microscopic examination of preserved organisms or calculations of biomass. Because biomass is lost during formaldehyde storage, these two objectives cannot coexist (Durbin and Durbin, 1978). Only after a

lengthy period of preservation and with the knowledge that the weight loss correction is approximative can samples intended for subsequent analysis be used for biomass measurements (Omori, 1978). It is typically easy to obtain both fresh samples for measuring biomass and stored samples for evaluation. It is frequently impractical to sort live plankton samples soon enough after collecting to get organisms in suitable condition for biomass estimation. A lack of natural feed can occasionally impair aquaculture activities, especially hatchery operations. Experiments on techniques of gathering and preserving algae were conducted in order to provide a supply during times of scarcity.

Fixation of the sample and subsequent storage are necessary. Numerous investigations have been made to determine how fixation and preservation affect phytoplankton, both in artificially created cultures and in wild populations. In contrast, fixation results in significant cell loss and alterations to cellular characteristics in delicate organisms like prasinophytes and cryptomonads (Murphy and Haugen, 1985). Creating a uniform process for phytoplankton fixation and storage is difficult. The simplest solution to this problem is to optimize the procedure according to the phytoplankton target, goal, and study period. Different types of preservatives are used to preserve the phytoplankton in long term storage methods. Formaldehyde, glutaraldehyde, transeau solution, ethyl alcohol and glycerin are used for preserving plankton. Despite the availability of a wide range of antimicrobial drugs, the preservation of phytoplankton grab samples has altered little in recent years. Formalin is the most often used preservative today. Long experience has demonstrated that this chemical reduces phyto- and zooplankton biodegradation in samples (Welch, 1948), although it falls short of being an ideal preservative in several critical ways: (a) conserved samples produce unpleasant fumes, (b) repeated contact with formalin-containing solutions promotes skin cracking and roughening, and (c) algal colors deteriorate fast, making identification more difficult as the samples age (Karlson 2017). The ideal plankton preservative should be: (a) non-volatile and non-irritating to human skin, eyes, and respiratory system, (b) chemically stable in solution and have a long shelf life, (c) inexpensive, (d) toxic to all micro-organisms at very low concentrations, (e) a good preservative of chlorophyll and other cell pigments, and (f) unaffected by large amounts of dissolved and suspended organic and inorganic matter commonly occurring in water samples (Karlson, 2017).

Tetraselmis sp. have been extensively fixed and preserved using conventional fixatives such Lugol's iodine, formalin, and methanol (Azma et al., 2011). The problem with these fixatives is that formalin and Lugol's change the fluorescence and structure of cells, and alcohol (methanol, ethanol) eliminates the lipophilic pigments, which prevents chlorophyll autofluorescence from occurring. (Marie et al., 2005).

The fixative most frequently employed in pathology is formaldehyde. Formaldehyde is poisonous, carcinogenic, extremely irritating, and a powerful sensitizer. While formaldehyde is toxic through all routes of exposure, has irritating fumes to the eyes, skin, and mucous membranes, and is a known human carcinogen, it is thought to be the best fixative for preserving the taxonomic and morphological characteristics of mixed marine plankton at a concentration of 4-5% in distilled water. However, their effectiveness is not assessed in terms of duration of storage and quality maintenance of plankton. Although alcohol is very flammable and generally safe to handle, prolonged contact with it can cause skin irritation.

The study has been designed to determine the effectiveness of different chemical preservatives of phytoplankton in lab conditions. Finding the right fixative or preservative concentration is essential to prevent the introduction of artifacts, morphological changes, and microbiological contamination during long-term preservation. The bias introduced by the preservative prohibits the measurement of the cells' actual sizes because almost all measurements on phytoplankton cells for ecological reasons are derived from preserved or prefixed cells. Calculations based on preserved cells, such as those for carbon content or biomass/biovolume, yield inaccurate findings that do not accurately depict the conditions of the environment from which the cells were harvested. The coastal phytoplankton populations of Bangladesh have not attracted much scientific attention to date. Literature has described the phytoplankton communities of Bangladesh's north-eastern coast and its south-eastern coast's Karnafuli estuary (Islam and Aziz 1975). Furthermore, there have been a few reports on phytoplankton diversity along Bangladesh's southwestern coast (Ahmed et al. 2010; Aziz, Rahman, and Ahmed 2012). *Biddulphia sp*., a phytoplankton species from Bangladesh's Naf estuary, was researched by Aziz (2005) in terms of taxonomy and biology.

There is no study has been found on the effectiveness of plankton preservation and fixation with different preservatives. For this reason, there is scope for working on this experiment.

In an investigation on preservation done by Mukherjee et al. in 2014, formaldehyde and Lugol's iodine were utilized for long-term preservation. Stoecker et al., 1994 conducted a study on the Preservation of marine planktonic ciliates: losses and cell shrinkage during fixation in this study they used formaldehyde and lugol's solution. Six different preservatives and fixatives are being used in the current study and they are formaldehyde, ethyl alcohol and glyserin, lugols solution (neutral), lugols solution (acidic), glutaeraldehde, transeau solution. There is no specific study is found on effectiveness of plankton preservation and fixation with different preservatives.

The study has the potential to expand phytoplankton research by offering significant information into the optimal preservation strategies. Researchers will have a better knowledge of how various preservatives influence phytoplankton samples, which will lead to better techniques in future studies.

1.2 Objectives of the study:

➢ Identification of the suitable chemical preservative for preserving phytoplankton samples in laboratory conditions; and

➢ Identification of the degree to which preservatives during long-term storage damage phytoplankton cells

1.3 Significance of the study:

• Outcome of the study may help the intensive study on microalgae in laboratory condition;

• Determination of microalgae preservative effectiveness may facilitate scientists in the selection of suitable agents for algal cell and tissue preservation; and

• Moreover, the findings will provide a new base of knowledge for algal biology and biotechnology sector.

Chapter Two

Review of literature

Microalgae are a varied collection of unicellular photosynthetic microorganisms that live in both freshwater and saltwater settings. Approximately 72,500 microalgae species have been discovered and approximately 30000 have been recognized (Guiry, 2012). Microalgae have potential uses as a food source in aquaculture because of their nutritional value (Ponis et al., 2006). Microorganisms have the potential to be beneficial as human food, chemical manufacturing, aquaculture, and solar energy bioconversion (Goldman, 1979). Microalgae are a most important food source for the nourishment of larvae of fish and bivalves, as well as larvae of mollusks, crustaceans, fish, or live prey employed in culture, like rotifers and artemia (Holme et al., 2009). The presence of lipids, proteins (amino acids), carbs, and vitamins in diverse microalgae species is one of the primary reasons for considering these organisms as a feed source for aquaculture animals (Southgate et al.,2003). Furthermore, the ingredient of highly unsaturated fatty acids (HUFAs), mainly, eicosapentaenoic acid (20: n-3) (EPA), arachiodonic acid (20: n-6) (ARA), docosahexaenoic acid (22: n-3) (DHA), and linolenic acid (18: n-3) (LA), is the most essential determinant of the nutritional composition of microalgae (Lavens et al., 1996).

In mariculture, live microalgae have traditionally been employed as food for bivalves (Brown et al., 1989). Nearly one-third of the costs of producing spit in commercial hatcheries are incurred in the generation of microalgae (Benemann, 1992). Over the past few years, substitution goods such as bacteria, dried algae yeast and numerous sorts of microcapsules, algal paste and slurry algal paste have been investigated in an attempt to create cost-benefit replacements and simplify hatchery-nursery procedures (Knauer and Southgate, 1999). In general, they found growth rates are lower and mortalities are higher than microalgae (Robert and Trintignac, 1997). Among different alternative foods, the preserved microalgae used in slurry form or paste become visible to be one the most promising, in spite of the fact that very little experimental studies have been conducted to estimate the value of nutrition of stored microalgae (McCausland et al., 1999).

Plankton samples are most commonly utilized for biomass estimations or microscopic study of organisms which are preserved in different condition. These two goals are mutually exclusive because biomass is lost during formaldehyde storage (Durbin and Durbin 1978). Samples are stored for further analysis which can be utilized for measurements of biomass only after a long term of preservation and with the understanding that the loss of weight adjustment is estimated (Omori, 1978). Getting both new samples for the measurement of biomass and samples that are stored for assessment is usually simple.

The genus *Tetracelmis* (*Chlorodendrophycea, Chloropkyta*) is most likely an early-diverging linkage of green algae (Turmel et al., 2016). It is an abnormal collection of organisms that are found in water but may also live in the nearby land surface that is washed into the aquatic environment, displaying transitional characteristics possibly possessed by early terrestrial life. The well-known members of the genus are quadriflagellates unicells, but only *Tetracelmis* species may form stalked colonies at different point in their cycle of life (Sym and Pienaar, 1993). The majority of Chlorodendrophyceae are found in marine habitats which remain as planktonic or benthic organisms and, in this environment, they can form dense colonies and cause different algal blooms in pools or bays. Several species can be found in freshwater settings (John et al., 2002). Several *Tetraselmis* species have been known for the endosymbiotic role for marine animals, including *Tetraselmis convolutae*, a facultative symbiont of the acoel flatworm Symsagittifera (Convoluta) roscoffensis (Serodio et al., 2011), and an undescribed *Tetraselmis* species isolated from the radiolarian Spongodrymus

Tetraselmis sp. retains some prasinophyte primitive characteristics and shares different ultrastructural properties with the core of Chlorophyta (Trebouxiophyceae, Ulvophyceae, and a phycoplast), including close mitosis and aphycoplast (Sym &Pienaar, 1993). Molecular evidence has verified the genus' evolutionary link with the core Chlorophyta (Marin, 2012).

Numerous studies have been conducted on the taxonomy and morphology of the genus Tetraselmis (Martin and Melkonian, 1994). *Tetraselmis* contains approximately 27 currently recognized species that include taxa previously ascribed to the genus Platymonas,

Prasinocladus, and Aulacochlamys (Sym and Pienaar, 1993). These species circumscriptions are based on light microscopical (LM) and electron microscopical (EM) characteristics such as cell size and shape, structure of anterior cell lobes, chloroplast morphology, the position of stigma, shape and position of pyrenoid (Butcher, 1952) ultrastructure of the pyrenoid and flagella hair scale (Marin et al., 1996).

Tetraselmis is relatively large (to 14 microns) for a unicellular alga. As a result, it is successfully used as feed for fish, bigger pods that are capable of swimming freely and brine shrimp. While the morphology of *Tetraselmis* species varies, spherical and oval shapes are frequent. It could have a reddish "eye" spot. (Kenneth Wingerter)

A thin cell wall known as theca covers the cells of this species of organisms and is cr (Sym & Pienaar, 1993). The flagella of this species are coated with hairs and pentagonal scales (Melkonian, 1990). Sexual reproduction technique is observed in this organism. Different species produce thick-walled cysts which is vegetative that can be heavily sculpted (Sym & Pienaar, 1993). Even though they are shorter, the flagella are the same length as the cell body. Due to the peculiar configuration of its flagella, *Tetraselmis* swims in a distinctive manner; it moves swiftly and usually appears unsteady, frequently moving in a straight line before abruptly changing course. The Pyramimonas genus, to which it is most closely related, shares similarities with it in terms of swimming behavior and physical makeup.

Tetraselmis species are economically important because their euryhaline and eurythermal character makes them excellent for mass cultivation (Fabregas et al., 1984). In aquaculture facilities, the genus is frequently used as feed for young mollusks, shrimp larvae, and rotifers (Azma et al., 2011). *Tetraselmis* has long been a staple genus in aquaculture and biotechnology because to its abundant growth rate and resistance. *Tetraselmis* is appreciated as an aquarium diet due to its high lipid content. It is particularly high in the essential fatty acids EPA and DHA. It is also high in specific amino acids (such as alanine), which encourage rapid growth and act as an appetite stimulant for marine animals. It is high in vitamins C and E. Even better, it contains sufficient carbohydrates to fulfill the metabolic energy required for the animals. Carbohydrates can account for up to 27% of the dry weight. The starches it manufactures are quite similar to those produced by land plants. *Tetraselmis* also contains a high concentration of color-enhancing xanthophyll carotenoids

(e.g., neoxanthin, antheraxanthin, violaxanthin, and lutein), which may assist the eating animal by scavenging harmful oxidizing molecules. Members of the genus *Tetraselmis* are commonly utilized as a component of the food for early developmental stages of bivalve spat in aquaculture (Wilkfors et al., 1984). For this use, a number of strains from culture collections are indicated, including Tetraselmis suecica CCAP 66/4 (Thompson et al., 1988).

Tetraselmis (at least a few species) is also notable for having antibiotic component. Specific phenolic components are thought to be responsible for algal extracts' antibacterial properties. *Tetraselmis suecica* supernatants and extracts have been proven to protect fish from pathogenic bacteria such as Aeromonas, Staphylococcus, and Vibrio while also lowering the populations of these germs in the tank water. These compounds might most likely be employed in a variety of ways to prevent disease in captive marine animals. For example, dosing brine shrimp with Tetraselmis immediately before feeding could prevent seahorses from Vibrio infections, to which they are particularly vulnerable.

The feeding value of *T. suecica* for bivalve larval and postlarval stages has been well studied. When combined with other algae species, this species was found to be a good diet for *Oedulis, C. gigas, Ruditapes decussatus*, and *Mytilus*, but it was found to be poor to moderate food for Ostreaedulis, Saccostrea commercialis, and Venerupis pullastra (Albentosa et al., 1993). Tetraselmis suecica, Pavlova lutheri, and Chlorella sp., three marine microalgae essential for aquaculture, have lately been successfully produced semi-continuously under controlled conditions in alveolar plate reactors (Tredici et al., 1996). It was discovered that *T. suecica* preservation at low temperature (+4°C) was effective in preserving cell viability and the stability of biochemical composition, notably the fatty acid profile (Tredici et al., 1996).

To maintain sample quality, preservation is required (to lessen cell deterioration and morphological change) (Sournia 1978). However, the effects of preservatives on certain phytoplankton taxa change over time (Throndsen , 1978). Due to its lower risk to human health when compared to regularly used alternatives like formaldehyde and gluteraldehyde, Lugol's iodine solution (Lugol's) is often regarded as the ideal preservative to utilize for researching phytoplankton species (Hallegraeff et al., 2003). Sample quality must be

maintained through preservation (to reduce cell deterioration and morphological change) (Hasle, G., & Sournia, A, 1978). However, the effects of preservatives on different phytoplankton taxa change with time (Throndsen, 1978). Numerous fixatives/preservatives, such as diluted formaldehyde (Stoecker et al. 1987), a modified Bouin's solution (Dolan & Coats 1990), and various amounts of acidic Lugol's solution (Sherr & Sherr 1993), have been employed to count micro plankton in seawater samples. Higher concentrations may lessen ciliate losses even though diluted acid Lugol's solution is advised for counting flagellates (Throndsen, 1978) and has been used to repair and keep cilia (Jerome et al., 1993). In many cases, live ciliate counting produces substantially higher estimates of ciliate abundance than preserved sample counting (Sorokin 1981). For instance, Dale and Burkill, (1982) found that live counting of ciliates from coastal waters produced up to 20% higher estimates of abundance than counting of samples maintained with 0.4% (final conc.) formaldehyde-free. Volume-based carbon conversion factors with a focus on cell size and numerical abundance are frequently used to estimate the biomass of ciliate assemblage. Estimates of cell biomass based on measures of cell volume may change due to shrinkage caused by fixation and preservation (Jerome et al., 1993).

Due to its lower risk to human health when compared to regularly used alternatives like formaldehyde and gluteraldehyde, Lugol's iodine solution (Lugol's) is typically recognized as the ideal preservative to utilize for researching phytoplankton species (Hallegraeff et al., 2003In addition to staining and enhancing the density of individual algal cells, Lugol's also speeds up settling when used with the Utermöhl analytical method (Choi and Stoecker, 1989). For microscopic examination of phytoplankton, nanozooplankton, microzooplankton, and mesozooplankton samples, a potassium-iodide and iodine solution (Lugol's iodine solution) is frequently employed as a fixative (Jaspers and Carstensen, 2009). Neutral Lugol's iodine solution is thought to be an ideal fixative for carbon- or nitrogen-stable isotope investigations since it is devoid of these elements. It has been hypothesized that the addition of sodium thiosulfate could increase the PCR success rate of the samples of phytoplankton that have been fixed in Lugol's iodine solution following molecular investigations (Mäki et al., 2017). Neutral Lugol's iodine solution is thought to be a good fixative for carbon or nitrogen stable isotope investigations since it is devoid of carbon and nitrogen.

Phytoplankton samples treated in Lugol's iodine solution have been the subject of molecular analyses (Mäki et al. 2017). According to a claim made by Auinger et al. (2008), the addition of sodium thiosulfate evaluated the preservation effectiveness of acidified to neutral Lugol's solutions in Baltic Sea samples and discovered both to be ineffective.

Formaldehyde is the most often used fixative in pathology. It is frequently used as a 10% solution of neutral buffered formalin (NBF). A formalin-formaldehyde solution is typically used as the fixative because formaldehyde is a gas at ambient temperature. Aqueous solutions of formaldehyde swiftly change from formaldehyde to methylene glycol; very little aldehyde is left behind (Fox et al., 1985). Buffered formalin is the fixative most frequently used for plankton samples and sediment trap studies because of its durability over extended periods of time (Lee et al., 1992).

The molecules of methylene glycol (molecular weight 48) and formaldehyde (30) penetrate cells and extracellular materials fast, frequently reaching a depth of 5 mm in about 2 hours. It is commonly accepted that a specimen should be placed in a formaldehyde solution for at least 24 hours for good structural preservation because formaldehyde's chemical reactions with proteins are slower than those of any other material used as a fixative (Fullmer, 1976).

Ethanol (Ethyl alcohol) is typically 95% concentrated. It is also an effective preservative and the preferred approach for long-term preservation and storage of most plankton. It is commonly diluted to 70-75% potency using distilled water. This is the smallest concentration at which preservation will be possible. In alcohol, samples become brittle, a lot of the color is removed, and there are evaporation concerns. According to some accounts, adding 1% glycerol to the samples helps keep them flexible and slows evaporation.

Chapter Three

Methodology

3.1. Study Area:

The targeted species used for this experiment was *Tetraselmis sp.* It is known as marine phytoplankton, which belongs to the Chlorodendrophyceae class. The isolated sample of *Tetraselmis sp.* (One liter of concentrated sample water) was collected from the Marine Fisheries and Technology Station, BFRI, Cox's Bazar. The BFRI collected this sample from the Bay of Bengal, Bangladesh. Then, the sample was brought in the Aquatic Ecology Laboratory of the Department of Fisheries Resource Management, CVASU and diluted to a definite volume of 1500 mL for adjusting to the experimental setup.

3.2. Experimental Design:

Six (06) different preservative chemicals were used in this experiment. The chemical preservatives were considered as factors for this experiment (F1-F6). Total 06 factors: F1) Formaldehyde, F2) Ethyl alcohol and glycerin, F3) Logul's solution (neutral), F4) Logul's solution (acidic), F5) Glutaraldehyde, and F6) Transeu solution were used in this experiment. For every factor, there were five different treatments (T1-T5) (Table 01). The duration of this whole experiment was about one month: August 2022 to September 2022.

Table-01: Factors and treatments used for plankton preservation

Factor	**T1**	**T2**	**T3**	**T4**	**T5**
Formaldehyde (F1)	1%	2%	3%	4%	5%
Ethyl alcohol and glycerin (F2)	1%	2%	3%	4%	5%
Logul's solution (neutral) (F3)	0.4	0.6	0.8	1	2
Logul's solution (acidic) (F4)	0.4	0.6	0.8	1	2
Glutaraldehyde (F5)	1%	2%	3%	4%	5%
Transeu solution (F6)	1%	2%	3%	4%	5%

3.3. Formulation of preservatives:

Formaldehyde, Ethyl alcohol and glycerin, Lugol's solution (neutral), Lugol's solution (acidic), Glutaraldehyde and Transeu solution were formulated to different concentration in this experiment.

Formaldehyde (F1)

(A) Preparation: Twenty (20) percent aqueous solution of formaldehyde (40 percent formaldehyde HCHO) neutralized with hexamethylenetetramine. Then, 100 g hexamethylenetetramine to 1 liter of the 20 percent solution was added

(B) Application: 2 mL in 100 mL sample

Ethyl alcohol and glycerin (F2)

(A) Preparation: 70% ethyl alcohol was mixed with 5% glycerin

(B) Application: 2 mL in 100 mL sample

Lugol's solution (Neutral) (F3)

(A) Preparation: It was brought in the commercial formulation.

(B) Application: 0.4 to 0.8 mL chemical was added to 200 mL of sample to give the sample a weak brown color. The mixture was shaken well.

Lugol's solution (Acidic) (F4)

(A) Preparation: 100 gm potassium iodide (KI) was dissolved in 1 liter of distilled water; then 50 crystalline iodine was dissolved and 100 mL of glacial acetic acid was added.

(B) Application: 0.4 to 0.8 mL fixative was added to 200 mL of sample to give the sample a weak brown color. The mixture was shaken well.

Glutaraldehyde (F5):

(A) Preparation: 25% aqueous general-grade glutaraldehyde

(B) Application: 1.0 mL of 25% aqueous general grade glutaraldehyde for each 100mL of the algal sample which to be preserved.

Transeau solution (F6)

(A) Preparation: Contained 6 parts water, 3 parts ethyl alcohol (95%), 1 part Formalin (commercial)

(B) Application: 1:1 ratio with sample

3.4. Initial count, preservation and storage

The initial cell count of *Tetraselmis sp.* was done to compare the final cell count in different weeks after employing treatments. Then the treatments of the relative factors (F1 to F6) were employed to the sample bottles. The airtight sample bottles were kept in dark condition in room temperature.

3.5. Counting and observation

Before counting and observation of the sample, the samples were stirred with a glass rod to ensure no sedimentation in the sample bottle. The counting and observation were done at 7 days interval up to 4 weeks (W1-W4). At the end of every week, the cells were counted in Sedgewick-Rafter (SR) cells under the microscope (Optika B190 TB; Camera Optika C-B3) and the same procedure was followed for the rest of the experimental duration (Equation 1 and Equation 2). The cell counts were normalized with the diameter of the SR cell and then expressed as percent intact and damaged cells to minimize deviations from visual cell counting.

Equation-1:

The cell or colony concentration (**C**) to unit per mL for a transect count is calculated using the equation below, followed by adjustments for any dilution or concentration factors.

$$C = \frac{(\mathbf{N \times 1000}\ mm^3)}{(\mathbf{L \times D \times W \times S})}$$

Where: **N** = number of cells/colonies counted

L = length of transect strip (mm) = 10

W = width of transect strip (mm) = 2

D = chamber depth (mm) =1

S = number of transects counted =2

Equation 2:

$$\text{Average intact or damaged cells} = \frac{\text{Intact cells or damaged cells}}{\text{Total cells}} \times 100$$

3.6. Data collection, analysis and interpretation

The cell count data was recorded every week in Microsoft Excel software. The data was categorized, analyzed and visualized with Microsoft Excel (version 2016) and SPSS (version 22). One-way ANOVA (Analysis of Variance) was performed to determine whether there was any significant variation among the factors' efficiency or not.

The research methodology was illustrated in (figure 01) below:

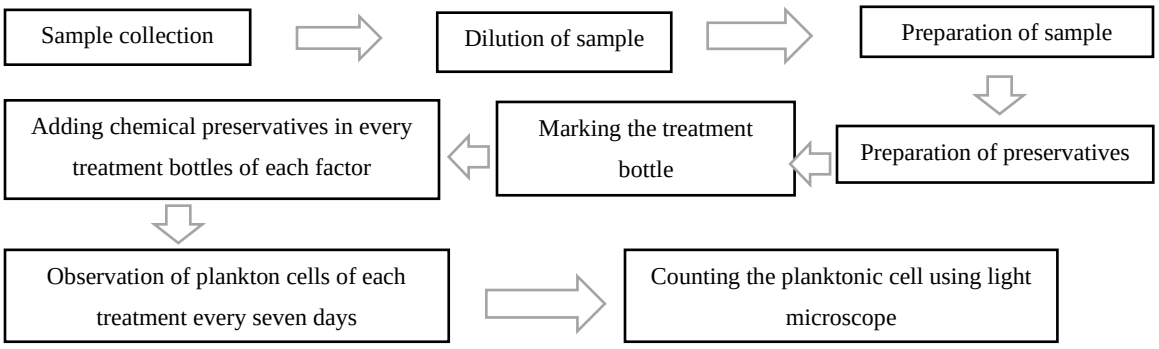


Figure-01: Flowchart of the research methodology

Photo Gallery

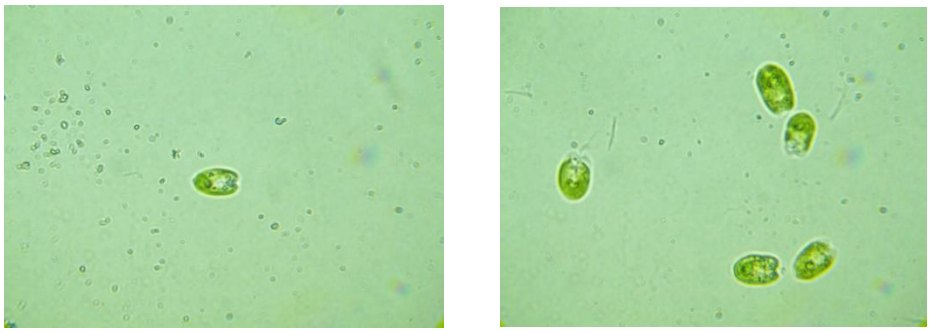


Plate-1: Phytoplankton (*Tetraselmis sp*)

Plate -02: Plankton sample

Plate-03: Sample bottle

Plate-04: Labeling the sample bottle and preservatives

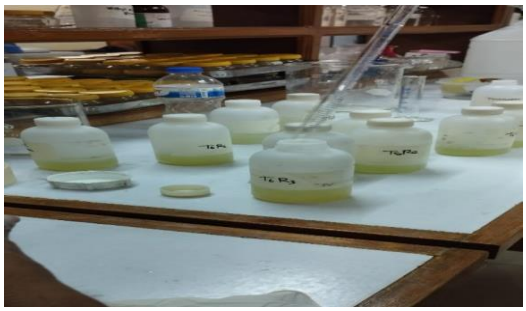


Plate-05: Preserving the plankton sample using different preservatives

Plate-06: Counting of plankton cells with a microscope

Chapter Four

Result

5.1. Preservation efficiency of the preservatives

5.1.1 Formaldehyde (Factor 1):

Among T1 to T5 of F1, the highest number of intact cells were found at F1T5 (26.66%), followed by F1T4, F1T1, and F1T2, and the lowest number of intact cells were found at F1T3 (10.34%) respectively. (Fig-02).

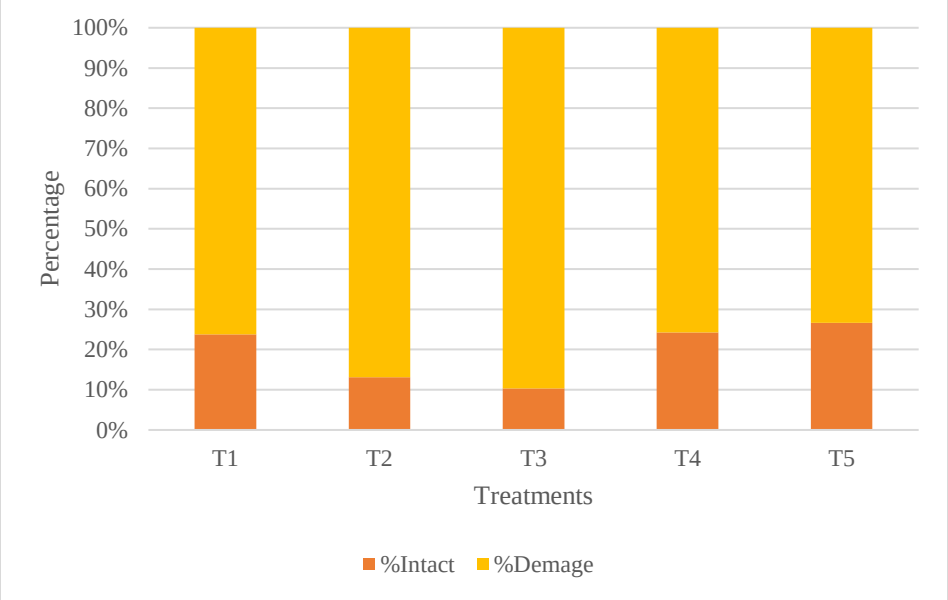
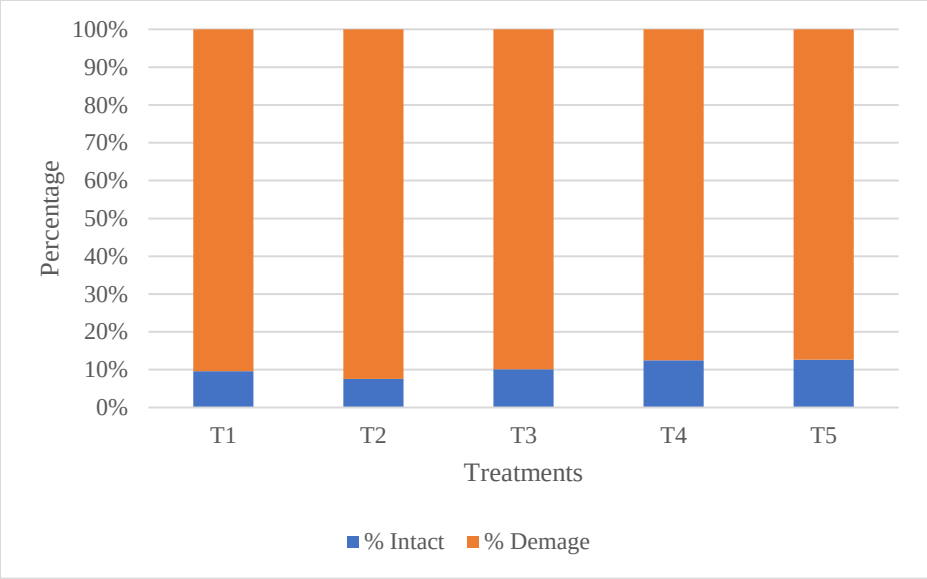


Figure-02: Percent comparison of intact and damaged cells among the treatments of formaldehyde

5.1.2 Ethyl alcohol and glycerin (Factor 2):

Among T1 to T5 of F2, the highest number of intact cells in case of percentage was found at F2T5 (12.64%), followed by F2T4, F2T3, F2T1, and the lowest number of intact cells in case of percentages was found at F2T2 (7.58%) respectively



(Fig-03) Figure-03: Percent comparison of intact and damaged cells among the treatments of Ethyl alcohol and glycerin

5.1.3 Lugols iodine (neutral) (Factor 3):

Among T1 to T5 of F3, the highest number of intact cell in case of percentages was found at F3T1 (27.07%), followed by F3T3, F3T2, F3T4 and the lowest number of intact cells was found at F3T5 (11.01%).

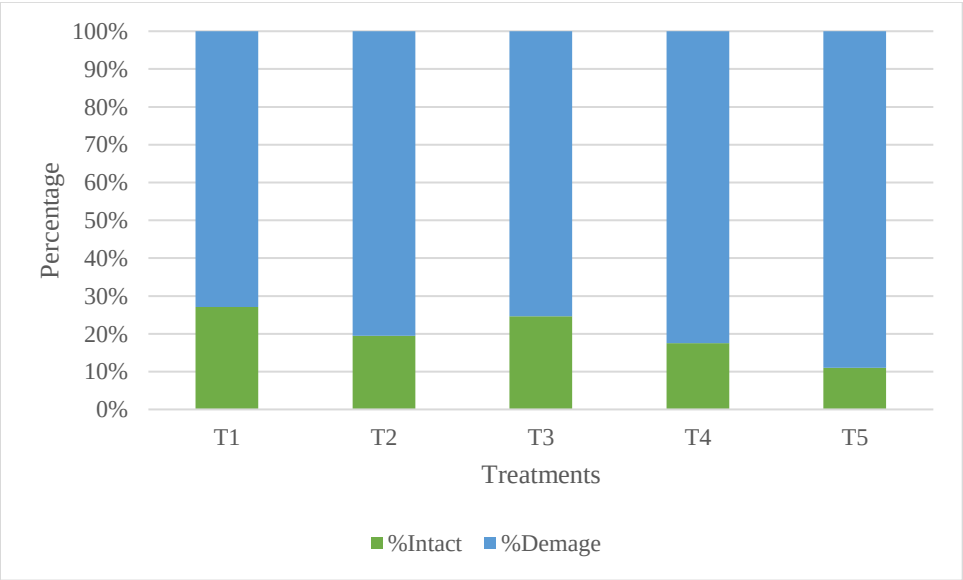


Figure-04: Percent comparison of intact and damaged cells among the treatments of Lugols iodine (neutral)

5.1.4 Lugol's solution (acidic) (Factor 4):

Among T1 to T5 of F4, the highest number of intact cells in case of percentage was found at F4T5 (22.10%), followed by F3T3, F3T2, and F3T4 and the lowest number of intact cells was found at F4T1 (13.83%). (Fig-05)

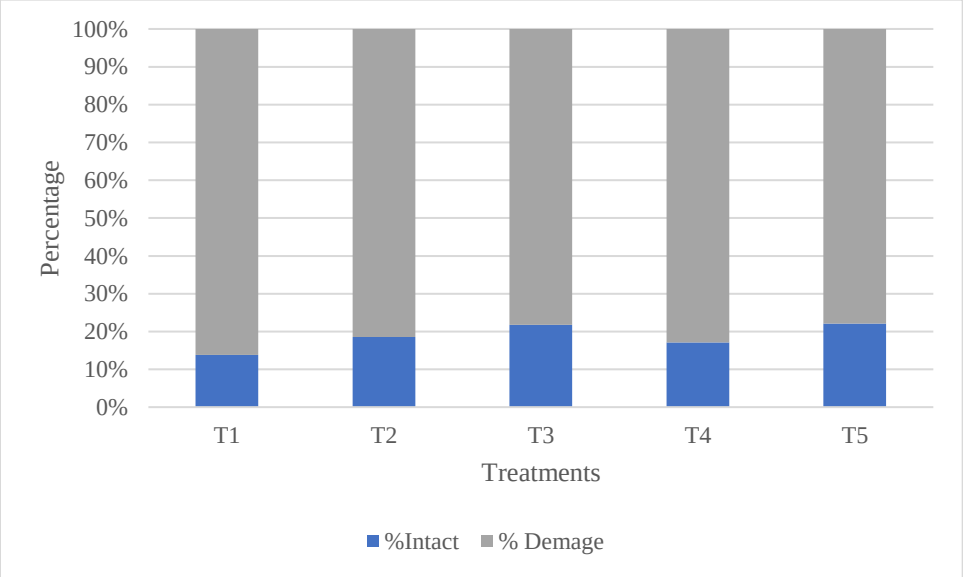


Figure-05: Percent comparison of intact and damaged cells among the treatments of Lugols iodine (acidic)

5.1.5 Glutaraldehyde (Factor 5):

Among T1 to T5 of F5, the highest number of intact cells in case of percentages was found at F5T2 (1.18%)**,** followed by F5T1, F5T3, F5T4 and the lowest number of intact cells were found at F5T5 (0%). (Fig-06)

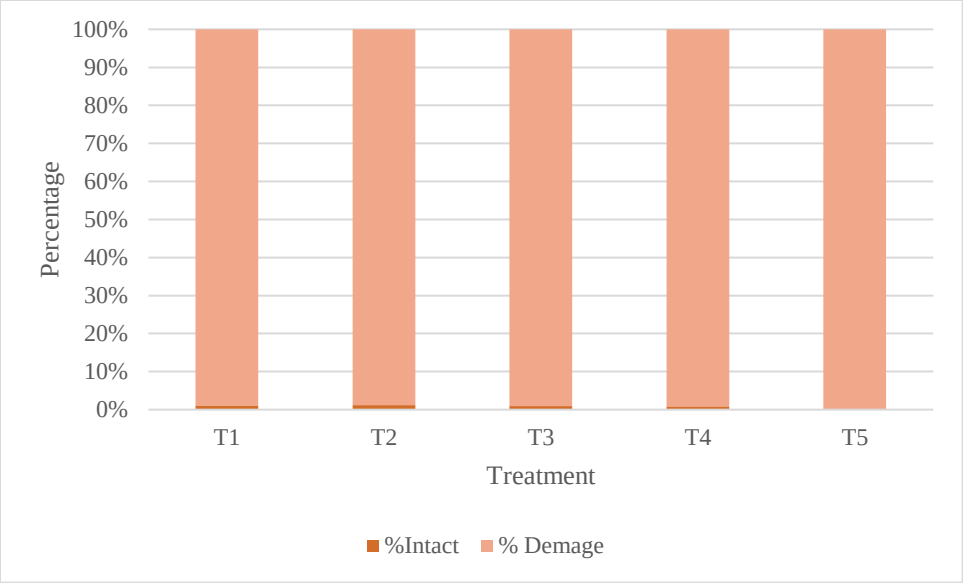


Figure-06: Percent comparison of intact and damaged cells among the treatments of glutaraldehyde

5.1.6 Transeau solution (Factor 6):

Among T1 to T5 of F6, the highest numbers of intact cells were found at F6T1 (27.54%), followed by F6T4, F6T5, F6T2 and the lowest numbers of intact cells were found at F6T3 (9.48%) (Fig-07).

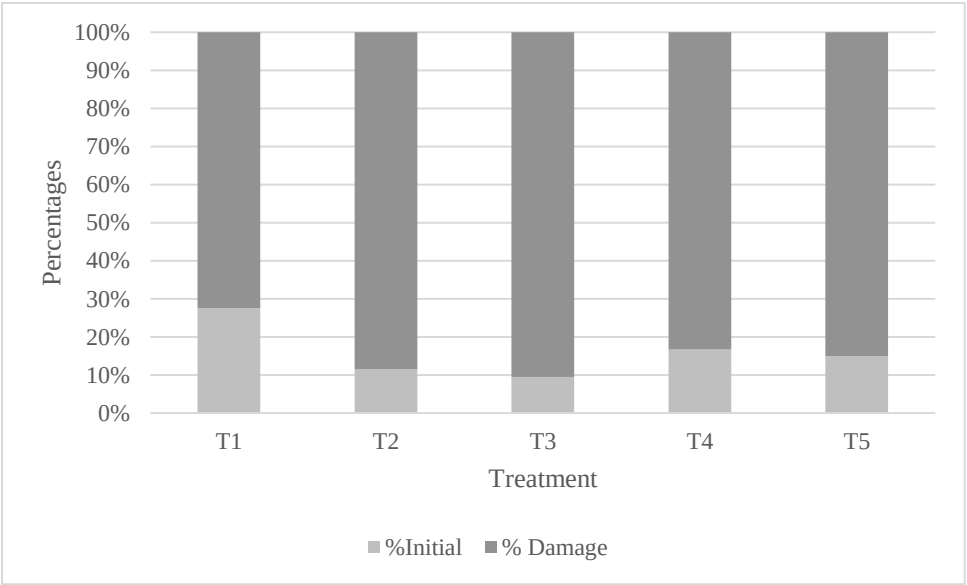


Figure-07: Percent comparison of intact and damaged cells among the treatments of Transeau solution

5.2 Comparative efficiency of the preservatives

The extent of intact cells (%) was highest in F3 (19.95%), followed by F1 (19.63%), F4 (18.67%), F6 (16.04%), F2 (10.49%) and the lowest was at F5 (0.83%). On the other hand, the result also showed that the extent of damaged cells (%) was highest in F5 (99.17%), followed by F2, F6, F4, F1 and lowest at F3 (80.05%). There were significant variations observed among the efficiency of the factors in terms keeping the cells intact with time ($p<0.05$).

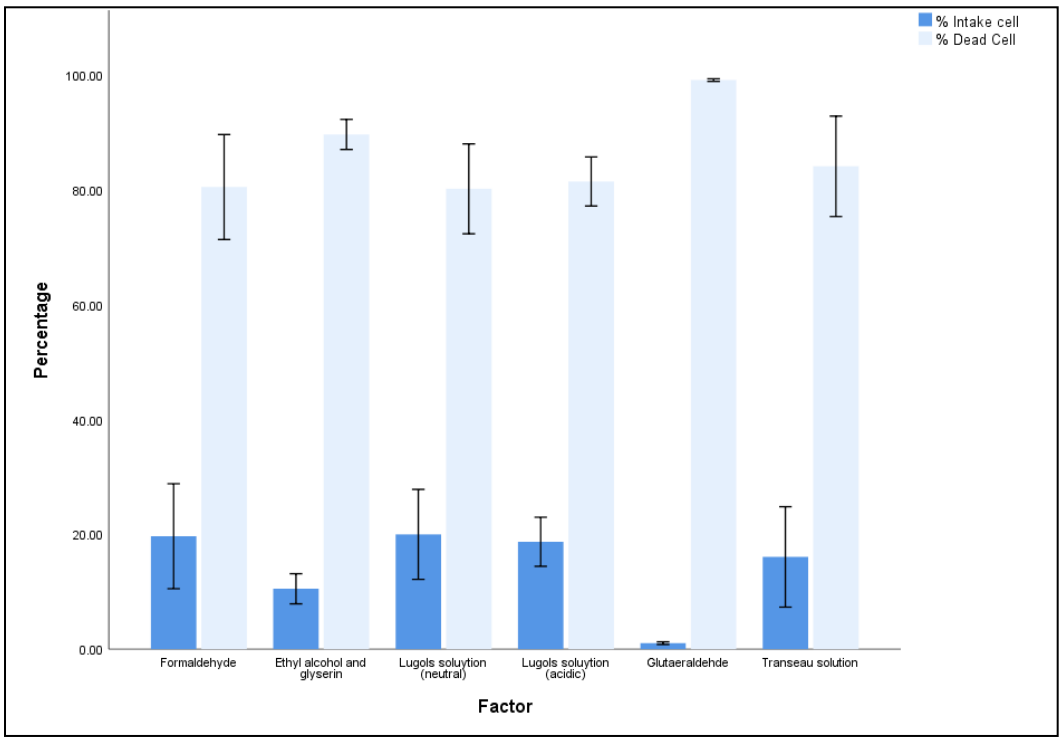


Figure-08: Comparison of efficiency among the preservatives

Chapter Five

Discussion

Preservation is the maintenance of a fixed condition for extended periods of time. Phytoplankton preservation is important because phytoplankton is a great source of food for maximum aquatic organism. Preserving phytoplankton assist to fulfil the scarcity of the food in aquaculture industry. Phytoplankton preservation also minimizes the food cost in the aquaculture sector. Preserving plankton also creates great scope for the researcher to research in the laboratory.

Different preservatives were used for preserving *Tetraselmis sp.* Formaldehyde, Ethyl alcohol and glycerin, Lugols iodine (neutral), Lugols solution (acidic), Glutaeraldehde, Transeau solution were used for conducting this experiment. Different concentrations of each preservative were used to find out the effectiveness of different preservatives that were applied in this experiment.

The result showed that the effectiveness of different preservatives which was used in this experiment to preserve phytoplankton (*Tetraselmis sp.*). Six different types of preservatives were used in this experiment and the most effective preservatives found in this experiment were neutral Lugol's solution, then formaldehyde.

Different concentration of 50 ml formaldehyde is observed in this experiment and as increasing level the concentration of formaldehyde, the deformation of plankton cells are observed and the number of damaged cells are increased with increasing concentration but in case of two concentration of formaldehyde, some dissimilarity is observed in those concentrations the amount of damaged cell is lower than the first concentration. This result partially agrees with previous work.

Mukherjee et al., (2014) found that the cell of phytoplankton did not show any variation if they were preserved with 0.5% formalin. When the concentration of formalin was increased then numerous types of cell rapture began to start. The maximum reduction of cell of plankton was found when plankton cells are preserved in 6.0% or higher concentration of formalin.

Mukerjee et al., (2014) also observed that cells remained unharmed up to a preservative dosage of 4.5%. Formalin concentrations of 5.0% or higher displayed frustular rupturing in cells kept for 30 days, however chain-forming phytoplankton, such as the *Cyanophyceae, Trichodesmiumerythraeum* did not exhibit chain fragmentation despite peripheral cell wall damage.

In this experiment the species which was observed did not contain chain-forming characters and this experiment is conduct for only 30 days.

Mukerjee et al., (2014) found that the decrease in cell surface area or the cell size using the composite preservative (0.5% formalin + 6.0% acidic Lugol's iodine) was attributed mainly to the higher concentration of acidic Lugol's iodine. It was quite obvious that acidic Lugol's iodine caused chain fragmentation. Using the second composite preservative solution (1.0% formalin + 1.5% acidic Lugol's iodine), no cell rupturing or chain fragmentation were observed and reduction in the average cell dimension was minimal; however, the profusion of microbial contamination rendered this composite solution unworthy for ecological practices.

The effect of eight various concentrations of acidic Lugol's iodine and formalin on cell surface area was studied. The decrease in cell surface area or cell size while employing the composite preservative (0.5% formalin + 6.0% acidic Lugol's iodine) was due primarily to the higher acidic Lugol's iodine content. The acidic Lugol's iodine clearly induced chain fragmentation. No cell rupturing or chain fragmentation were detected when the second composite preservation solution (1.0% formalin + 1.5% acidic Lugol's iodine) was used, and the average cell dimension was reduced to a minimum. The third composite preservative (1.5% acidic Lugol's iodine + 7.0% formalin) caused widespread frustule rupturing due to cell enlargement and corrosion. In general, the rest of the preservative composite concentrations displayed cell breakage, frustular silica corrosion, and chain shortening by fragmentation without contamination, as expected with higher preservative concentrations. The preserved specimens showed no degradation, distortion, or microbiological contamination in the case of the composite preservative solution containing 2.0% (v/v) formalin + 2.5% (v/v) acidic Lugol's iodine. Furthermore, the cells

that stayed intact were 68.3% the size of the nonpreserved cells on average, demonstrating that this composite preservative solution should be appropriate for long-term preservation.

The result did not show such kind of combination of different preservatives and for this reason, there is no similar result was found in the previous study of Mukerjee et al. (2014).

The result also found that in the case of *Tetraselmis sp.* the mean count cells of this species were declining from the beginning of the first day of the experiment which agrees with the previous study.

Williams et al., 2016 conducted a study about the preservation of marine plankton with different logul's solutions on different types of marine phytoplankton the time duration ranged from 1 to 8 months, and for every species of plankton, during preservation they observed that Day 1 mean cell count was used as a baseline against which each of the subsequent counts were tested for significant differences. Some results showed statistically significant increases in cell count when compared to Day 1.

The result also showed that two Logul's solution were prepared for this experiment and they were Logul's solution (acidic), Logul's solution (neutral). Between these two solution mean cell count was lower in neutral logul's solution than in acidic logul's solution. The result obtained from the experiment does not agree with the previous work of Williams et al. (2016).

Williams et al. (2016) discovered that in the instance of *Tetraselmis suecica* (Microflagellate), observed mean cell counts were considerably lower on all days in Cneut (the sole exception being Day 169), while Lacid numbers were significantly lower from Day 113 onwards. Counts in Cneut were considerably lower than Cacid and Lacid on all analysis days (with the exception of Day 1 in Cacid) until Day 169, and findings in Cacid were significantly lower than Lacid on the majority of days.

The result showed that the most effective preservative was Lugo'ls solution (neutral) and the worst is Glutaraldehyde which also partially agrees with some previous work that were mentioned above.

Mukherjee et al. (2014) discovered that both formaldehyde and acidic Lugol's iodine have negative impacts on preserved phytoplankton with different preservatives.

Williams et al. (2016) observed that in different types of Lugol's solution, the neutral logul's solution is comparatively better than other Logul's solution and this study is only conducted on the effect of Lugols solution.

Stoecker et al., (1994) conducted a study on preserving marine ciliate and found that during the counting of ciliates, acidic Logul's solution is more effective than formaldehyde.

Lepesteur, (1993) conducted a study and in that study and there were different method of preservation was used freezing and thawing, the use or non-use of cryoprotectants (DMSO and/or glycerol), and chemical fixation. These methods were tested on 3 freshwater and marine algal species. Different intensity parameters and 2 properties were considered

The experiment was done only for the marine water algal species and it was used for single species. Only one preservation technique had been employed which was preserving plankton samples with different types of chemicals.

Liu et al., (2023) found large changes in plankton bio volume and abundance among different size classes, which may indicate a distinct effect of acid Lugol's solution on various plankton size classes. They emphasized that the effect of storage time should be taken into account when interpreting or comparing data of plankton communities acquired from samples with various storage durations.

There was no observation of cell size classes in this experiment and did not show the effects of Lugols solution on different classes. The result only agrees with the storage time of preserving plankton samples in numerous durations of storage.

Durán-Campos, (2019) used Aliquots of 500 mL preserved with Lugol solution in glass bottles.Samples were kept in the dark until cell counting, following the recommendations of Edler and Elbrächter (2010).

In this experiment 50 mL of samples were being preserved with formaldehyde, Lugols solution and other solution and the sample bottles used for this experiment were stored in dark conditions during the experiment.

Dolgin (2019) found that chemical fixatives were used to retain cells with minimum morphological changes and to improve cell structural integrity prior to dehydration. Although successful, these methods included the use of hazardous substances such as formaldehyde and glutaldehyde. Four phytoplankton species were selected to test various treatments qualitatively in order to identify which fixation procedure would result in the best qualitative cell preservation for SEM examination. The results showed that no single treatment was the most effective for all of the species evaluated.

The result showed that different preservatives that were used in this current experiment partially agreed with the previous work and in this experiment, few treatments was comparatively more effective than other treatment used in this experiment which totally disagreed with the current findings.

The study provides knowledge about the best preservatives among different preservatives that were used in this experiment. This experiment paves the way for researchers to make decisions about the effectiveness of the preservatives. It also provides information about the morphological change of the phytoplankton. This would assist the researcher in future research.

Chapter Six

Conclusion

The present study has been done on the effectiveness of different preservatives on the preservation and fixation of the *Tetraselmis sp.* phytoplankton sample. This experiment will assist the researcher in understanding the best preservatives for preserving phytoplankton among different preservatives and it also provide knowledge about the impact of the chemical on the specific plankton species. Different preservatives were used in this current experiment and among those preservatives, neutral Lugol's solution is the most effective comparatively other preservatives which were used in this experiment. The experimental data which is found from this experiment will help different aquaculture industries. It helps to provide information about the technique of preservation for the plankton species. The study can suggest which preservatives are best preservatives. The study helps to find out the amount of intact cells during the experiment. The study emphasizes the use of best preservatives for research purposes. Lugol's solution (acidic) was found most effective preservatives and glutaraldehyde was found less effective preservatives among different preservatives which was used in this experiment. Glutaraldehyde which was used in this experiment showed adverse effects on *Tetraselmis sp.* and in this chemical preservative the maximum cells of the species had been ruptured and for this reason, this species was less suitable among all other preservatives. The study helps the researcher by providing information about the properties of a different chemical that was used in this study. The study provides information about the storage time of plankton preservation in laboratory conditions. The study's outcome provides information on the importance of choosing appropriate preservatives and fixative during working with *Tetraselmis sp.* phytoplankton samples. In conclusion, this study significantly contributes to the understanding of effective preservation and fixation methods for *Tetraselmis sp.* phytoplankton samples.

Chapter Seven

RECOMMENDATIONS AND FUTURE PERSPECTIVES

The present investigation reveals information about the effectiveness of different preservatives on the preservation and fixation of phytoplankton (*Tetraselmis sp*.) samples. However, this experiment has some limitations. Based on the findings of the study on the effectiveness of different preservatives on the preservation and fixation of phytoplankton (*Tetraselmis sp*.) samples, here are some recommendations for further research and practical applications:

- The samples should be stored under appropriate conditions during the preservation process.
- Consistent temperature and light exposure should be maintained.
- A time-course analysis should be conducted to determine the optimal duration for preserving *Tetraselmis sp*. samples. This will help identify the most effective preservation time point that maintains cell viability and morphology without significant degradation.
- Investigating the long-term storage effects of the tested preservatives would be beneficial. Assessing the stability of phytoplankton samples preserved using different methods over extended periods will ensure the reliability of stored samples for future research and comparisons.

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Appendix

Preservation efficiency of the preservatives

Formaldehyde:

Treatment	Intial	Intact	Damaged	%Intact	%Damaged
T1	50765	12050	38715	23.7368	76.2632
T2	60325	7925	52400	13.1372	86.8628
T3	72245	7475	64770	10.3467	89.6533
T4	36243	8800	27443	24.2806	75.7194
T5	39656	10575	29081	26.6668	73.3332

Ethyl alcohol and glycerin:

Treatment	Initial	Intact	Damaged	%Intact	%Damaged
T1	32145	3100	29045	9.6438	90.3562
T2	16476	1250	15226	7.58679	92.4132
T3	12587	1275	11312	10.1295	89.8705
T4	13243	1650	11593	12.4594	87.5406
T5	11465	1450	10015	12.6472	87.3528

Lugol's Iodine (neutral):

Treatment	Initial	Intact	Damaged	%Intact	%Damaged
T1	29543	8000	21543	27.0792	72.9208
T2	27956	5450	22506	19.4949	80.5051
T3	18367	4525	13842	24.6366	75.3634
T4	27385	4800	22585	17.5278	82.4722
T5	40634	4475	36159	11.0129	88.9871

Lugls solution (acidic):

Treatment	Initial	Intact	Damaged	%Intact	%Damaged
T1	50264	6950	43314	13.827	86.173
T2	41276	7675	33601	18.5943	81.4057
T3	27365	5950	21415	21.7431	78.2569
T4	36745	6275	30470	17.0772	82.9228
T5	29745	6575	23170	22.1046	77.8954

Glutaeraldehde:

Treatment	Initial	Intact	Damaged	%Intact	%Damaged
T1	17834	200	17634	1.12145	98.8785
T2	10545	125	10420	1.1854	98.8146
T3	10233	100	10133	0.97723	99.0228
T4	5763	50	5713	0.8676	99.1324
T5	7543	0	7543	0	100

Transeau solution:

Treatment	Initial	Intact	Damaged	%Intact	%Damaged
T1	22143	6100	16043	27.5482	72.4518
T2	27453	3150	24303	9.48933	90.5107
T3	28453	2700	25753	9.48933	90.5107
T4	19572	3275	16297	16.7331	83.2669
T5	14398	2150	12248	14.9326	85.0674

Comparative efficiency of the preservatives

Factor	Intact cell (%)	Damaged Cell (%)
Formaldehyde	19.63	80.37
Ethyl alcohol and glyserin	10.49	89.51
Lugols soluytion (neutral)	19.95	80.05
Lugols soluytion(acidic)	18.67	81.33
Glutaeraldehde	0.83	99.17
Transeau solution	16.04	83.96

Brief Biography of the Author

This is Abu Obyada Md. Mayaze, son of Md. Sirajul Islam and Sultana Fatima from Shreepur Upazila under Gazipur district of Bangladesh. He passed the Secondary School Certificate Examination in 2013 from Adamjee Cantonment Public School, followed by Higher Secondary Certificate Examination in 2015 from Adamjee Cantonment College. He obtained his BSc. Fisheries (Hons.) in 2021 from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. Now, he is a candidate for the degree of MS in Fisheries Resource Management under the Department of Fisheries Resource Management, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. He is passionate to qualify himself as a competent researcher, and thus to develop the fisheries sector of Bangladesh.