Study of Environmental DNA from Goan Estuaries

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Declaration

I hereby declare that the data presented in this Dissertation report entitled, "Study of Environmental DNA from Goan Estuaries" is based on the results of investigations carried out by me in the Biotechnology Discipline at the School Of Biological Science and Biotechnology, Goa University under the supervision of Prof. Sanjeev C Ghadi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities / College will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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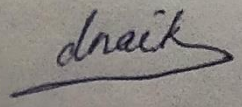

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Study of Environmental DNA from Goan Estuaries" is a bonafidework carried out by Mr Ankit Anuroop Naik under my supervision in partial fulfilment of the requirements for the award of the degree of Master's in Biotechnology in the Biotechnology Discipline at the School of Biological Science and Biotechnology, Goa University/College.

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Preface

The dynamic nature of estuarine ecosystems presents both challenges and opportunities for environmental researchers seeking to understand the intricate interplay between organisms and their surroundings. Estuaries are often regarded as critical transitional zones where freshwater and marine environments converge, harboring both rich biodiversity and serving as a vital ecological niche. Within these complex ecosystems, the use of environmental DNA (eDNA) has emerged as a powerful tool for non-invasive monitoring and assessment.

This research endeavor delves into the study of eDNA fromGoa estuaries, aiming to create an understanding of the diverse microbial ecosystem present in them. This study focuses on the isolation and amplification of eDNA, the comparison of various isolation methods, the detection of the presence of humic acid in extracted DNA, and the determination of eDNA degradation rates.

This research endeavor represents a concerted effort to unravel the molecular mysteries veiled within estuarine ecosystems. Through rigorous experimentation and meticulous analysis, this study endeavours to contribute to the burgeoning field of environmental genomics and foster a deeper appreciation for the intricate web of life that thrives within these vital coastal habitats.

Acknowledgement

I extend my heartfelt gratitude to the divine for granting me the fortitude to navigate through the intricacies of my dissertation. I am immensely thankful to the School of Biological Science and Biotechnology for entrusting me with the invaluable opportunity to delve into research within their esteemed discipline. A special appreciation goes to our esteemed dean, Professor Benard F. Rodrigues, whose gracious permission paved the way for the execution of my dissertation. I extend my heartfelt gratitude to my dissertation guide, Senior Professor Sanjeev C Ghadi, whose unwavering guidance, constructive suggestions, and invaluable advice have been instrumental in shaping this research endeavor. Your mentorship has been a beacon of inspiration throughout this journey.

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Lastly, I would like to acknowledge the support of my family, whose love, encouragement, and understanding have been my pillars of strength. This research would not have been possible without their unwavering belief in my abilities and their enduring support.

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Abbreviations

Abbreviation	Full Form
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
UV	Ultraviolet
PWG	Plant Working Group
CBOL	Consortium for the Barcoding of Life
HS	Humic substances
TB	Toluidine Blue
HDPE	High Density Polyethylene
mL	millilitre
NaOH	Sodium Hydroxide
Mins	minutes
M	meter
µL	Microliter
L	Liter
°C	Degree Centigrade
RPM	Revolution per minute
EDTA	Ethylene diaminetetraacetic acid
HCL	Hydrochloric acid
Na-EDTA	Disodium salt of EDTA
CTAB	Cetyl-trimethylammonium bromide

NaCl	Sodium chloride
EEO	Electroendosmosis
Sec	Seconds
PCR	Polymerase Chain Reaction
MgCl2	Magnesium chloride
MQ	MilliQ water
mM	millimolar
M	Molar
DGGE	Denaturing Gradient Gel Electrophoresis
ANOVA	Analysis Of Variance
Ng	Nanogram
ET-OH	Traditional method of eDNA extraction
H0	Null hypothesis
H1	Alternative hypothesis
SS	Sum of squares
dF	Degrees of freedom
MS	Mean square
F	Calculated statistic
DS	Double stranded

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Abstract

Environmental DNA (eDNA) provides insights that contribute to studies of species distribution and ecology of an area. This study focuses on the isolation of eDNA from Goan estuaries. Various methods for eDNA extraction from water samples, such as the traditional method, a kit-based method, and a chemically modified method, were used in the present studies to compare for eDNA. It was determined that the CTAB-based method exhibited better eDNA yield that served as a template source for PCRamplification. Further, the kit-based method demonstrated higher yield and pure eDNA compared to the traditional method but with associated humic acid contamination. Additionally, the presence of humic acid in eDNA extracts, particularly in samples extracted *via* traditional methods, was highlighted, emphasizing the need for robust purification protocols to mitigate potential contaminants. The study also investigated the effects of storage temperature on eDNA integrity over time. It was observed that samples stored at room temperature exhibited significant decreases in eDNA concentration compared to those stored at 4˚C, emphasizing the importance of controlled storage conditions. Moreover, statistical analysis revealed a significant decrease in eDNA concentration at different storage temperatures, highlighting the influence of temperature on eDNA stability.

Chapter 1

Introduction

Introduction

Background

Environmental DNA, commonly referred to as eDNA, is a general term that describes all genetic material present in the environment. This includes DNA that exists outside an organism, such as in mucus and feces from macro-organisms, as well as DNA from microorganisms' surrounding environment or media (Minamoto, 2022). Over the past decade, scientists have been utilizing eDNA from not only microorganisms but also plants and vertebrates (Pedersen et al., 2015). The development of advanced eDNA testing tools with high sensitivity and specificity has led to an increased use of this method for the the presence and diversity of target organisms (Barnes et al.,2021).

The concept of eDNA and the term itself originated from microbiology, where the focus was on targeting DNA from abundant live and dead microbes present in environmental samples(Turner et al., 2014). However, the scope of eDNA analysis expanded to encompass DNA from larger organisms, including animals and plants, which is referred to as microbial eDNA. While microbial eDNA has been studied in various fields such as human forensics, agricultural transgenics, paleogenetics, and fecal pollution source tracking since 1991, its application to aquatic macrofauna was first documented in 2008(Turner et al., 2014).

Skin flakes, urine, feces, eggshells, hair, saliva, insect exuviae, regurgitation pellets, feathers, leaves, root cap cells, and, in rare instances, pollen can cause deposition of eDNA in the surrounding environment (Pedersen et al., 2015). Additionally, plasmid and chromosomal DNA can be secreted by living

prokaryotes in their surrounding environment. Research on plants and bacteria has shown that dead cells can be rapidly lysed and can release their DNA into the surrounding environment.(Pedersen et al., 2015).

Various extreme and diverse eco-niches, such as estuaries, mangroves, salt pans, and coastal sediments, serve as habitats for numerous microorganisms that harbor immense potential as sources of industrially important enzymes and other bioactive molecules(Solomon et al. 2016). The distribution of these species within a habitat and niche is critical for understanding their biological dynamics and determining extinction risks, which forms effective conservation policies (Takahara et al., 2012). However, achieving precise estimates of distribution can be challenging, especially in complex microhabitats such as aquatic systems characterized by intricate topology and dense vegetation. This is where environmental DNA (eDNA) has emerged as a promising tool for documenting the distributions of aquatic vertebrate species (Takahara et al., 2012).

The concentration of a particular type of eDNA is primarily dependent on the presence and distribution of various species in an environment. The relationship between the source organism and the concentration of eDNA at the point of measurement is intricately intertwined with various environmental processes, creating a complex interplay of factors.(Harrison et al., 2019). The journey of eDNA from its source to its detection point is governed by a series of dynamic processes, including its origin, transport, and decay. These processes collectively influence the physical state of eDNA, which in turn dictates the mechanisms and consequences of its interactions within the natural environment. (Harrison et al., 2019).eDNA is quite fragile and can be easily degraded by the action of

bacterial/fungal exonucleases, enzymes, and various chemicals present in the environment(Takahara et al., 2012).

The utilization of eDNA entails detecting small, species-specific DNA fragments in environmental samples. It is a quick and effective way to measure species richness in natural communities is through multispecies detection using high-throughput sequencing and DNA extracted from environmental samples. eDNAmetabarcoding is frequently used to assess bacterial and fungal taxonomic richness, or the richness of microorganisms, and it is an effective supplement to traditional culture-based techniques(Deiner et al., 2017).Conventional taxonomic identification methods hinge upon capturing live or deceased animals, often resulting in habitat disturbance or destruction. Conversely, eDNA analysis relies solely on the genetic remnants an organism leaves in its environment. While eDNA may not provide insights into population metrics like sex ratios, it serves as a highly effective tool for detecting endangered, invasive, elusive, or rare species(Deiner et al., 2017). However, using an effective procedure to extract whole community DNA from environmental materials is a crucial precondition for a successful metagenomic investigation(Hassan et al., 2018). This novel approach provides various benefits, including increased accuracy and lower survey costs, as well as the ability to find rare or invasive species that would otherwise go unnoticed (Takahara et al., 2012).

Aqueous eDNA has garnered significant interest due to its simplicity and sensitivity in detecting rare aquatic macrofauna, including invasive or endangered vertebrates and invertebrates. Compared to traditional methods of direct observation, which often have low detection probabilities, limited sampling seasons, high costs, and pose risks to sensitive species, eDNA analysis offers

distinct advantages. It provides a non-invasive and efficient means of detecting rare organisms, even in challenging environments, thereby enhancing conservation efforts and facilitating biodiversity assessments in aquatic ecosystems.

The application of eDNA holds immense promise for revolutionizing biodiversity monitoring and management practices in aquatic ecosystems. By providing rapid, cost-effective, and non-invasive means of species detection, eDNA technology represents a significant advancement in the field of conservation biology, offering valuable insights into the spatial distribution and abundance of aquatic vertebrate species(Takahara et al., 2012).

Estuarine coastal ecosystems present an advantageous scenario for examining the correlation between biodiversity and the environment using eDNA techniques. This is particularly relevant as numerous estuaries experience extensive pollutant influence, leading to adverse impacts on benthic communities(Bernardino et al., 2019).Surface water is often prioritized over deep waters for eDNA extraction owing to several critical factors that influence the distribution and concentration of eDNA(Pilliod et al., 2014).Pilliod et al., reported that primary rationale lies in the proximity of surface water to potential sources of eDNA, including aquatic organisms and their biological activities such as excretions, shedding, or the release of sloughed-off cells. Moreover, the accumulation of decaying organic matter in surface water contributes to a richer eDNA reservoir compared to deep waters. Another significant aspect is the dynamic nature of surface water, characterized by mixing, turbulence, and water movement, which facilitate the dispersal of eDNA throughout the water column. This enhanced dispersion

increases the likelihood of capturing a more representative sample of eDNA, thus improving the chances of detecting target species(Barnes et al, 2021).

Despite the extensive success of eDNA applications and the growing recognition of its potential applications, there remains considerable room for improvement in our fundamental understanding of eDNA and its associated methodologies(Shea et al., 2023). A mounting body of evidence highlights the influence of collection and laboratory processing methods on eDNA results, underscoring the need for increased attention to eDNA methodologies and the development of best practices.(Deiner et al., 2017).Moreover, there is a growing acknowledgment of the complex ecology inherent to eDNA itself, Recent studies have revealed that eDNA exhibits both particulate and solute-like propertiesin the environment, suggesting a heterogeneous and polydisperse nature(Barnes et al., 2021). This complexity necessitates a deeper understanding of the origin, state, transport, and fate of genetic materials released into the environment to fully harness the utility of eDNA analysis (Goldberg et al., 2015)

The current work focuses on extracting eDNA from estuaries in Goa, utilizing a simple, modified and cost-effective protocol. This study also aims to detect the presence of humic acids in the samples. Furthermore, this research also seeks to estimate the rate of eDNA degradation post-extraction. By implementing novel methodologies and protocols, this study endeavours to enhance our understanding of eDNA dynamics in estuarine environments, contributing valuable insights to environmental monitoring and biodiversity conservation efforts.

Aim and Objective

Aim:-To study eDNA isolated from estuarine waters of Goa

Objectives

1. Isolation and comparison of various methods used for isolationofeDNA from estuary
2. PCR amplification of the isolated eDNA using selected primers
3. Detection of the presence ofhumic acid in the extracted DNA
4. Determination of the degradation rate of eDNA

Hypothesis

Based on the literature work carried out, we hypothesize that the CTAB-based extraction method will yield higher concentrations of eDNA compared to other extraction methods, owing to its superior efficiency in aquatic environments. Furthermore, we state that the presence of humic acid content in the extracted eDNA should exhibit a negative correlation with eDNA concentration, indicating a potential interference of humic acid with eDNA content. Additionally, we anticipate that storage temperature will significantly influence eDNA degradation, with samples stored at lower temperatures demonstrating higher stability over time compared to those stored at room temperature. These hypotheses form the basis for our investigation into the molecular dynamics of eDNA in estuarine ecosystems and provide a framework for interpreting our experimental findings.

Scope

The findings in this study aims to comprehensively investigate various aspects of eDNA dynamics in Goan estuaries. This includes optimizing extraction methods to enhance efficiency and address challenges like humic acid contamination, developing purification protocols to mitigate contaminants without compromising yield, improving recovery rates of PCR-amplified products from gel extraction methods, and studying the long-term stability and degradation dynamics of eDNA under different conditions. Additionally, it seeks to explore spatial and temporal variability in eDNA concentration, the impact of salinity on extraction efficiency, and integrate molecular and ecological approaches to assess biodiversity and ecosystem health.

Chapter 2

Review of Literature

Review of Literature

eDNA is extracted from environmental samples for various studies such as targeting specific species, broadening sampling diversity, and improving taxonomic resolution without the constraints of traditional techniques (Ruppert et al., 2019). One significant drawback of existing methods is the potential for DNA shearing, which results in a considerable reduction in DNA yield and compromises the suitability of the extracted DNA for metagenomic library construction. Therefore, there is an urgent need to develop novel extraction techniques that address these limitations and streamline the process of metagenomic/eDNA recovery (Shamim et al., 2017)

Recent advancements have introduced kit-based methodologies, which offer the potential to eliminate humic acid from the produced eDNA, addressing this particular issue in extraction procedures. However, commercial DNA extraction kits can be costly, particularly if you need to process a lot of samples. Large-scale eDNA projects may not be feasible due to the high cost of buying kits for every sample in research involving substantial sampling.(Barnes et al., 2021). Certain commercial kits also have the potential to include contaminants or inhibitors that could impair the accuracy and dependability of eDNA results by interfering with subsequent analysis. These pollutants or inhibitors may be a result of the reagents or kit parts employed during the extraction procedure (Jane et al., 2015)Although kit-based techniques provide standardized procedures, they might not always be the best for particular sample kinds or environmental circumstances. Changes in sample matrices, such as soil, water, or silt, may necessitate adjusting kit

instructions, which could result in inconsistent results or decreased DNA extraction efficiency (Barnes et al., 2021).

Recovering eDNA suitable for PCR amplification and metagenomic library construction poses significant challenges, primarily due to the presence of humic and fulvic acids, the major contaminants found in soil and sediments which flow into rivers.(Shamim et al., 2017) These substances interfere with downstream eDNA purification processes, complicating the extraction of high-quality eDNA. Ensuring the recovery of high molecular weight eDNA is crucial, particularly for metagenomic library construction(Bag et al., 2016)

Furthermore, it is imperative to recover large quantities of pure eDNA to ensure adequate representation of all genomes within a particular community. This requires rigorous purification methods to remove contaminants and impurities, thereby enhancing the accuracy and reliability of downstream analyses(Bertrand et al., 2005).

The Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) recommended that portions of two plastid genes, rbcLa and matK, be selected as the standard plant DNA barcodes, with the knowledge that additional markers may be required.(De Vere et al., 2015). For the reasons described above, the rbcLa primer is recommended for polymerase chain reaction (PCR). ITS (or ITS2) is another frequently utilized DNA segment in plant molecular systematics at the generic and species levels due to its ability for resolving inter- and intraspecific relationships(Cheng et al., 2016). Research suggests that accurate species identification requires a combination of barcodes from both the biparentally inherited nuclear genome and the uniparentally inherited plastid

genome. ITS is the most widelyusedmarker (Fazekas et al., 2009). Therefore, in this study, two universal primers, rbcLa and ITS2, were utilized to amplify the eDNA.

One of the primary hurdles in eDNA sample analysis is the presence of extraneous substances from the surrounding environment within the sample. (Deiner et al., 2017).Recent studies state that, the persistence of residual lysozyme in the extracted eDNA solution might hinder PCR reactions, diminishing amplification efficiency or yielding false-negative outcomes in subsequent analyses (Goldberg et al., 2015). The optimization of lysozyme concentration and incubation duration for distinct sample types or environmental conditions poses a challenge, often necessitating extensive experimentation to ensure proficient eDNA extraction(Franklyn et al., 2017).

Owing to the challenges related to humic acid co-precipitation with eDNA, conventional extraction techniques have primarily concentrated on obtaining clean water samples or directly extracting eDNA from the species itself.Therefore detecting the presence of humic acid before and after eDNA extraction is crucial to determine the efficiency of the protocol followed. A straightforward and swift method was introduced by Sheng et al., for the determination of humic substances (HS) at microgram levels in natural waters. This assay relies on the interaction between a dye, Toluidine Blue (TB), and HS molecules, resulting in the formation of a TB-HS complex. This complex induces a reduction in absorbance at 630 nm, allowing for the detection of Humic acid levels in the sample. (Sheng et al.,2007).

Hence, there is a pressing need for an efficient, cost-effective, and rapid method to recover highly purified metagenomic DNA from environmental samples, including those from estuarine, mangrove, and salt pan ecosystems(Shamim et al., 2017). Various methodological combinations are expected to yield differing levels of efficiency in eDNA analysis (Hinlo et al., 2017). However, given that eDNA detection frequently hinges on identifying minute quantities of extensively degraded DNA, prioritizing methods that optimize eDNA recovery becomes paramount. By doing so, the likelihood of successful detection is enhanced, thereby maximizing the efficacy of the analysis. Additionally, methods that achieve this objective in a cost-effective manner are particularly desirable, as they ensure the efficient utilization of resources without compromising the integrity of the results(Hinlo et al., 2017). Still, there is a rush in the creation, enhancement, and optimization of fresh or existing eDNA extraction techniques from various sample types.(Hassan et al., 2018).

Salt concentrations, particularly high levels of salts, can impact the efficiency of eDNA extraction by influencing the binding of DNA to various extraction matrices or interfering with enzymatic reactions involved in the extraction process (Gilbert et al., 2011). Hence in this study, Sodium hydroxide was used to increase a pH to about 8 to extract eDNA from estuaries of Goa.

The optimum concentration of extracted eDNA and its recovery is restricted by environmental factors leading to eDNA degradation. This degradation frequently leaves only modest amounts of genetic material intact, especially in warm, tropical climates. Many such as environmental conditions, microbial activity, and the stability of DNA fragments impact the rate of degradation of extracted eDNA (Barnes et al., 2021). Furthermore, the ability of DNA to spread across media,

including water, and the different times it takes for degradation depending on environmental factors can influence the inference of fine-scale spatiotemporal trends of species and communities(Ruppert et al., 2019). The longevity of eDNA is intricately linked to a combination of factors encompassing the physical, chemical, and biological characteristics of its micro-environment (Pedersen et al., 2015). Initially, the turnover rate of eDNA in marine and freshwater habitats was believed to be notably swift, ranging approximately from 6.5 to 25 hours (Pedersen et al., 2015).

Research conducted by Takahara et al., (2012) stated that although storing water samples in freezers is preferred, access to freezers may not always be feasible. Additionally, the freeze-thaw cycle, which occurs during storage and retrieval from freezing conditions, has been shown to affect DNA detection. However, the above study primarily focused on measuring eDNA concentration after a single freeze-thaw cycle, leaving a gap in understanding the effects of multiple freeze-thaw cycles over time (Takahara et al., 2012).

Research conducted by Yamanaka et al,(2016) investigated the impact of sample processing, including time and storage method before filtration, on eDNA recovery. However, these studies have typically utilized limited storage methods (e.g., ambient temperature, frozen) and were conducted within relatively short timeframes (up to four hours). (Yamanaka et al., 2016)

Our study aims to address various gaps by examining the effect of three storage methods (room temperature, refrigerated, and frozen) on eDNA over an extended period of time (18 days) present in estuarine water. We aim to investigate two scenarios: first, the extraction of eDNA from water samples stored at various

temperatures, and second, the effect on extracted eDNA when stored using the same three storage methods.

Chapter 3

Methodology

Methodology

1) Isolation of eDNA

Sample collection

5L water samples were collected from the Goan estuaries Zuariand Mandovi using HDPE canisters.Surface water samples were collected from three different sites(Location1:-LZ1,Location 2:- LZ2, Location 3:-LZ3 for Zuari estuary and LM1:- Location 1, LM2 :-Location 2, LM3 :- Location 3 for Mandovi estuary), each separated by 500m .Parameters like temperature, salinity and pH were analysed.Water samples were immediately stored in the cold room at 4°C within 2 hours of collection. Minimum amount of water sample required to extract eDNA was determined.

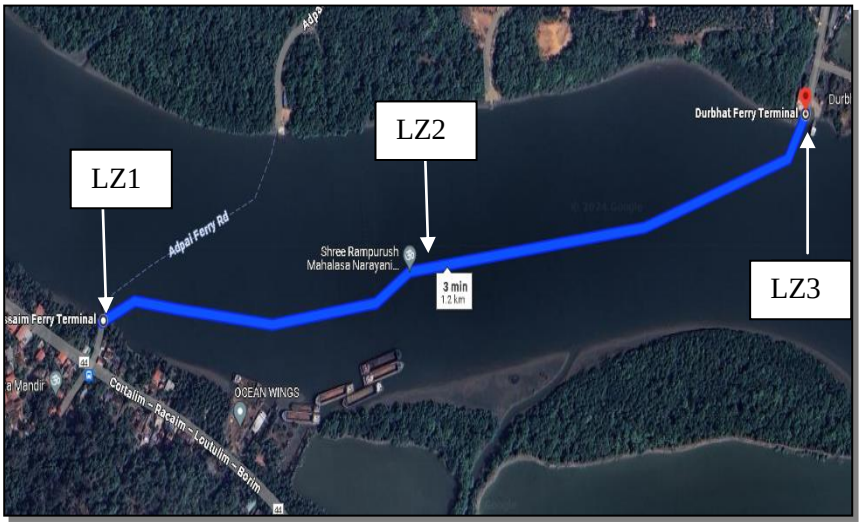


Figure 3.1:- Different location sites for Sampling:-Zuari estuary-goa

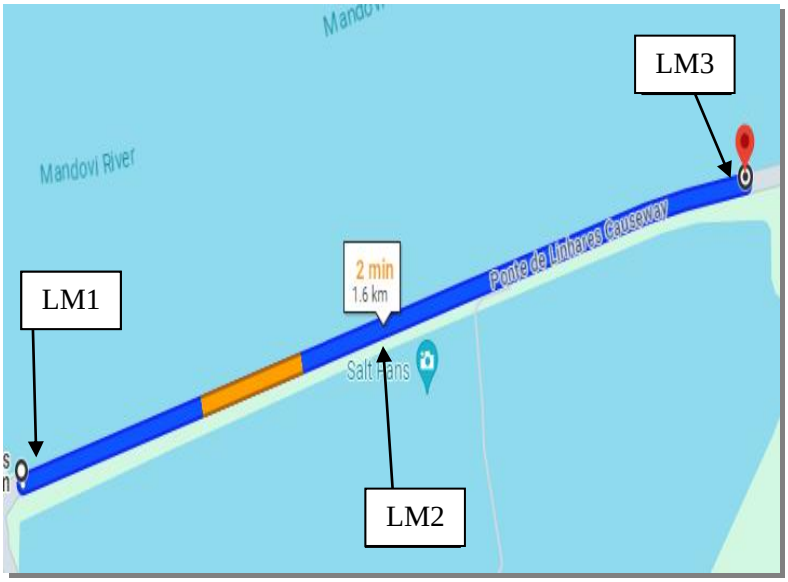


Fig 3.2 :- Different location sites for Sampling:-Mandovi estuary-goa

2) Extraction of eDNA using various methods

2.1) Et-OH method of extraction (Traditional Method)

Following filtration of the collected water samples, the filter paper was rolled up with clean forceps and put inside a 50 mL centrifuge tube containing 5 mL of NaOH-ethanol solution (Table 1.1).The tube was incubated at 80°C for 10 minutes in a water bath. The membrane filter was gently removed and placed in a 2 mL sterile syringe using clean forceps. The eluate was collected in the same 50mL. The tube was then centrifuged at 10,000 rpm for 10 mins. After removing the supernatant, 100µL of elution buffer (Table 1.2) was added to the denatured DNA to make it soluble, and the mixture was then kept at -20°C until further use(Vingataramin& Frost, 2015).

Table 3.1) :- NaOH-ethanol based extraction solution(Vingataramin& Frost, 2015)

Component	Volume (ml)	Concentration
2M NaOH	5.5	240mM
96% ethanol	35	74%
0.025 EDTA	5	2.7mM
Final volume	45.5	

Table 3.2) :- Tris-EDTA Based Elution Buffer(Vingataramin& Frost, 2015)

Component	Volume (ml)	Final concentration
5M tris HCL pH (8)	5	50mM
0.5M EDTA	0.01	0.1mM
Triton X100	0.5	1%
Tween 20	0.25	0.5%
Distilled water	44.24	
Final volume	50.0	

2.2) Isolation using NucleospineDNA water kit

eDNA was isolated using NucleoSpin® eDNA water kit distributed by Takara Bio USA,Inc. following the user manufacturers protocol. The isolated eDNA was then kept at -20°C until further use.

2.3) CTAB chemical method (modified protocol)

In this method, a filtration process was employed wherein 1.5L of water was carefully filtered through a 0.22-µm filter membrane. Following this, a volume of 5 mL of extraction buffer, composed of 1% CTAB, 3% SDS, 100 mM TrisHCl, 100 mM NaEDTA, and 1.5 M NaCl at pH8, was introduced to the filter. The resulting mixture was incubated for a period of 60 mins at 70°C while gently vortexing. Subsequently, the sample was centrifuged at 4500g for 15 mins.The supernatant obtained was used for subsequent steps (Hassan et al., 2018).

4 mLof isopropanol was added to the supernatant followed by incubation on ice for 20 mins. This was followed by centrifugation(4500g), at 4°C for 15 mins. The resulting pellet was resuspended in 400 µL of 70% ethanol and centrifuged at 4°C, for 10 mins at 4500g. The pellet obtained was air dried and resuspended in 100µL TE buffer.The isolated eDNA was stored at -20°C until further use(Hassan et al., 2018)

All isolated eDNA obtained using three distinct methods were run on a 0.7% low EEO agarose gel and observed using a UV transilluminator. The purity of the isolated eDNA was assessed using a spectrophotometer, and the concentration was determined using the Qubit 2.0 Fluorometer.

3) PCR Amplification

PCR amplification for the extracted eDNA was carried out on a thermal cycler (Agilent SureCycler 8800) using rbcLa and ITS2 primers . The resultant PCR products were then subjected to gel extraction and/or DGGE.

Table 3.3 :-PCR Parameters

3.3.1) Forprimer rbcLa and ITS2 primers

Steps	Temperature(˚C)	Time	Cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec	40
Annealing	56	30 sec	
Extension	72	45 sec	
Final extension	72	10 min	1

Table 3.4:- Primer Sequence

Primers used	Orientation	Sequences	Reference
rbcLa	Forward Primer	5′-ATGTCACCACAAACAGAGACTAAAGC-3′	(Asahina et al, 2010)
	Reverse primer	5′-GCAGCAGCTAGTTCCGGGCTCCA-3′	
ITS2	Forward Primer	5′-ATGCGATACTTGGTGTGAAT-3′	(Gu et al, 2013)
	Reverse primer	5′-GACGCTTCTCCAGACTACAAT-3′	

Table 3.5:-PCR reaction mixture component(LZ1)

Sample (L 1)	Reaction mixture for rbcLa	Reaction mixture for ITS2
Sterile MQ	28.8	29.8
5X Taq buffer	10	10
MgCl2 (25mM)	2	2
dNTP(10mM)	1	1
Template (~50 ng)	4	4
Forward primer	1.3	1.1
Reverse primer	1.9	1.1
Taq polymerase	1	1
Total Volume	50	50

Table 3.6:-PCR reaction mixture component(LZ2)

Sample (L2)	Reaction mixture for rbcLa	Reaction mixture for ITS2
Sterile MQ	25.8	26.8
5X Taq buffer	10	10
MgCl2 (25mM)	2	2

dNTP(10mM)	1	1
Template (~50 ng)	7	7
Forward primer	1.3	1.1
Reverse primer	1.9	1.1
Taq polymerase	1	1
Total Volume	50	50

Table 3.7 :- PCR reaction mixture component(LZ3)

Sample (L3)	Reaction mixture for rbcLa	Reaction mixture for ITS2
Sterile MQ	28.8	29.8
5X Taq buffer	10	10
MgCl2 (25mM)	2	2
dNTP(10mM)	1	1
Template (~50 ng)	4	4
Forward primer	1.3	1.1
Reverse primer	1.9	1.1
Taq polymerase	1	1
Total Volume	50	50

3.1) Gel extraction

Inorder to isolate amplified bands of interest from the gel, two gel extraction protocols were implemented. Gel extractionwas carried out using GeNei Gel Extraction Kit, while the other method employed the use of Clone Well II E-gel manufactured my ThermoFisher Scientific-IN.

3.2) Denaturing Gradient Gel Electrophoresis

To effectively separate the bands obtained from PCR amplification, Denaturing Gradient Gel Electrophoresis (DGGE) was carried out, following the protocol outlined by (Strathdee & Free, 2013).

Initially, 20µL of required amplified band was loaded onto the gel. Electrophoresis was conducted for a duration of 6 hours until completion, maintaining a voltage of 100V. Following electrophoresis, the gel was stained using the silver nitrate/formaldehyde staining as mentioned in the paper.

Table 3.8 :- Gradient setup for denaturing gradient gel electrophoresis (Strathdee & Free, 2013)

Content	Amount (mL)	Components
Plug region	1.0	0% DGGE solution
Main gel	6.9	0% DGGE solution
	4.1	80% DGGE solution
Main gel	1.4	0% DGGE solution
	9.6	80% DGGE solution
Stacker gel	4 .0	0% DGGE solution

4) Detection of Humic Acid in the extracted eDNA samples

To ascertain the presence of humic acids both before and after eDNA extraction, a protocol outlined by (Sheng et al., 2007) was followed. To estimate the sensitivity of the procedure, estuary water from three different locations and a pure bacterial DNA sample was used as controls. Karl Pearsons correlation analysis was used to determine the relationship between eDNA concentration and humic acid presence.

5) eDNA degradation rate

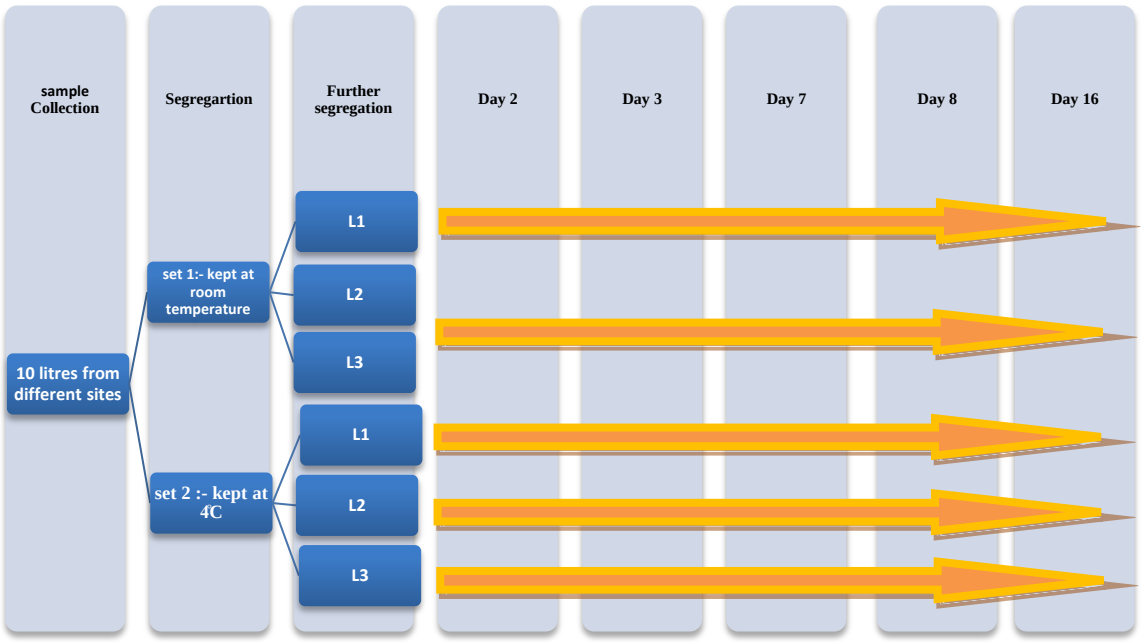
5.1) Effect of temperature and time during storage of water samples containing eDNA

To evaluate the impact of temperature on eDNA present in watersamples, a protocol proposed by (Hinlo et al., 2017) was followed. Water samples totalling approximately 10L from three distinct locations was collected. Each set of water samples weresubsequently further divided into two sets. One set was maintained at room temperature, while the other was kept at 4 ˚C.

Periodically (as mentioned in figure), eDNAwas extracted from approximately 500 mL of water sample using the chemically modified method. The concentration of the extracted eDNA was determined using Qubit 2.0 fluorometer.

(Note:- room temperature is take to be 30˚C ± 2˚C).

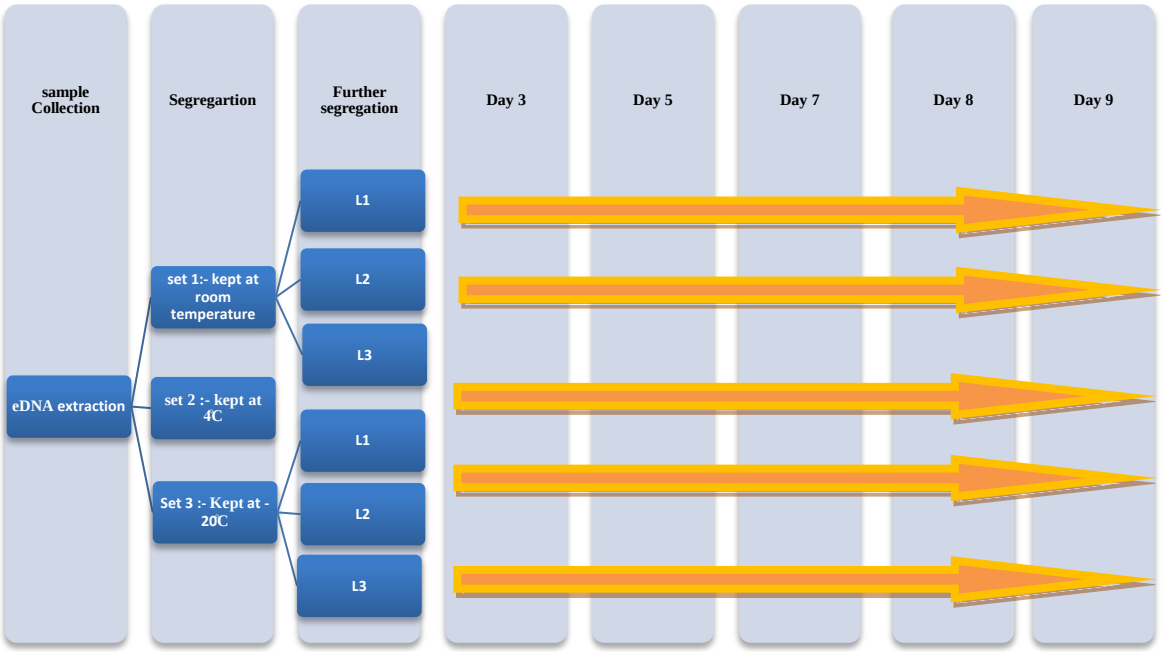
Figure 3.3:-Flow diagram depicting the setup for eDNA degradation rate experiment(5.1)



5.2) Effect of temperature and time during storage of the extracted eDNA

To investigate the impact of storage method and duration on the concentration of the isolate eDNA following extraction, a methodology akin to the one described above was employed. After extracting eDNA from samples collected at three distinct locations, it was divided into three sets. The first set of three samples was stored at room temperature, the second set at 4˚C in a refrigerator, and the third set at -20˚C. Subsequently, at intervals mentioned in the diagram, the concentration of the stored eDNA was determined using Qubit 2.0 Fluorometer.

Figure 3.4:- Flow diagram depicting the setup for eDNA degradation rate experiment (5.2)



5.3) Effectof temperature and time during storage of water samples containing eDNA

To validate the prior experimental findings, estuary water was used for comparisons on both Day 1 and Day 2. This study sought to compare the DNA yield of samples filtered within 24 hours (Day 1) with samples filtered at 24 hours (Day 2) as many researches carried out on eDNArecommend filtering water samples within 24 hours of collection for best results.Water samples were collected from three distinct locations, divided into three sets and each set was subjected to three different temperature conditions: room temperature, 4˚C, and -20˚C. The filtration and eDNA extraction procedures were carried out as mentioned above.

Filtration of water samples on Day 1 occurred promptly within 1.5 hours post-collection, while filtration and eDNAextraction of Day 2 samples were carried out 24 hours after the completion of Day 1 eDNAextraction procedures.

5.4) Data analysis

To ascertain the statistical significance of the obtained data, we conducted a Repeated Measures ANOVA. In order to mitigate the risk of Type I error inherent in Experiment 1A, a post hoc analysis was performed on Excel subsequent to the ANOVA. For Experiments 1B and 1C, a standard repeated measures ANOVA was conducted without any additional adjustments.

6) Comparative Analysis of eDNA Yield from Various Water Bodies

Previous researches state thateDNA concentration can act as an indirect link above the biodiversity of the desired area(Ficetola et al., 2008), (Goldberg et al., 2015) and (Thomsen et al., 2012) . Therefore, to compare the biodiversity across diverse water ecosystems, eDNA was extracted from three distinct water bodies: seawater(Caranzalim sea :- Location1:-LS1, Location 2:- LS2, Location 3:-LS3) estuary (Zuari), and freshwater lake(Carambolim lake :- Location1:-LF1, Location 2:- LF2, Location 3:-LF3). The concentrations of eDNA were quantified using the Qubit 2.0 fluorometer.

Figure 3.5:- Different location sites for Sampling:-Caranzalem beach-goa

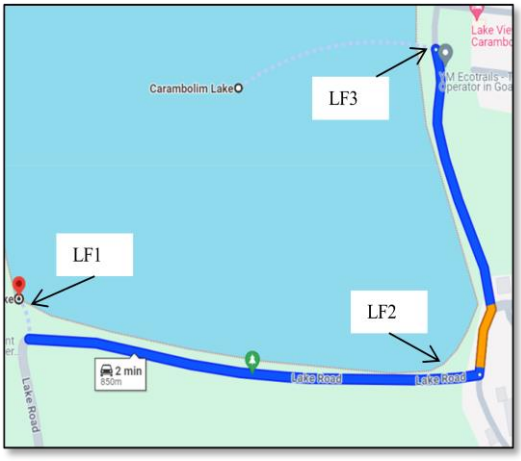


Figure 3.6:- Different location sites for Sampling:-Carambolim lake-goa

Chapter 4

Analysis and Conclusion

Results obtained

1) Isolation of eDNA

1.1) Sample collection

Samples were collected from three distinct sites in the Zuari and Mandovi estuaries. Parameters such as salinity and temperature were measured as mentioned below.Due to the lack of conclusive results obtained from analyzing the Mandovi water sample for eDNA concentration using traditional, kit-based, and CTAB methods, the focus of the study was redirected towards the water sample from the Zuari estuary instead of the Mandovi estuary.

Table 4.1.1:-Parameters and coordinates measured during sample collection

Sampling sites	Co-ordinates		Temperature (˚C)	Salinity (‰)
	Latitude	Longitude		
LZ1	15°22'06.4"	73°57'50.8"	24	21
LZ2	15°22'07.9"	73°58'09.1"	18	15
LZ3	15°22'12.7"	73°58'28.9"	22	22
LM1	15°29'57.2"	73°50'19.2"	26	20
LM2	15°30'05.4"	73°50'53.3"	23	25
LM3	15°50’20”	73°84’99”	27	24

It was determined that around 200 mL of water sample was the minimum amount required for eDNA. Subsequently, to ensure proper visualization of the bands on the agarose gel during electrophoresis, it was found that a minimum of approximately 500 mL of water was necessary.

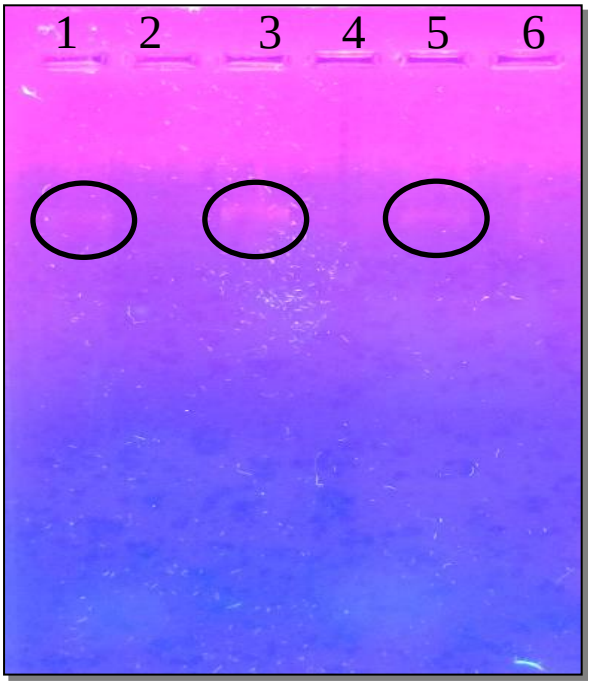


Fig 4.1.1:- eDNA extracted from different volumes of water sample Lane 1:- 200 mL , Lane 3:- 500 mL, Lane 5:- 500mL water sample

2) Extraction of eDNA sample

The eDNA was extracted using the three methods. eDNA extracted using traditional methodstends to have a very high concentration of eDNA as compared to the other two methods.Subsequently, when the eDNA was analyzed for its purity, eDNA extracted using the kit method showed comparatively higher purity. When the resulting eDNA samples were run on a gel, bands were observed for the CTAB-chemical method (Fig 1.4). However, no bands were detected in any

of the three locations (Zuari) from which eDNA was extracted using the traditional and kit-based methods. Notably, eDNA isolated via the traditional method exhibited a visible brown precipitate, indicating highhumic acid content.

Table 4.1.2:- eDNA concentration from Zuari estuary

Methods utilized	eDNA (ng/µL)		
	LZ1	LZ2	LZ3
CTAB Method	14.20	7.60	11.70
Nucleospin Kit Method	4.60	1.80	3.40
ET-OH method	30.20	20.60	7.12

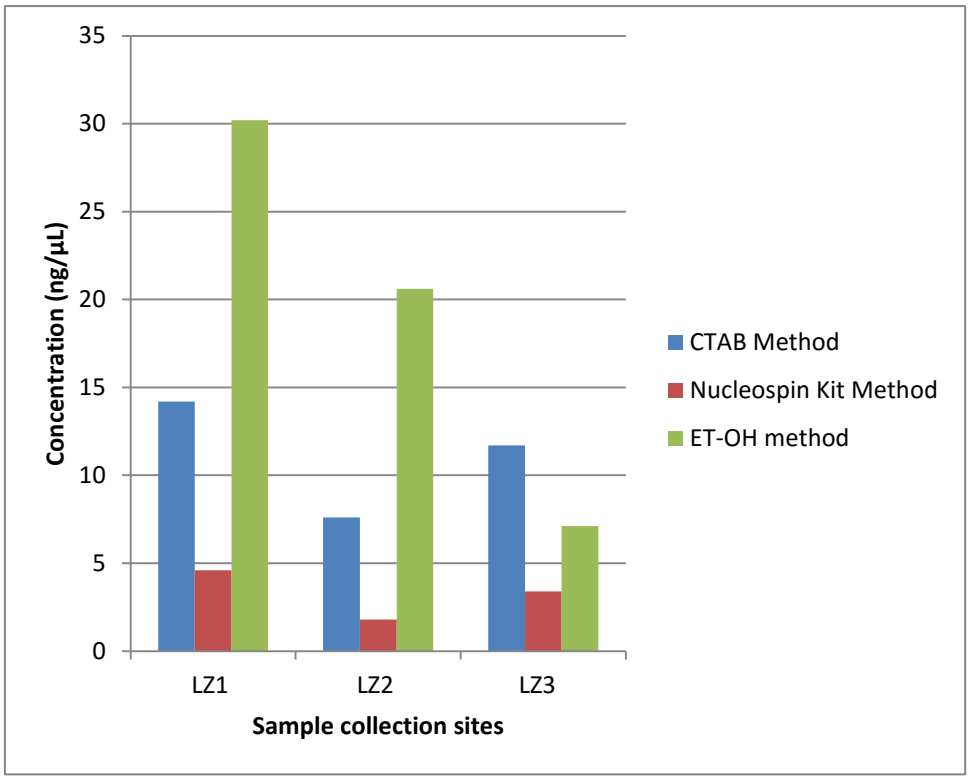


Figure 4.1.2 :- Graph depicting concentration of eDNA extracted using various methods

Table 4.1.3:- Purity of eDNA

Meth	Absorbance ratio (260/280)		
	LZ1	LZ2	LZ3
CTAB Method	1.50	1.16	1.23
Nucleospin Kit Method	1.75	1.50	1.68
ET-OH method	1.10	1.30	1.38

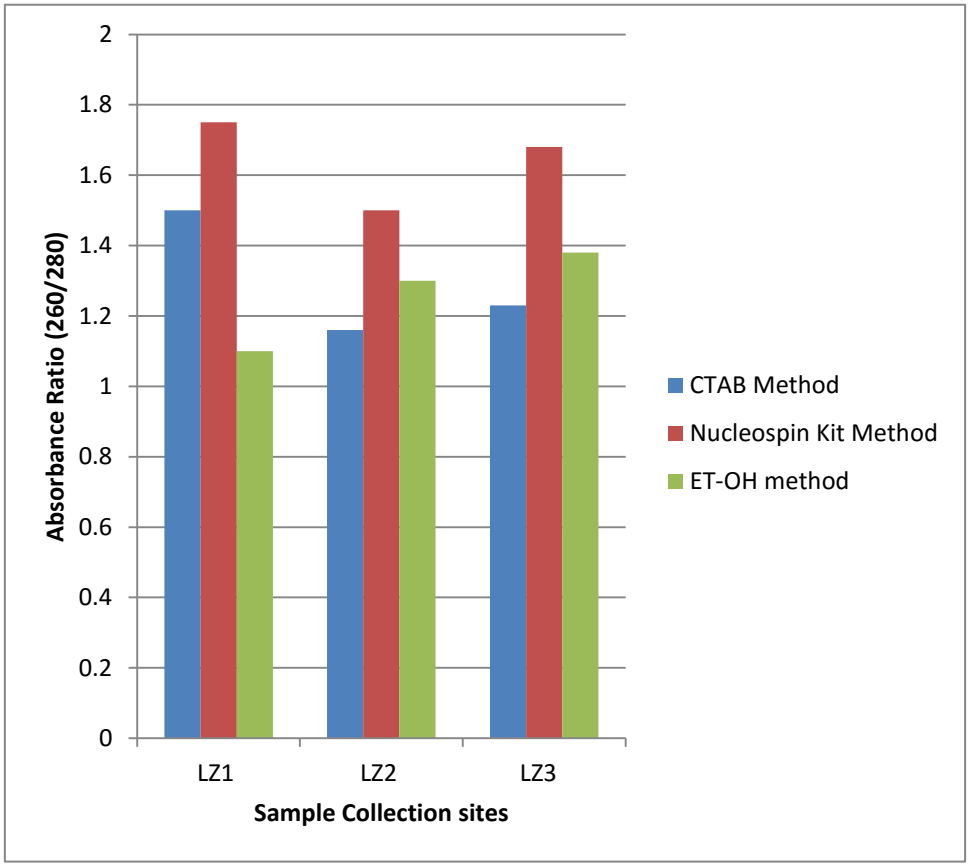


Figure 4.1.3:-Graphdepictingpurity of eDNA extracted using various

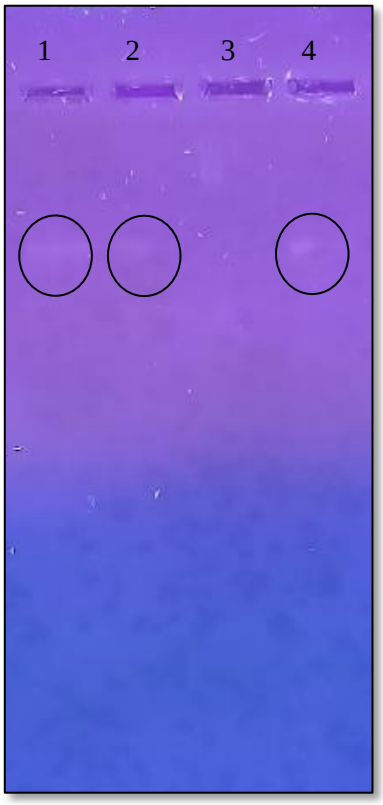


Figure 4.1.4:- 0.7% Agarose gel showing isolated eDNA bands(CTAB method) :- well 1,2,4

3) PCR Amplification

The bands obtained using CTAB method showed amplification for a 50ng reaction using rbcLa and ITS2 primers. The isolatedeDNA obtained via Et-OH and via the kit method did not show any amplification using any of the above primers.

Table 4.1.4:-PCR bands obtained for eDNA extracted using CTAB method

Sample sites	Amplified Bands Observed	
	rbcLa (50 ng)	ITS2 (50 ng)
LZ1	Yes	Yes
LZ2	No	Yes
LZ3	Yes	Yes

Table 4.1.5:-PCR results using primer rbcLa

Methods utilized	PCR Amplification using rbcLa		
	LZ1	LZ2	LZ3
CTAB Method	Yes	No	Yes
Nucleospin Kit Method	No	No	No
ET-OH method	No	No	No

Table 4.1.6:- PCR results using primer ITS2

Methods utilized	PCR Amplification using ITS2		
	LZ1	LZ2	LZ3
CTAB Method	Yes	Yes	Yes
Nucleospin Kit Method	No	No	No
ET-OH method	No	No	No

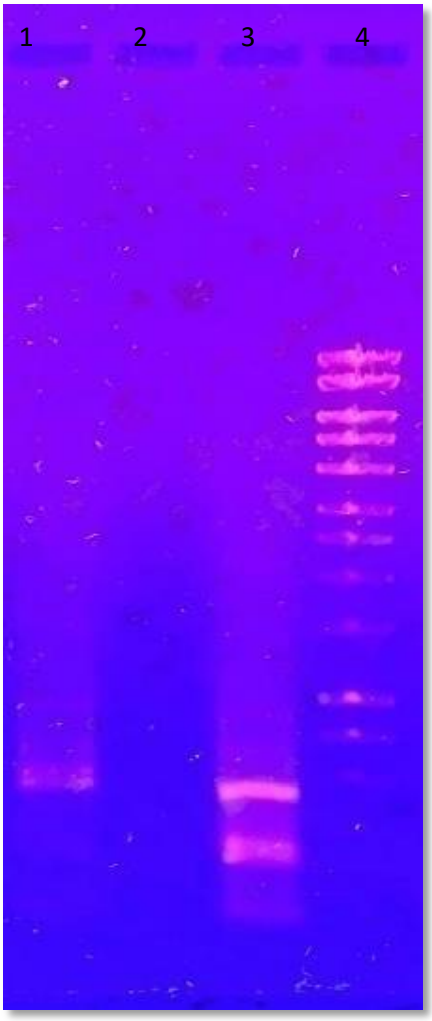


Figure 4.1.5 :-PCR amplified fragments amplified using rbcLa primer. lane 1:- LZ1, lane 2:- LZ2, lane 3:- LZ3, lane 4:- 1 kb Ladder.

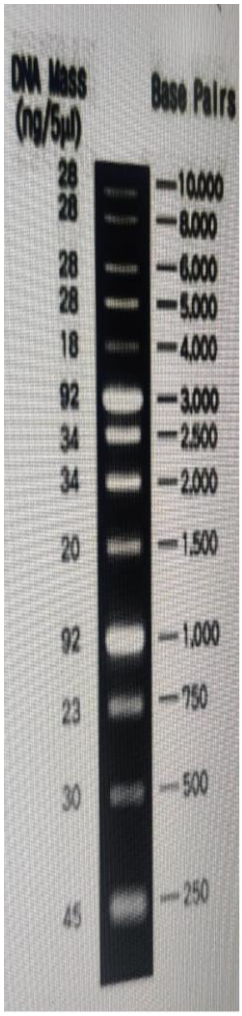


Figure 4.1.6:- Molecular weight of 1 kb DNA ladder

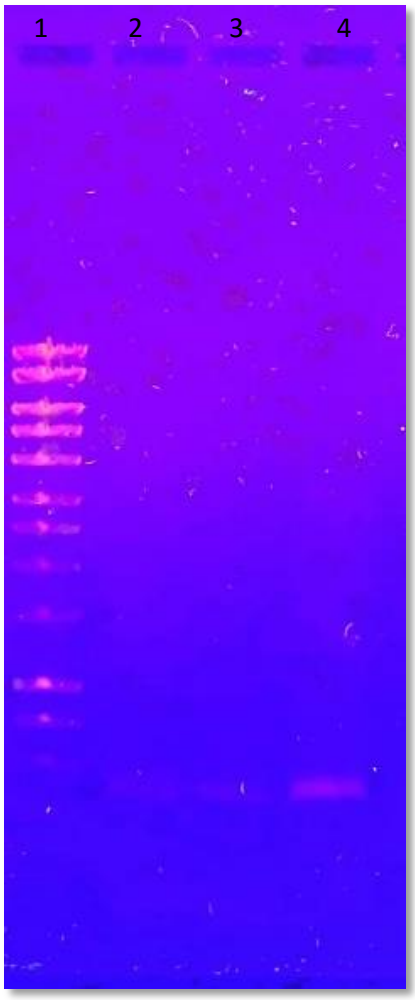


Figure 4.1.7 :-PCR amplified fragments amplified using ITS2 primer. lane 1:- LZ1, lane 2:- LZ2, lane 3:- LZ3, lane 4:- 1 kb Ladder.

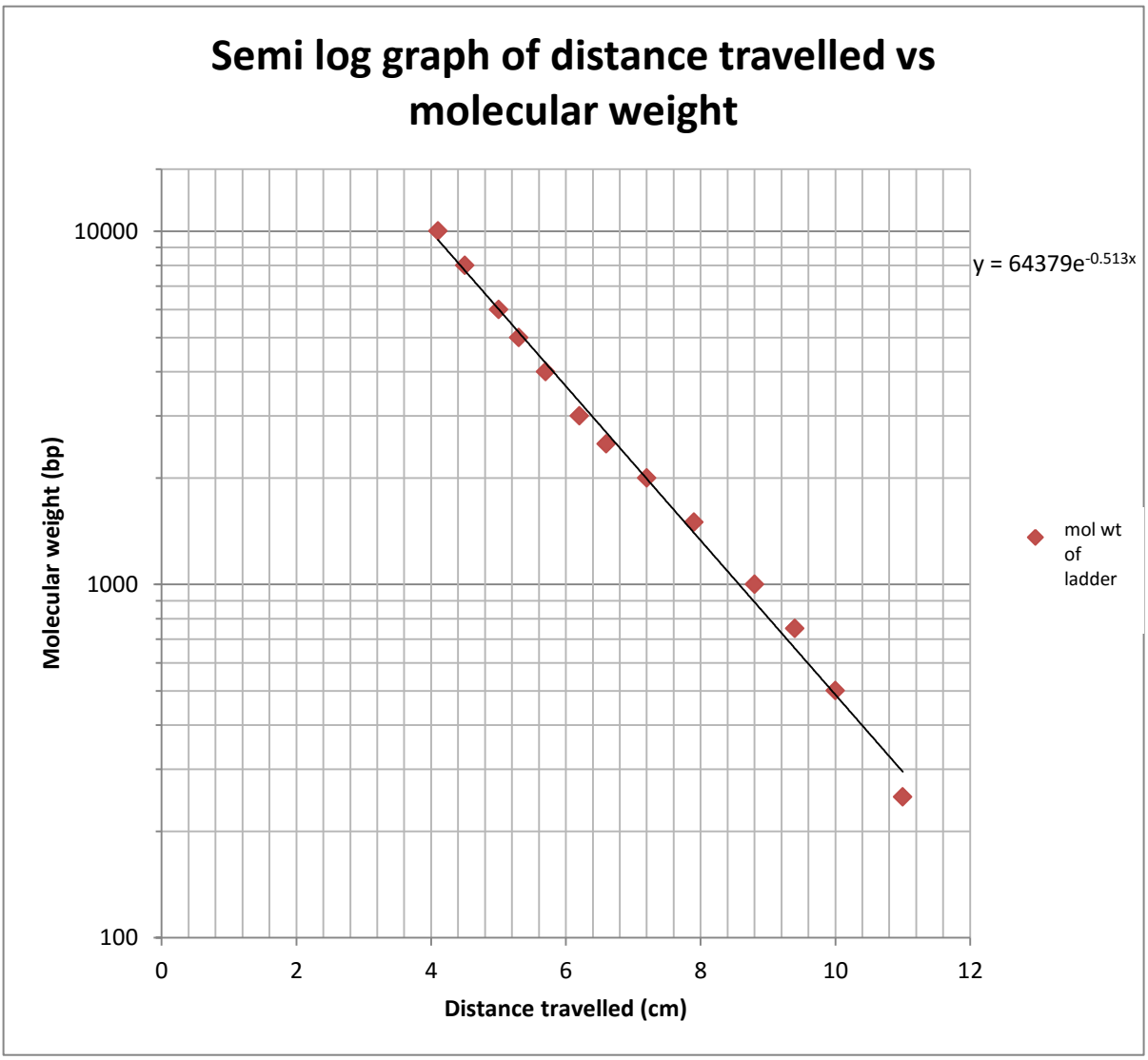


Figure 4.1.8:- Semi log graph of distance travelled vs molecular weight of the ladder

Table 4.1.7:- Molecular weight of the amplified bands obtained via semi log graph

Amplified bands to be sent for sequencing	Molecular weight (bp)
rbcLa (LZ1)	380.90
ITS2 (LZ3)	327

3.1) *Gel extraction*

Following PCR, amplified bands of eDNA were effectively separated using both the conventional silica-based extraction method and the Clone Well II E-gel.

Figure 4.1.9:- PCR products electrophoresed through the E-gel.

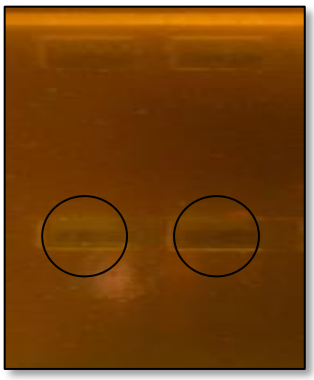


Figure 4.2 :- PCR products entering the 2nd well for extraction.

3.2) Denaturing gradient gel electrophoresis

DGGE method was used to separate obtained amplified bands. However, due to the procedure involving temperature variation as an additional factor for proper separation of the eDNA, a clear separation was not achieved, leaving a smear on the gel.Thesmear along the lane comprised of the multiple bands of eDNA.

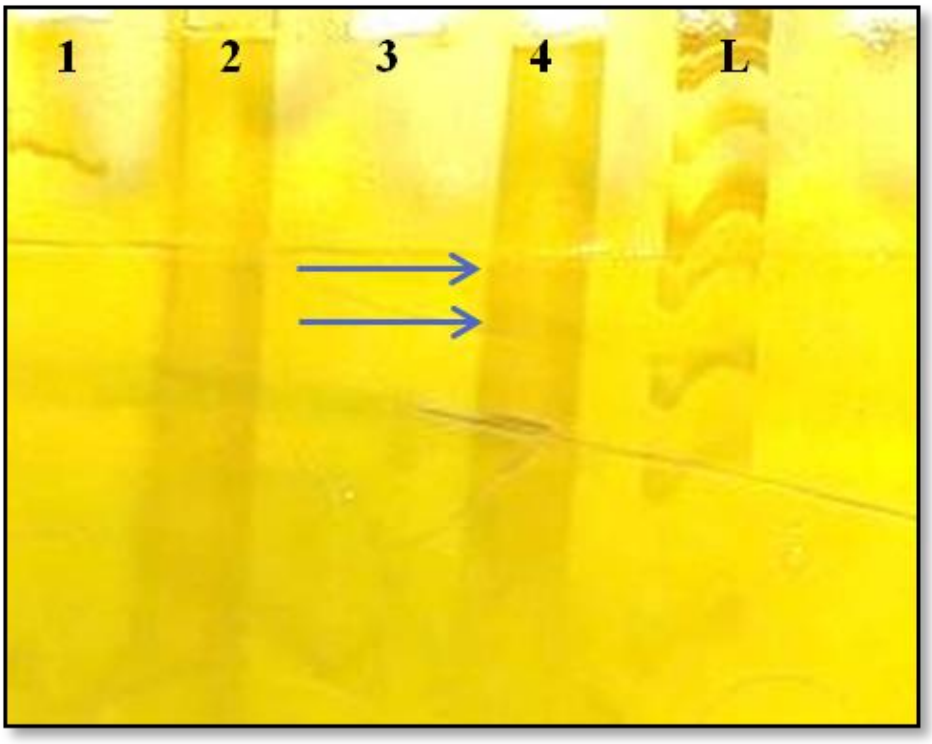


Figure 4.2.1:- PCR products electrophoresed on a DGGE gel L:- 1kb Ladder

4) Detection of HumicAcid in the extracted eDNA

As a result of a significant drop in absorbance, it was found that eDNA extracted using the conventional approach typically had a high concentration of humic acid. eDNA isolated via chemical and kit methods, on the other hand, contain far less humic acid. The results were likewise shown by the controls, with the pure DNA isolate showing the lowest drop in absorbance and estuarine water showing the largest fall in absorbance. The pure DNA isolate's absorbance barely decreased, indicating that the method is still insufficiently sensitive to accurately detect the humic acid level.

Table 4.1.8:- Result of humic acid detection

	location	toluidine blue (ml)	sample (ml)	Absorbance(A) (630nm)	Delta Absorbance (A(blank)-A) nm	Average (nm)	Standard Deviation
Blank/Reference		1	2	0.32	0		
Control DNA Sample		1	2	0.32	0.005		
Estuary water	LZ1	1	2	0.176	0.149	0.147	0.002
	LZ2	1	2	0.178	0.147		
	LZ3	1	2	0.180	0.145		
Traditional method of eDNA extraction	LZ1	1	2	0.253	0.072	0.060	0.012
	LZ2	1	2	0.278	0.047		
	LZ3	1	2	0.265	0.060		
Kit Method of eDNA extraction	LZ1	1	2	0.300	0.025	0.025	0.004
	LZ2	1	2	0.295	0.03		
	LZ3	1	2	0.304	0.021		
Chemical method of eDNA extraction	LZ1	1	2	0.296	0.029	0.024	0.008
	LZ2	1	2	0.310	0.015		
	LZ3	1	2	0.298	0.027		

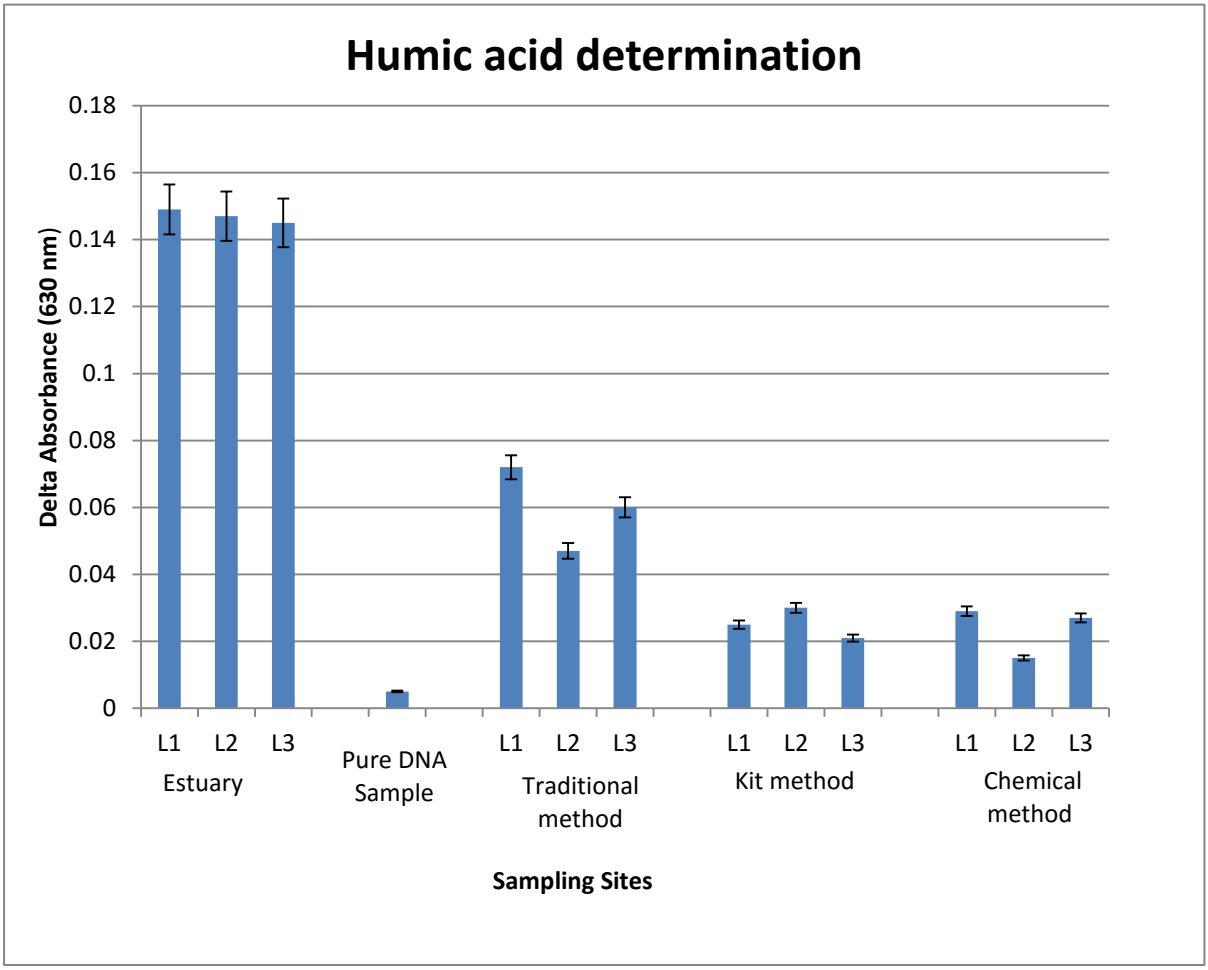


Figure 4.2.2:- Graph depicting delta absorbance of different samples

Data Analysis:-

Karl Pearsons correlation analysis method was used to find outwhether there is a relationship between the eDNA concentration and the delta absorbance which corresponds to the amount of Humic acid present. The following table was constructed to suit the method.

Table 4.1.9:- Average eDNA concentration and delta Absorbance of the eDNA extracted via various methods

Samples	Average eDNA concentration(ng/µL)	Average Delta Absorbance (nm)
Traditional method	2.78	0.060
Kit based method	4.23	0.025
Chemical method	6.85	0.024

Table 4.2:- Karl Pearsons correlation analysis

	Average eDNA concentration	Average Delta Absorbance
Average eDNA concentration	1	
Average Delta Absorbance	-0.80	1

From the above data, correlation coefficient (r) is found out to be -0.80. This depicts that there is a very strong negative correlation between the eDNA concentration and Delta Absorbance. Hence this acts as an indicative factor that presence of humic acid decreases the concentration of eDNA.

5) eDNA Degradation Rate

5.1) eDNA of the water samples kept at room temperature showed a significant decrease in the eDNA concentration as compared to the eDNA concentration of water sample kept at 4˚C.

Table 4.2.1:-Result of eDNA degradation (5.1)

Day(s)	Location	DNA concentration of water Samples kept at	
		Room Temperature	4˚C
2	LZ1	12.10	15.40
	LZ2	10.10	12.00
	LZ3	8.50	9.80
3	LZ1	11.90	15.40
	LZ2	9.70	12.00
	LZ3	8.30	9.70
7	LZ1	10.20	15.20
	LZ2	8.90	11.90
	LZ3	7.10	9.40
8	LZ1	10.00	15.00
	LZ2	8.70	11.80
	LZ3	6.80	9.50
10	LZ1	9.30	14.80
	LZ2	8.00	11.30
	LZ3	6.20	9.20
12	LZ1	8.60	14.70
	LZ2	7.50	11.40

	LZ3	5.60	9.10
14	LZ1	6.60	14.50
	LZ2	5.80	11.00
	LZ3	4.70	8.70
16	LZ1	4.70	14.00
	LZ2	2.90	10.40
	LZ3	1.50	8.10
18	LZ1	1.60	13.50
	LZ2	0	9.20
	LZ3	0	7.50

Table 4.2.2:- Average Results of eDNA Degradation rate

Day(s)	Average eDNA concentration of samples at room temperature	Average eDNA concentration of samples at 4˚C
2	10.23	12.40
3	9.97	12.40
7	8.73	12.16
8	8.50	12.10
10	7.83	11.77
12	7.23	11.73
14	5.70	11.40
16	3.03	10.83
18	0.53	10.07

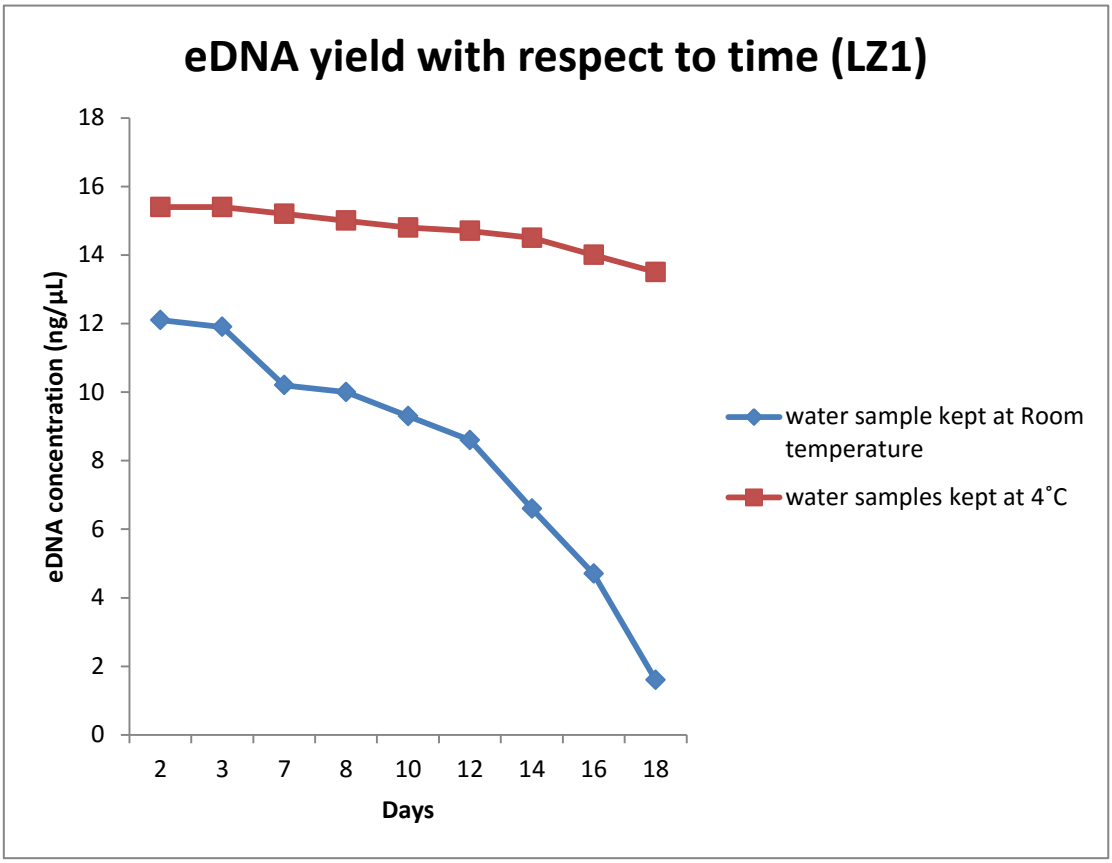


Figure 4.2.3 :- Line graph depicting eDNA yield with respect to time for LZ1 sample

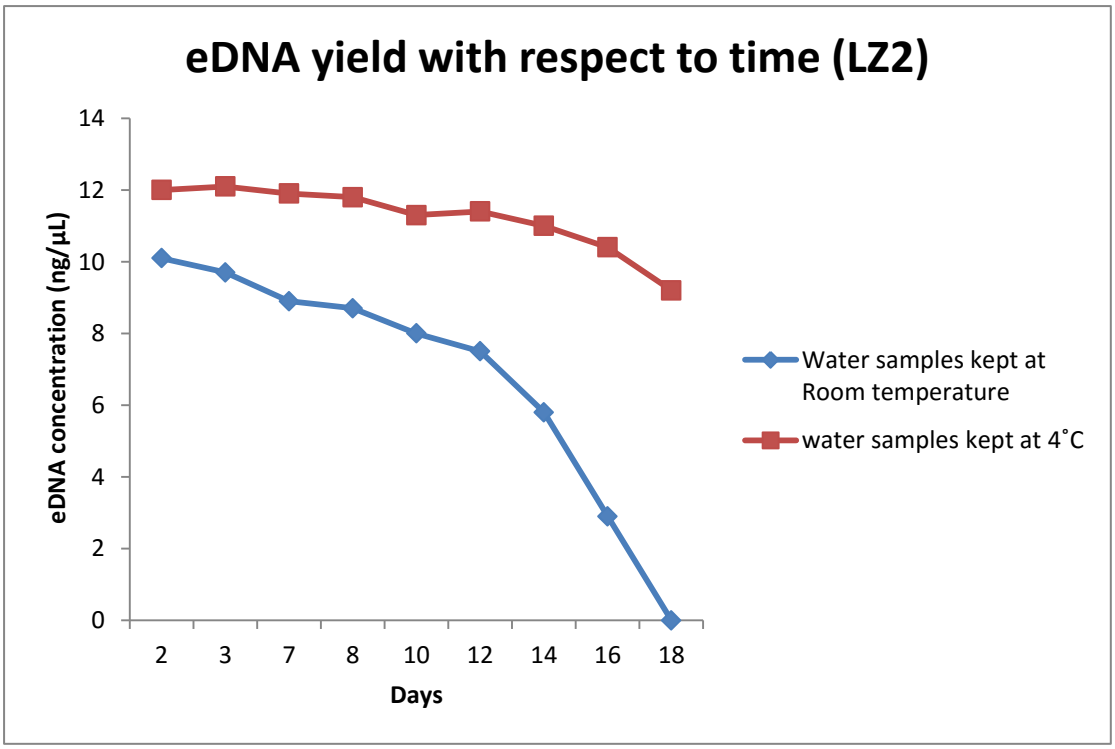


Figure 4.2.4 :- Line graph depicting eDNA yield with respect to time for LZ2 sample

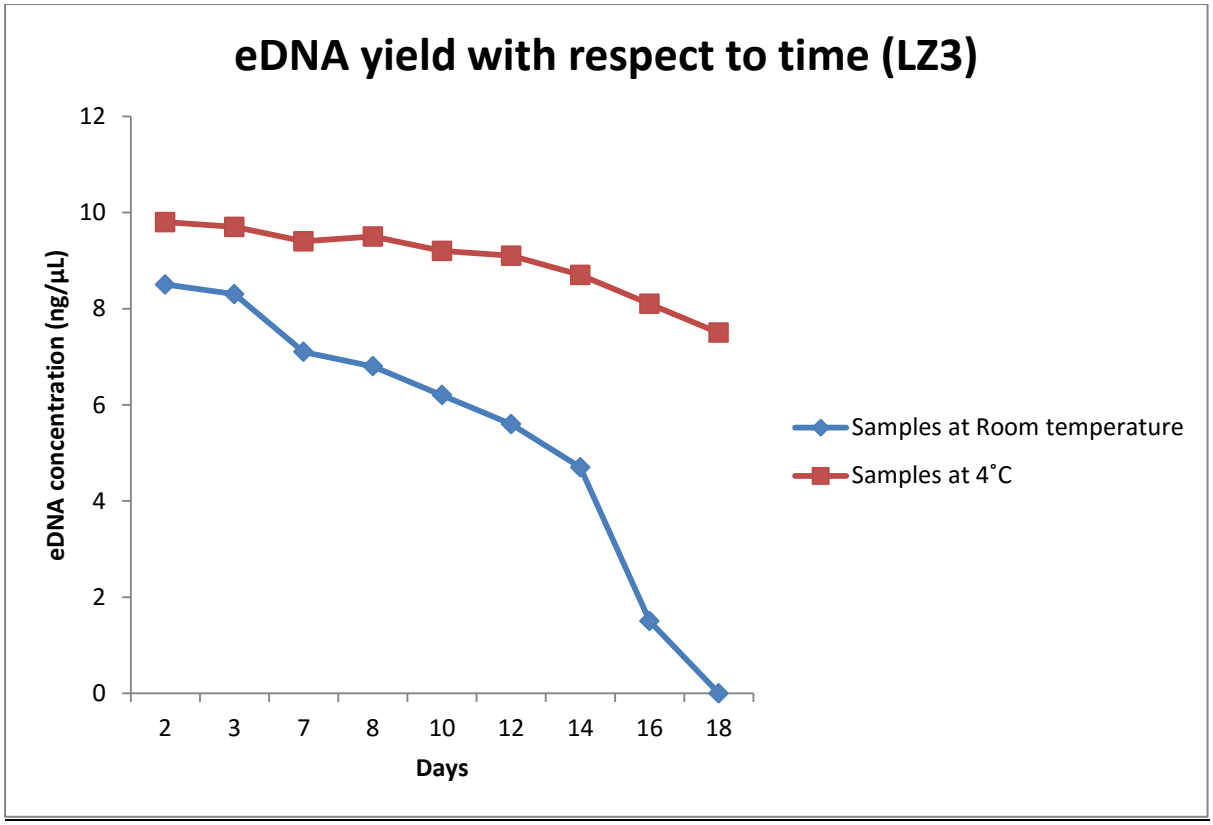


Figure 4.2.5 :- Line graph depicting eDNA yield with respect to time for LZ3 sample

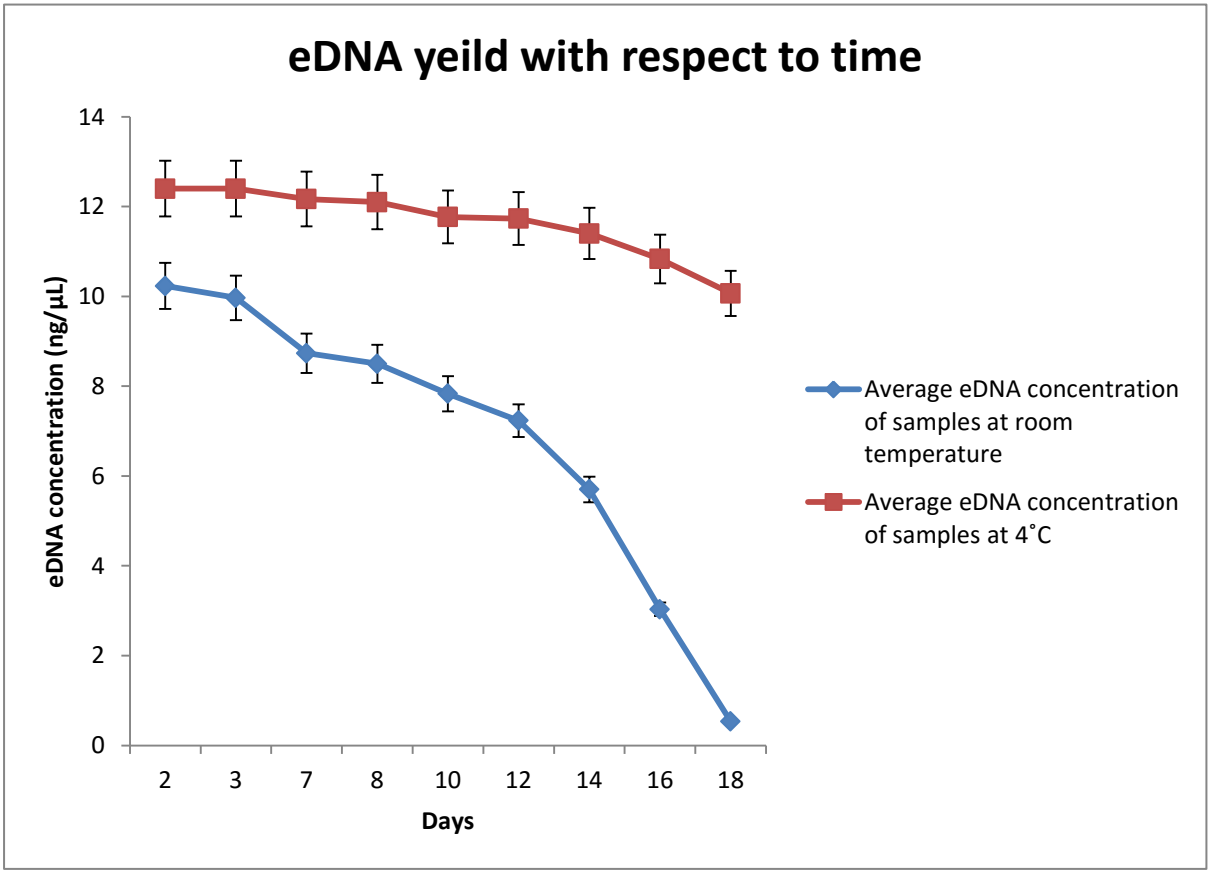


Figure 4.2.6 :- Line graph depicting average eDNA yield with respect to time

Data Analysis:-

From the above data 2 hypothesis were deduced out

Null Hypothesis (H0): There is no significant difference in eDNA concentration between samples stored at room temperature and those stored at 4˚C over time.

Alternative Hypothesis (H1): There is a significant difference in eDNA concentration between samples stored at room temperature and those stored at 4˚C over time.

Using ANOVA:- 2 factor without replication(repeated ANOVA on excel), following results were obtained.

Table 4.2.3:- Results of ANOVA :- two factor without replication (a)

ANOVA: Two-Factor Without Replication				
Days	Count	Sum	Average	Variance
2	2	22.63	11.32	2.35
3	2	22.37	11.18	2.96
7	2	20.90	10.45	5.89
8	2	20.60	10.30	6.48
10	2	19.60	9.80	7.74
12	2	18.96	9.48	10.13
14	2	17.10	8.55	16.24
16	2	13.86	6.93	30.41
18	2	10.60	5.30	45.44
Average	9	61.76	6.87	10.55

eDNA concentration of samples at room temperature				
Average eDNA concentration of samples at 4˚C	9	104.90	11.65	0.61

Table 4.2.4:- Results of ANOVA :- two factor without replication (b)

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	64.74	8	8.10	2.65	0.0951	3.44
Columns	103.21	1	103.20	33.769	0.0004	5.3177
Error	24.44	8	3.06			
Total	192.38	17				

To further minimise the risk of type1 error, post hoc analysis was carried out using bonferroni adjustment on excel. Following are the results.

Table 4.2.5:- Results of Bonferroni adjustment, utilising t-test

t-Test: Two-Sample Assuming Unequal Variances(for bonferroni adjustment)		
	Average eDNA concentration of samples at room temperature	Average eDNA concentration of samples at 4˚C
Mean	6.87	11.65
Variance	10.54	0.61
Observations	9	9
Hypothesized Mean Difference	0	
Df	9	
t Stat	-4.30	
P(T<=t) one-tail	0.000991	
t Critical one-tail	1.84	
P(T<=t) two-tail	0.001982	
t Critical two-tail	2.26	
bonferroni adjustment	0.05/2= 0.025	
Significant?	TRUE	

The preceding results show that computed F is bigger than F critical. To clarify our findings, the P-value obtained is less than the significance level(0.05). Also the P value for two tail was found out to be lesser than the bonferroni adjustment. The 3 readings indicate that our alternate hypothesis—which states that there is a significant variation in eDNA content between samples maintained at room temperature and those stored at 4˚C over time:- should be accepted in place of our null hypothesis.

5.2) When the extracted eDNA was stored at room temperature, the eDNA concentration decreased dramatically compared to when it was kept at 4°C andat -20°C. eDNA stored at 4 °C showed a considerable decline beginning on Day 7. eDNA at -20°C did not demonstrate any significant decline during the experiment. At room temperature, eDNA decomposed completely by day nine.

Table 4.2.6:-Result of eDNA degradation (5.2)

Day(s)	Location	eDNA concentration after extraction and storage at		
		Room temperature	4˚C	-20˚C
3	LZ1	13.20	15.00	15.80
	LZ2	10.50	11.50	12.40
	LZ3	9.30	9.70	10.30
5	LZ1	9.80	14.60	15.70
	LZ2	7.20	11.20	12.10
	LZ3	8.10	9.10	10.00
7	LZ1	7.20	14.00	15.50
	LZ2	4.80	10.40	11.90
	LZ3	5.70	8.70	9.80
	LZ1	3.80	13.60	15.30

8	LZ2	1.90	10.00	11.80
	LZ3	2.40	8.40	9.60
9	LZ1	1.50	13.30	15.00
	LZ2	0	9.60	11.50
	LZ3	0	7.80	9.30

Table 4.2.7:-Result of eDNA degradation (5.2)

Day(s)	Average eDNA concentration after extraction and storage at		
	Room temperature	4˚C	-20˚C
3	11.00	12.07	12.83
5	8.37	11.63	12.60
7	5.90	11.03	12.40
8	2.70	10.67	12.23
9	0.50	10.23	11.93

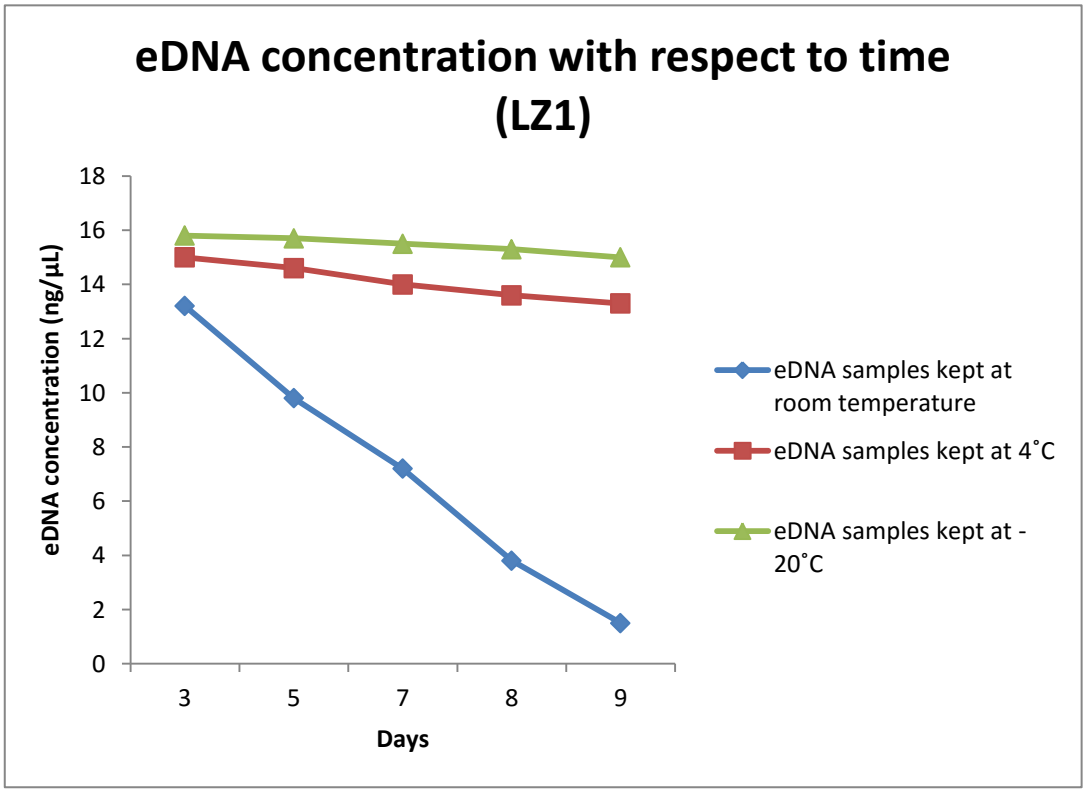


Figure 4.2.7 :- Line graph depicting eDNA concentration with respect to time for LZ1 sample

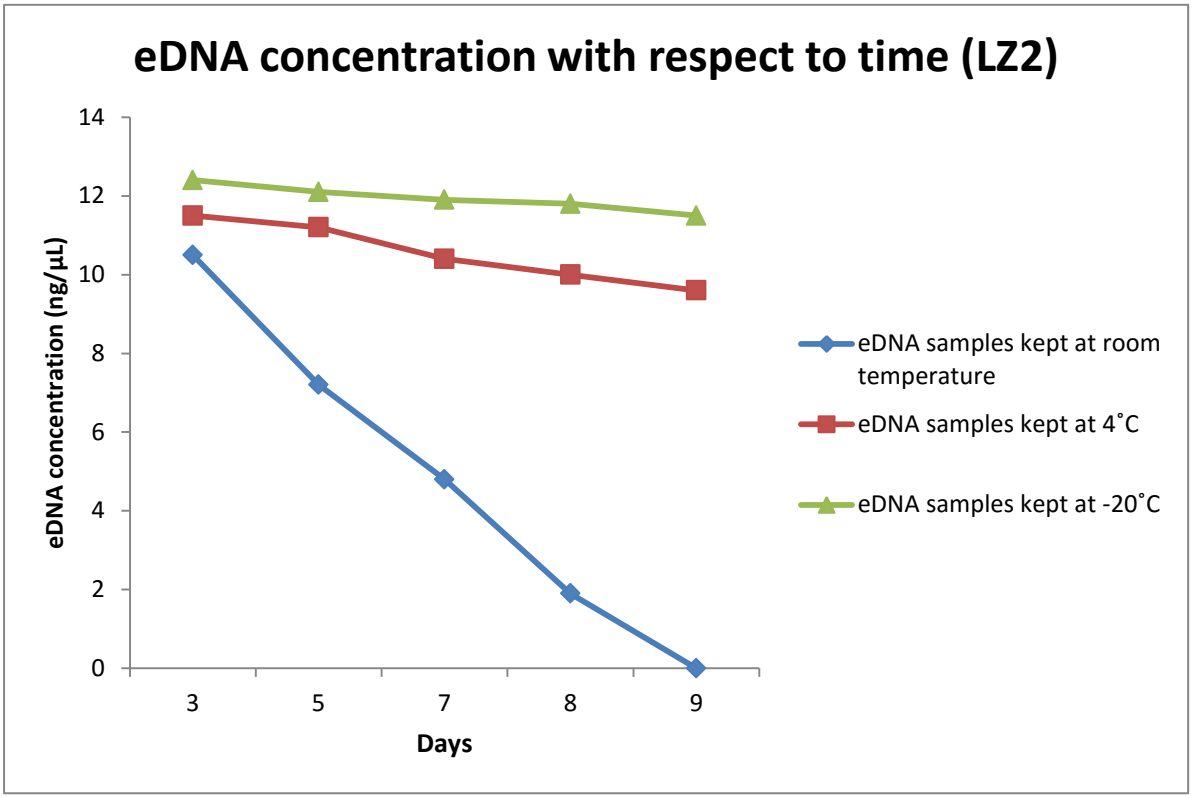


Figure 4.2.8 :- Line graph depicting eDNA concentration with respect to time for LZ2 sample

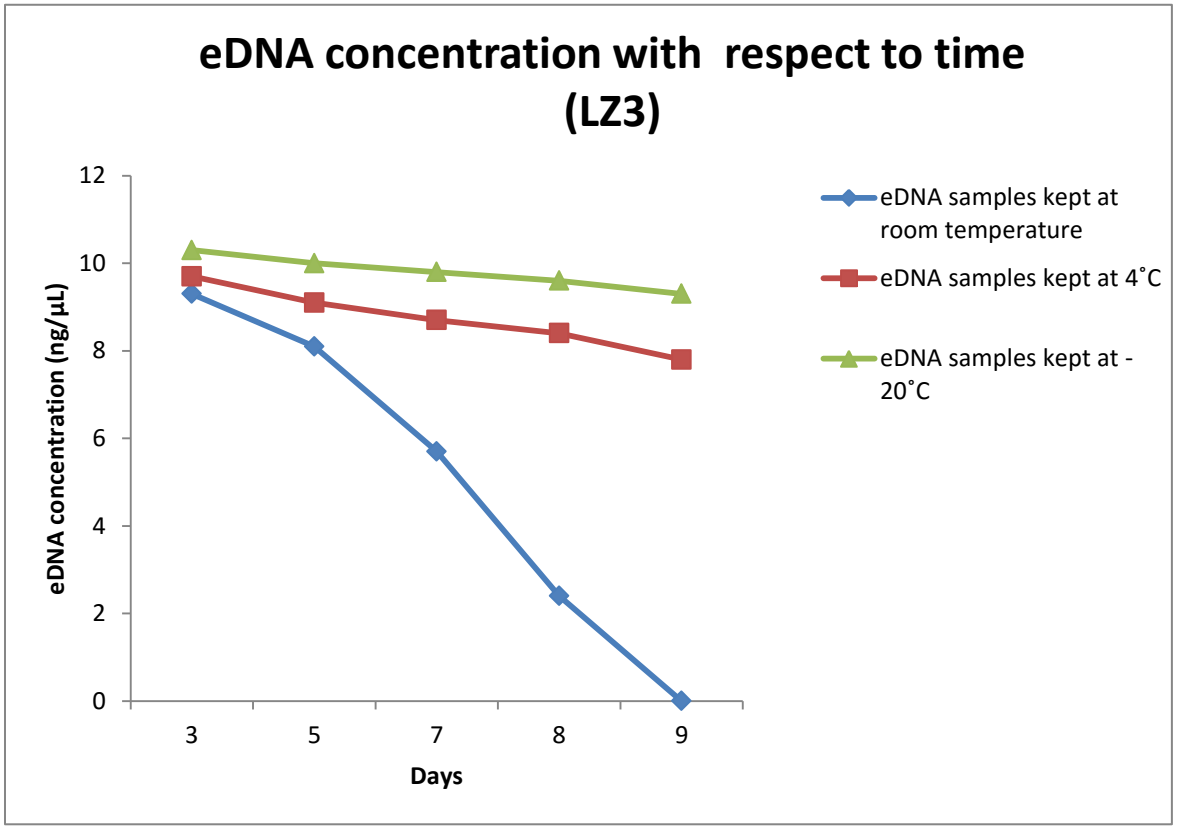


Figure 4.2.9 :- Line graph depicting eDNA concentration with respect to time for LZ3 sample

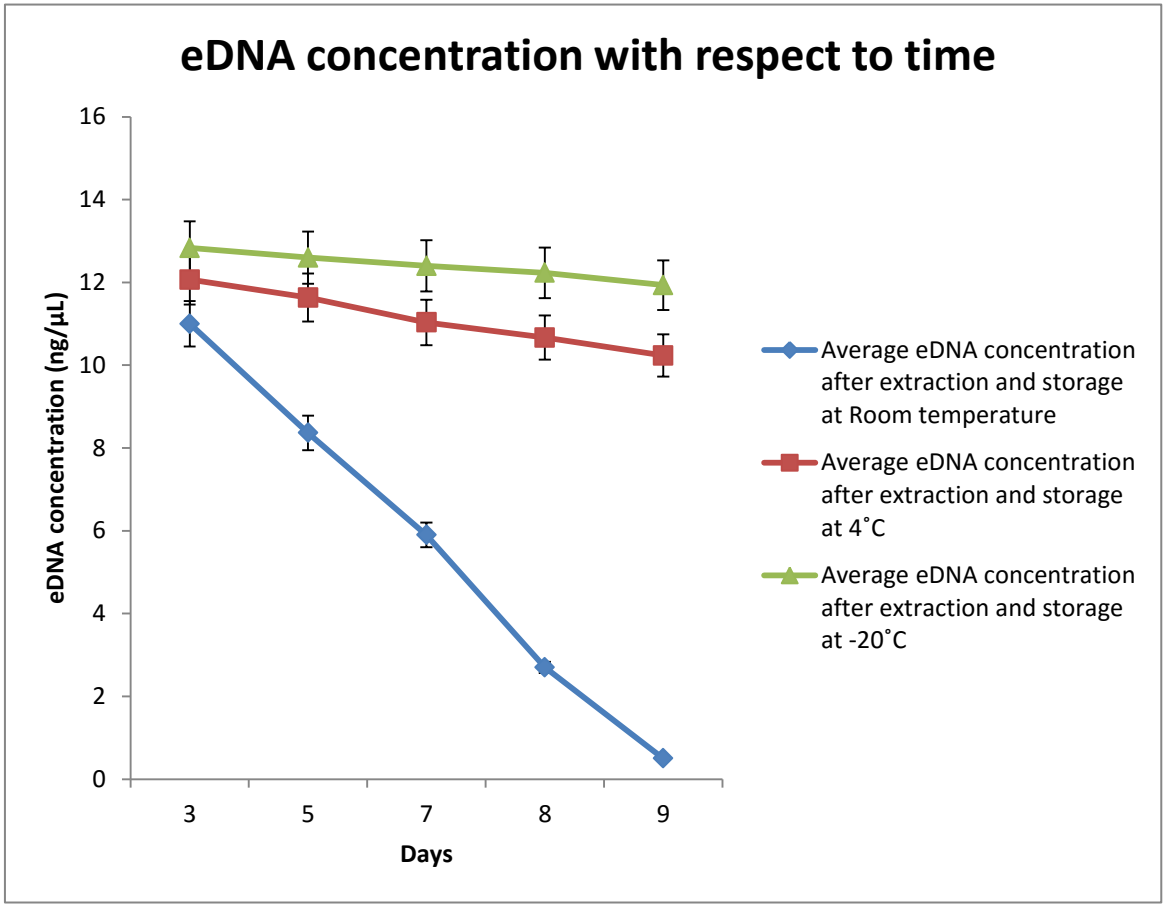


Figure 4.3 :- Line graph depicting average eDNA concentration with respect to time

Data Analysis:-

From the above data 2 hypothesis were deduced out

Null Hypothesis (H0): There is no significant difference in eDNA concentration among samples stored at room temperature, 4°C, and -20°C over time.

Alternative Hypothesis (H1): There is a significant difference in eDNA concentration among samples stored at room temperature, 4°C, and -20°C over time.

Using ANOVA:- 2 factor without replication(repeated ANOVA on excel), following results were obtained

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Table 4.2.8:- Results of ANOVA :- two factor without replication (a)

Days	Count	Sum	Average	Variance
Day 3	3	35.90	11.97	0.85
Day 5	3	32.60	10.86	4.92
Day 7	3	29.30	9.78	11.74
Day 8	3	25.60	8.53	26.12
Day 9	3	22.60	7.55	38.05
Room temperature	5	28.47	5.69	17.82
4˚C	5	55.63	11.13	0.54
-20˚C	5	61.96	12.39	0.12

Table 4.2.9:- Results of ANOVA :- two factor without replication (b)

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	37.38	4	9.34	2.05	0.180	3.84
Columns	126.85	2	63.42	13.888	0.0025	4.458
Error	36.54	8	4.57			
Total	200.76	14				

The above data show that the calculated F-value is greater than F critical. In addition, to specify our readings, the P value was compared to the same significance level utilized in standard ANOVA. The p value was found to be less than the significance level (0.05). Based on the two situation listed above, we reject our null hypothesis and accept our alternate hypothesis, which states that there is a substantial variation in eDNA content over time between samples stored at room temperature, 4°C, and -20°C.

5.3) *Effect of storage method and time on eDNA concentration for a period of 24 hours post collection*

The eDNA concentration in the water sample stored at room temperature decreased after 24 hours of sample collection. No significant reduction ineDNAconcentration was observed when the samples were stored at 4˚C and -20˚C.

Table 4.3:-Result of eDNA degradation (5.3)

Day(s)	Location	eDNA concentration (ng/µL)
1	LZ1	15.90
	LZ2	12.40
	LZ3	10.40
After 24 Hr storage at 4˚C		
2	LZ1	15.40
	LZ2	12.00
	LZ3	9.80

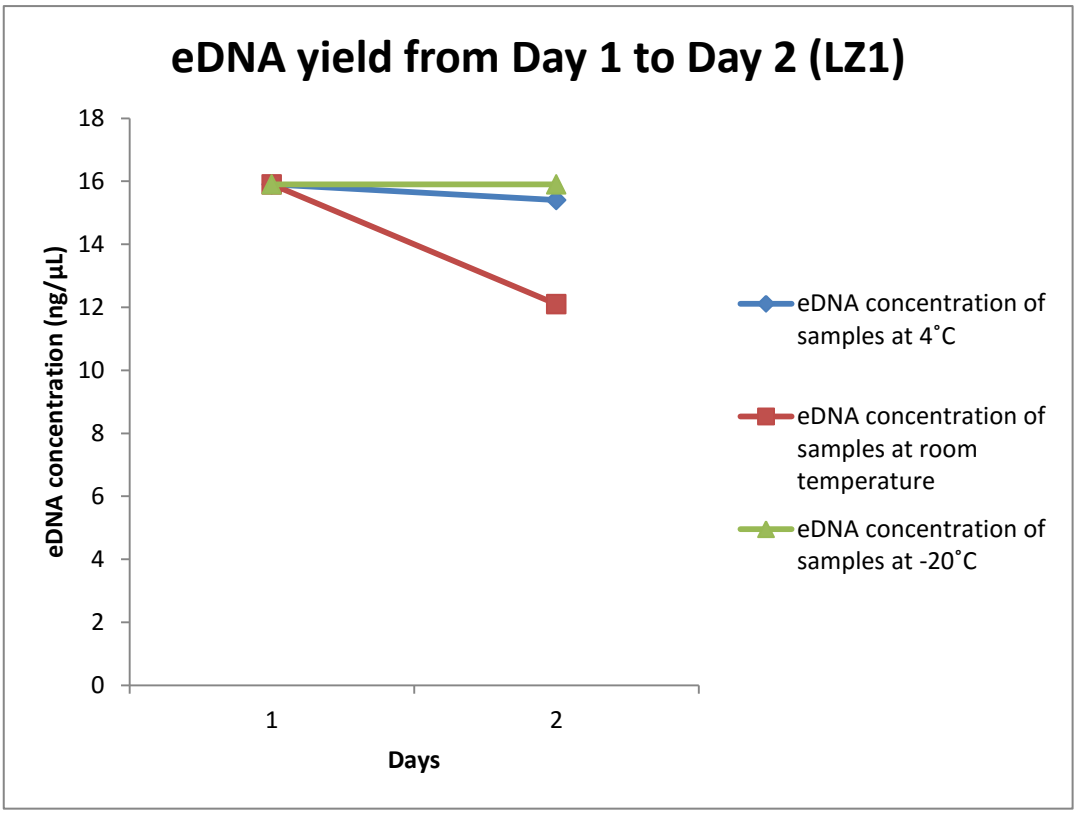

Figure 4.3.1 :- Line graph depicting eDNA yield from Day 1 to Day 2 for LZ1 sample

Table 4.3.1:-Result of eDNA degradation (5.3)

Day(s)	Location	eDNA concentration (ng/µL)
1	LZ1	15.90
	LZ2	12.40
	LZ3	10.40
After 24 Hr storage at Room temperature		
2	LZ1	12.10
	LZ2	10.10
	LZ3	8.50

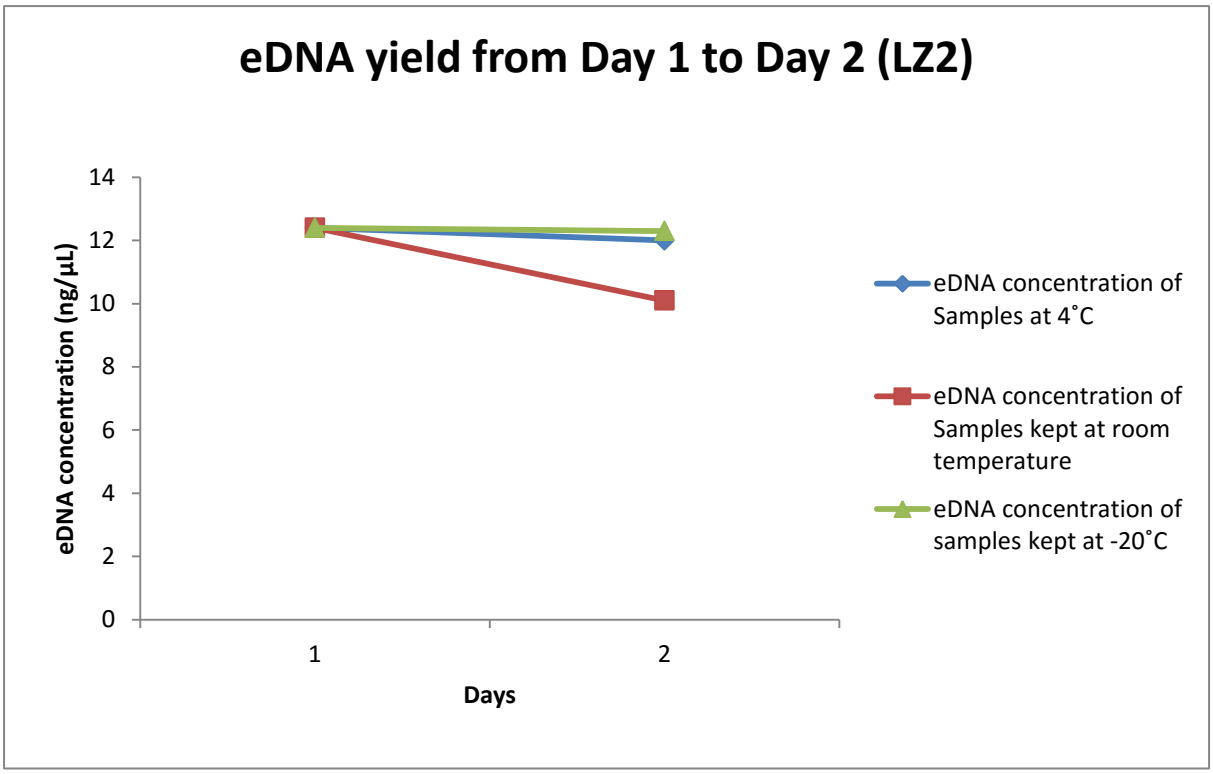

Figure 4.3.2 :- Line graph depicting eDNA yield from Day 1 to Day 2 for LZ2 sample

Table 4.3.2:-Result of eDNA degradation (5.3)

Day(s)	Location	eDNA concentration (ng/µL)
1	LZ1	15.90
	LZ2	12.40
	LZ3	10.40
After 24 Hr storage at -20˚C		
2	LZ1	15.90
	LZ2	12.30
	LZ3	10.40

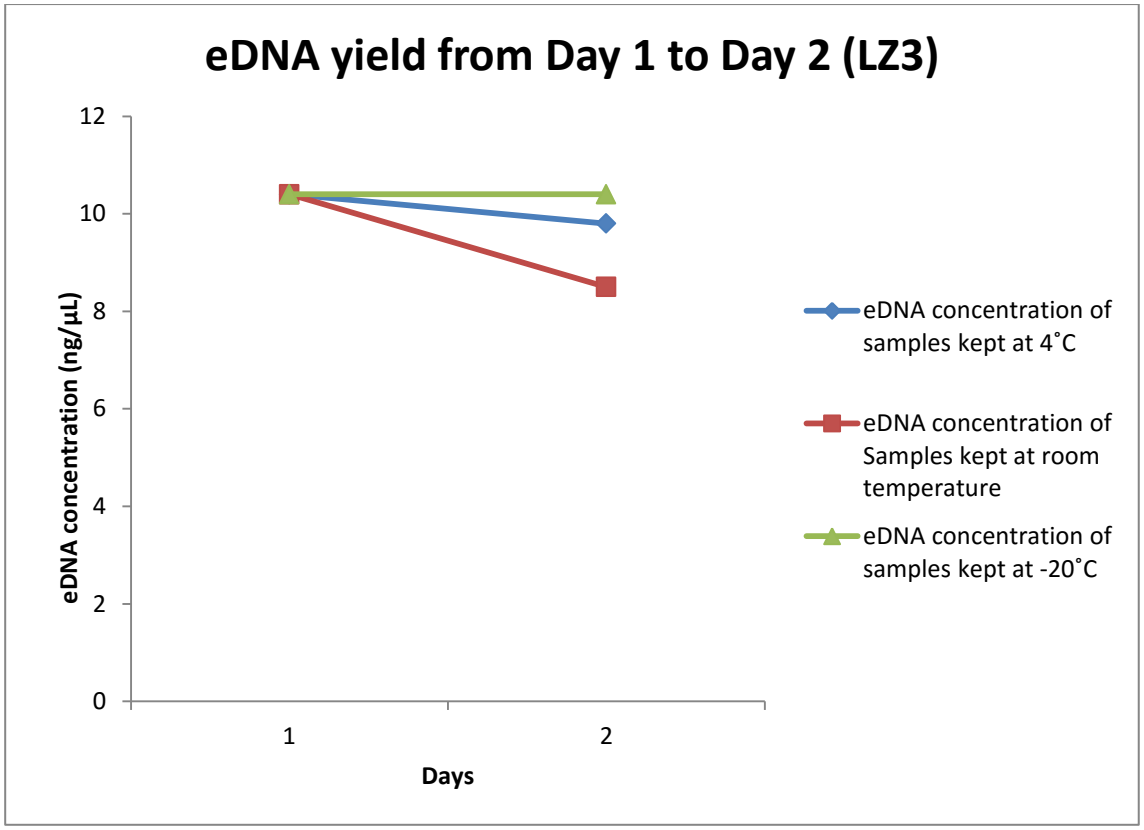

Figure 4.3.3 :- Line graph depicting eDNA yield from Day 1 to Day 2 for LZ3 sample

Table 4.3.3:-Result of eDNA degradation (5.3)

Day(s)	Average eDNA concentration of water samples kept at		
	Room Temperature	4˚C	-20˚C
1	12.90	12.90	12.90
2	10.23	12.40	12.87

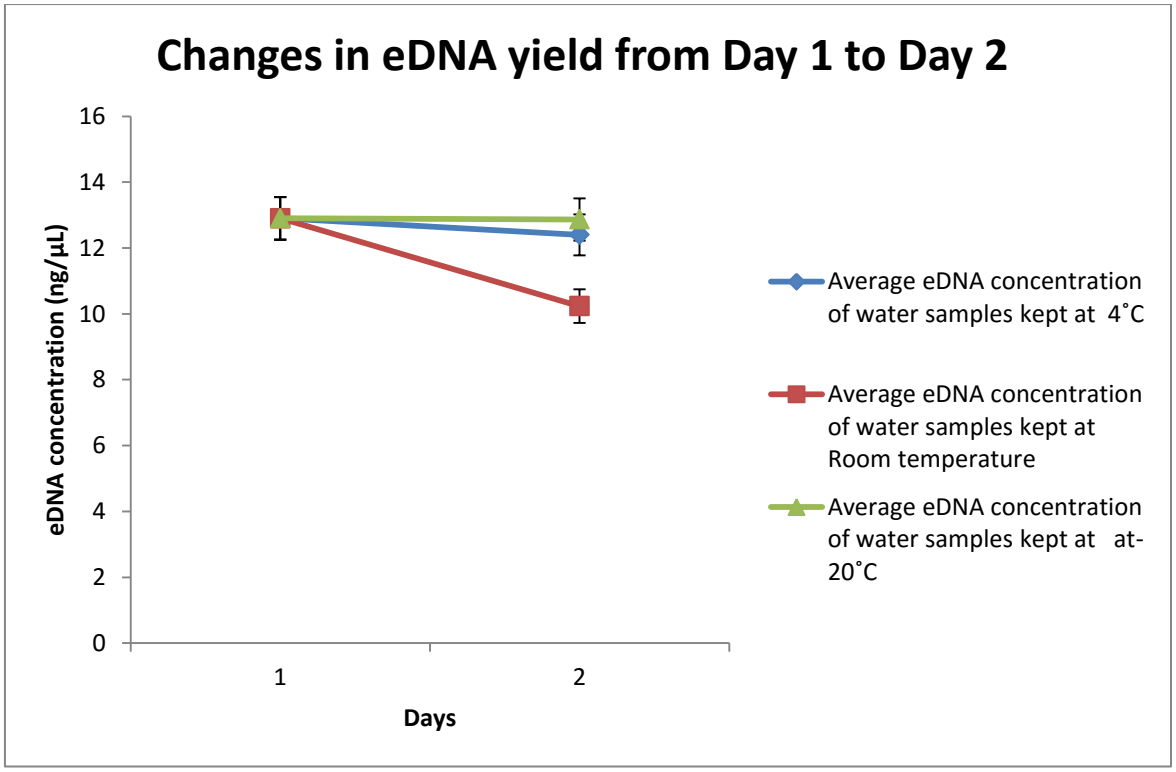

Figure 4.3.4 :- Line graph depicting Average eDNA yield from Day 1 to Day 2

Data Analysis:-

From the above data 2 hypothesis were deduced out

Null Hypothesis (H0): There is no significant difference in eDNA concentration between samples stored at room temperature, those stored at 4˚C and -20˚C over the period of 24 hours.

Alternative Hypothesis (H1): There is a significant difference in eDNA concentration between samples stored at room temperature, those stored at 4˚C and -20˚C over the period of 24 hours.

Using ANOVA:- 2 factor without replication(repeated ANOVA on excel), following results were obtained.

Table 4.3.4:- Results of ANOVA :- two factor without replication (a)

ANOVA: Two-Factor Without Replication				
SUMMARY	Count	Sum	Average	Variance
1	3	38.70	12.90	0
2	3	35.49	11.83	1.98
4˚C	2	25.30	12.65	0.12
Room temperature	2	23.13	11.56	3.56
at-20˚C	2	25.77	12.88	0.000544

Table 4.3.5:- Results of ANOVA :- two factor without replication (b)

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	1.71	1	1.71	1.73	0.32	18.51
Columns	1.98	2	0.99	1	0.5	19
Error	1.98	2	0.99			
Total	5.67	5				

From the above data, the calculated F was found out to be lesser than F critical. To specify our readings, the P-value was compared with the level of significance (0.05). It is observed that P- value is greater than the significance level. Hence, we accept our null hypothesis stating that there is no significant difference in eDNA concentration between samples stored at room temperature, those stored at 4˚C and -20˚C over the period of 24 hours

6) Comparative Analysis of eDNA Yield from Various Water Bodies

eDNA concentration of sea water samples were found to be the lowest as compared to the eDNA concentration of the freshwater and estuary water samples. However, there was no significant difference between the eDNA concentration of the estuary water samples and freshwater samples.

Table 4.3.6:- Parameters and coordinates measured during sample collection

Location	Sampling sites	Co-ordinates		Temperature (˚C)	Salinity (‰)
		Latitude	Longitude		
Caranzalem sea	LS1	15°46’38”	73°80’39”	31	30
	LS2	15°47’06”	73°80’49”	26	34
	LS3	15°47’65”	73°80’63”	28	31
Zuari estuary	LZ1	15°22'06.4"	73°57'50.8"	24	21
	LZ2	15°22'07.9"	73°58'09.1"	18	15
	LZ3	15°22'12.7"	73°58'28.9"	22	22
Carambolim Lake	LF1	15°48’69”	73°92’57”	20	0
	LF2	15°48’633”	73°92’95”	18	0
	LF3	15°48’91”	73°92’96”	15	0

Table 4.3.7:- Comparison of eDNA concentration of different water bodies

Water sample	Concentration of eDNA extracted via (ng/µL)		
	Traditional method	Kit based method	Chemical based method
Freshwater	3.24	7.10	8.4
Estuary	2.57	9.48	8.97
Seawater	2.98	4.50	4.80

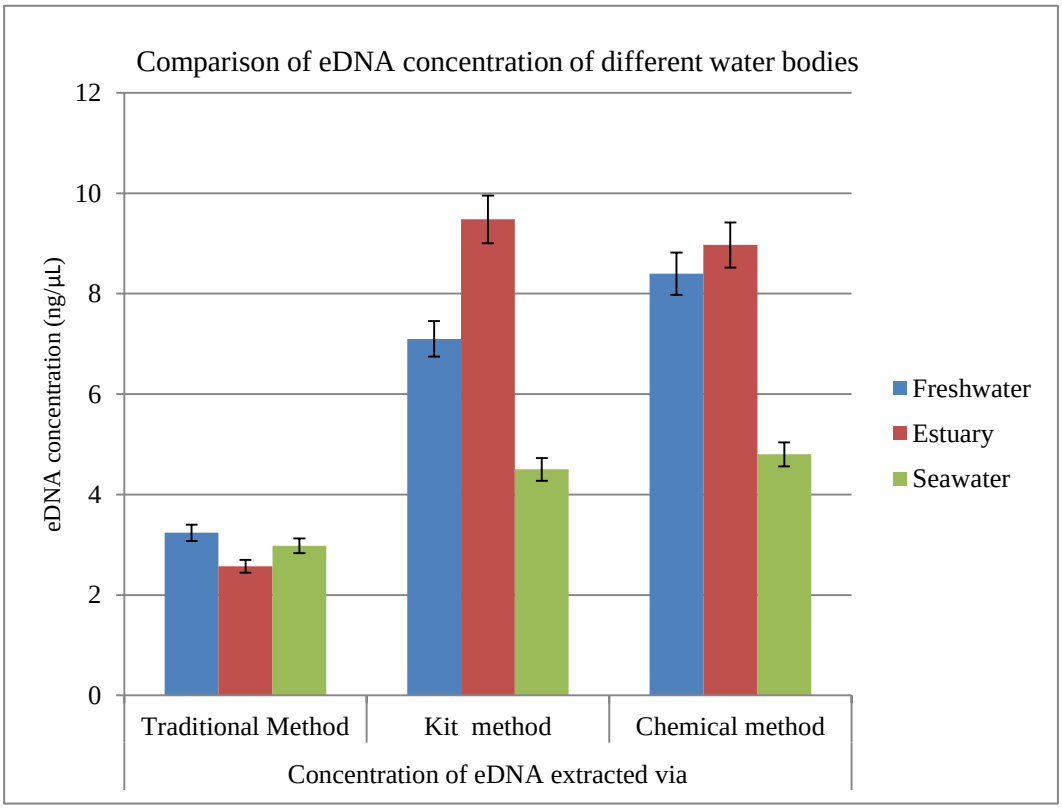

Figure 4.3.5 :- Graph depicting comparison of eDNA concentration of different water bodies

Table 4.3.8:- Comparison of eDNA concentration of 2 different estuaries

Samples	Average eDNA concentration of Mandovi (ng/µL)	Average eDNA concentration of Zuari (ng/µL)
eDNA extracted via Traditional method	2.78	19.30
eDNA extracted via Kit method	4.23	9.80
eDNA extracted via Chemical method	6.85	11.17

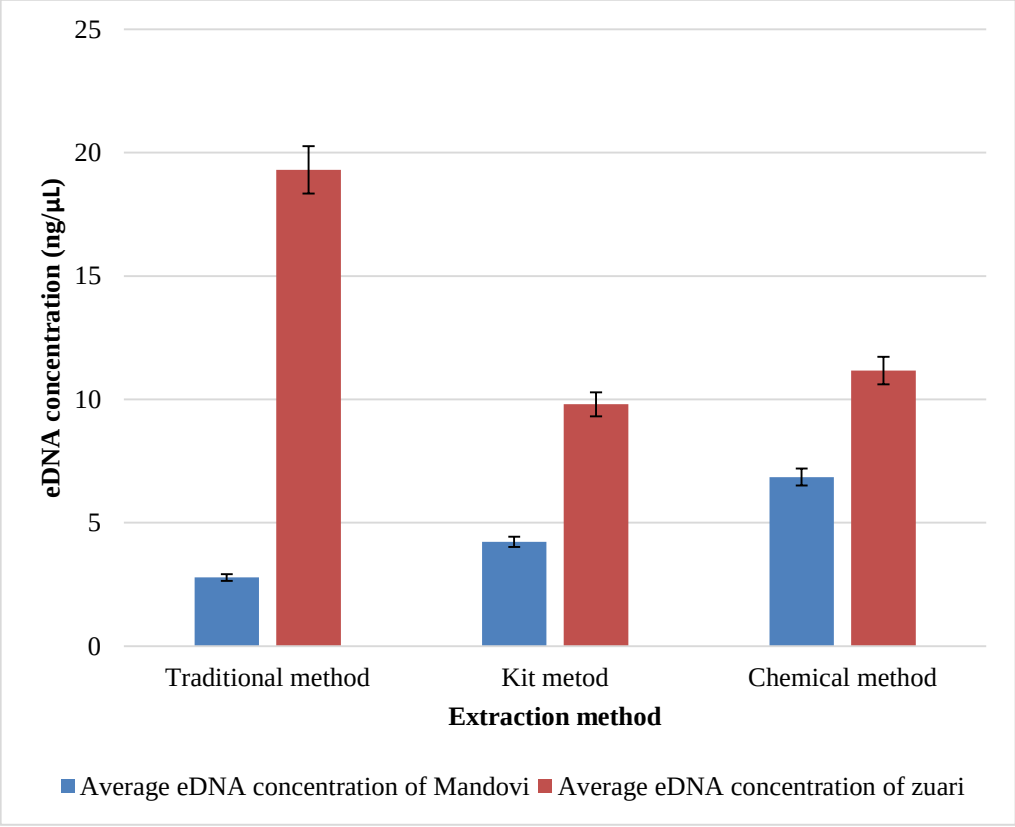

Figure 4.3.6 :- Graph depicting comparison of eDNA concentration of different estuaries

Conclusion

Environmental DNA (eDNA) was extracted, amplified, and degradation dynamics with respect to time and temperature were studied. Our findings provide valuable insights into the efficacy of different extraction methods, the influence of storage conditions on eDNA stability, and factors affecting eDNA concentration in estuarine waters.

Results indicated that a minimum water sample volume of approximately 500 mL is necessary for effective eDNA extraction and visualization of bands on agarose gel. The CTAB-based extraction method emerged as superior in terms of both extraction efficiency and subsequent amplification. Conversely, the kit-based extraction method yielded lower concentrations of eDNA but exhibited enhanced purity, while the Et-OH method though contaminated with humic acid produced higher eDNA yield.

Furthermore, gel extraction methods were successful in recovering PCR-amplified products, DGGE results emphasized the importance of temperature control and gradient gel mixtures for optimal band separation. The presence of humic acid in eDNA, particularly in samples extracted via traditional methods, underscores the need for robust purification protocols to mitigate potential contaminants. Our observations of a negative correlation between eDNA concentration and delta absorbance, as determined by Karl Pearson's coefficient, suggest the same.

Regarding eDNA degradation studies, our results indicate the critical role of storage temperature in preserving eDNA over time. Samples stored at room temperature exhibited significant decreases in eDNA concentration compared to

those stored at 4˚C, emphasizing the importance of controlled storage conditions. Additionally, statistical analysis revealed significant decreases in eDNA concentration at different storage temperatures (-20˚C, 4˚C, room temperature), determining the influence of temperature on eDNA stability.

Notably, our work carried with eDNA concentrations across different water sources revealed lower concentrations in seawater compared to freshwater and estuarine water samples. However, no significant difference was observed between eDNA concentrations in estuarine and freshwater samples, suggesting additional factors such as salinity may influence eDNA extraction efficiency.

Chapter 5

Future Prospects

Future Prospects.

1. Quantification of humic acid and its correlation to eDNA concentration and effect of humic acid on PCR amplification
2. Application studies on the isolated eDNA

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Appendix

1) 2 M NaOH solution

Amount to be added

= molecular weight × Molarity × volume to be prepared ÷ 1000

= 40 X 2 X 20 /1000

= 1.6 g in 20 mL distilled water

2) 0.025 M EDTA solution

Amount to be added

= molecular weight × Molarity × volume to be prepared ÷ 1000

= 292.24 X 0.025 X 20 / 1000

= 0.14612 g in 20 mL Distilled water

3) 74% ethanol (100 mL)

Take 74 mL of 100% ethanol and 26 mL of distilled water to make 100 mL of 74% ethanol

4) 6M HCL solution

Amount to be added

= molecular weight × Molarity × volume to be prepared ÷ 1000

= 36.46 X 6 X 20 / 1000

= 4.37 mL in 15.63 mL distilled water

5) 1% Triton X 100

1 mL of triton X 100 in 99 mL distilled water

6) 0.5% Tween 20

0.5 mL of tween 20 in 99.5 mL distilled water.

7) 1% CTAB

   1gm of CTAB in 100 ml distilled water

8) 50X TAE Buffer

   242 g Tris Base in distilled water

   57.1 mL glacial acetic acid

   100 mL 0.5 M EDTA solution (pH 8.0)

   Adjust the volume to 1L

9) 1X TAE Buffer

   Dissolve 10 mL of 50X TAE buffer in 490 mL Distilled water

10) 0.7% Agarose

   0.7 g Agarose powder in 100 mL 1X TAE Buffer

11) 0% Denturant composition:- 100 ml

   2ml of 50X TAE + 27 ml ofAcrylamide/NN Methylene bisacrylamide (37.5:1) + 71 ml of Sterile distilled water

12) 80% Denturant Composition :- 100 ml

   33.6 g urea + 32 ml Formamide + 2ml of 50X TAE + 27 ml ofAcrylamide/NN Methylene bisacrylamide (37.5:1) + 71 ml of Sterile distilled water

13) 10 % APS

   0.1 g of ammonium persulphate in 1 mL of distilled water.

14) Gel loading and running solution

   1L 1X TAE

15) Silver Staining solution

15.1) Fixative solution:- 2.5 mL Glacial acetic acid + 50 mL ethanol + 447.5 mL sterile distilled water

15.2) stain solution :- 0.3 g silver nitrate powder in 300 mL fixative solution

15.3) Developing solution:- 2.7 mL formaldehyde + 197.3 mL sterile distilled water + 6 g NaOH

16) Gel loading buffer

Bromophenol blue + Xylene cyanol to final concentration of 0.05% in 10 mL of 70% glycerol.