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Forensic Extraction And Microscopy Techniques For Diatom Analysis – Review

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ABSTRACT

Diatoms are a type of eukaryotic algae that are present in nearly all aquatic environments. They have a small size due to which they can enter the internal organs (brain, spleen, and bone marrow) like an individual with water. Thus, diatoms have forensic significance in the cases of drowning death as these help in revealing the information about the cause of death. For this purpose, diatoms have been extracted from both reference water and a biological sample and examined under a microscope to observe their morphological features. To increase the accuracy of the results, advanced techniques for accurate examination of diatoms have been developed. Furthermore, diatoms from samples have been identified and classified using artificial intelligence methods like convolutional neural networks and DNA barcoding, which can assist in determining the approximate geographical location of crime and cause of death. This paper offers a comprehensive overview of all methods developed for the forensic analysis of diatoms.

Keywords: Diatoms; Forensic significance; Drowning death; Microscopic examination; Artificial intelligence; DNA barcoding

1. INTRODUCTION

Diatoms are non-motile unicellular algae found in almost every moist environment. There are around 100,000-200,000 species of diatoms which are present in different shapes like elliptical, oval, circular etc. They are also present in filamentous (branched or unbranched) forms in colonies. In some cases, they appear as small seaweeds due to mucus or a gelatinous layer around them [1], [2], [3]. The cell wall of diatoms is made up of silica and cellulose and is called frustule. The shape of this frustule plays an important role in species identification as it generally appears as a soapbox in which two overlapping layers are present. The upper layer of it is called epitheca and the lower one is called hypotheca [4]. The presence of silica in the cell wall makes it inert and indestructible, which results in the accumulation of cell walls left behind by diatoms to form diatomaceous earth. This finds significant industrial applications, such as filtration, abrasive cleaning, and insecticides [5]. They also play a vital role in carbon dioxide fixation [6]. In addition to this, diatoms have applications in fields like nanotechnology, medicine, forensics, and environmental science [7].

Diatoms belong to the class Bacillariophyta and kingdom Protista. Diatoms are categorized into two groups: centric and pennate diatoms, depending on their structural traits. Centric diatoms are symmetrical radially and are not motile. The majority of these are found in coastal water bodies. On the other hand, pennate diatoms exist in freshwater environments and exhibit bilateral symmetry. They have a structure called a raphe that allows limited movement [8], [9], [10]. Diatoms aren't naturally found in the human body; they are only discovered in the corpses of the drowned and dead. In the drowning case, these are therefore used as traces of evidence [11]. The wide diversity of diatom species is specific to different geographical locations as the shape and structure of frustules vary according to environmental factors (like water quality, temperature, pollution etc). This helps to narrow down the investigations to that particular area [12]. Diatoms enter the human body through different routes but in case of death after drowning, inhalation is the main route for entry. After entering the body, they spread to different visceral

organs [13]. This facilitates the determination of the cause of death and the post-mortem time frame at the moment of death [14].

In 1861, the scientist guy observed that the drowning victim's body had been invaded by both artificial and natural watery detritus [15]. Hofmann concluded that diatoms were among the debris that had entered the victim's body after finding them in the lung fluid of one drowning victim in 1896 [16]. Following the discovery of diatoms in the victims' bodily fluids and visceral organs, Incze confirmed that the diatoms enter the victim's lungs and travel to other organs [17]. In 1949, Tamaska expanded on Incze's research by identifying diatoms in the drowned individual's bone marrow cells [18]. In 1966, Porawski went on to say that the existence of diatoms can indicate the cause of death. Based on his findings, the identification of whether drowning occurred before or after the death can be determined by looking for diatoms in another organ [19].

In 1972, Timerman provided clarification by describing what happened to diatoms within the body. He said that water and diatoms are breathed in and get into the lungs of the drowning victim. A persistent flow of water puts pressure on the lungs, causing alveoli to break. Diatoms and water are transferred to other organs by the circulation of the lungs. The tiny size of diatoms (20–200µm) permits them to infiltrate and accumulate in the organs that are viscerally situated. Since the diatoms lodge inside the organs, this type of drowning is referred to as diatom-associated drowning (dad) [20]. Additional research on several organs, including the brain, femur, kidney, stomach, liver, lungs, and sternum, was conducted in 1993, 1997, and 2000. These studies led to the conclusion that diatom functions as evidence to unravel the enigma of drowning [21]. In 1999, researchers such as Sidari and ludes established certain standards for verifying the reliability and appropriateness of the diatom analysis. According to their suggestion, at least 20 diatoms per 100 pellets must be present in the 10 gm of sample material taken from the drowning victim's body to consider it as death due to drowning [22], [23]. Krastic (2002) concluded that in after-death drowning cases, diatoms are identified in the lungs in greater concentrations than in distant organs. Ultimately, the qualitative and quantitative analysis of diatoms is critical in cases of submerged deaths [24].

2. EXTRACTION OF DIATOMS

In suspected drowning cases, quantitative and qualitative analysis of diatoms is done with the samples from the deceased person's body. The post-mortem biological samples collected are lungs, bone marrow, heart, liver, long bones, and blood. These samples are stored using preservatives to use for long-term examination. A water sample is also collected from the crime scene to perform a comparative analysis of diatoms. This water sample is preserved using Lugol's iodine solution or ethanol [25]. The procedures for these biological samples are as follows:

2.1 ACID DIGESTION

It's one of the oldest and global techniques used to extract diatoms from samples. This method treats the sample with strong acids (like h_2so_4, hno_3, and h_2o_2) for at least 24 hours. This process of breaking down the organic material in the sample leads to a detailed examination of frustules morphology. After that, the residue goes through a centrifuge for 15 minutes at 3000 rpm. The deposited particle is not disturbed during the removal of the supernatant. This mixture is then washed with distilled water and ethanol three times each. The pellet then collected from the bottom of the centrifuge tube is transferred to slide. This slide is observed under an oil immersion microscope of 10x, 40x and 100x magnification [26], [27], [28].

The biggest limitation of this experiment is that prolonged treatment of strong acids on the sample can lead to the destruction of valves of diatoms which can show false results [29], [30].

2.2 ENZYMATIC METHOD

This technique is based on the application of the proteinase-k enzyme along with Tris-HCL buffer. The collected postmortem sample is mixed with the 500 ml proteinase (10mg/ml) and 100 ml Tris-HCL buffer (0.01m) and incubated at 50°C overnight. Then this solution is diluted with 100 ml distilled water and centrifuged at 3000 rpm for 15 minutes. This allows the sedimentation of diatoms on the base. After this supernatant is removed and suspended pellets are transferred to the slide. This slide is observed under a light microscope. This method was found to be more rapid and effective than digestion but not cost-effective [31], [32].

2.3 FILTER MEMBRANE METHOD

This method was well suited for the extraction of diatoms from blood. In this method, a nitrocellulose membrane with a pore size of 5µm is used for filtration. A sample solution of 5ml blood in 10ml of 5 % sodium dodecyl sulphate (SDS) is prepared. This is filtered through the membrane of 47mm diameter. The filter is changed when

its pores start getting clogged due to the clogging of blood.

Following full filtering, 10 millilitres of boiling nitric acid is utilized to digest these filters for around ten minutes. The end product is subsequently diluted up to fifteen times with purified water and processed through a cell membrane with a diameter of 25 mm. This filtrate is then dried and examined under a microscope. When extracting from soft tissue, the solution obtained from the digestion of acid is mixed with 150 millilitres of purified water and filtered through a 47 mm-diameter membrane. Petroleum ether or isopropyl alcohol is used to break down the remaining fatty acid residue on the filter membrane [33], [34].

2.4 SOLUENE -350

In this method, soulene-350, NCS and PROTOSOL are used as solubilizers to digest the organic parts of tissue from the sample. After this ultrasonic irradiation is done on the sample for 300 min to accelerate the digestion of organic components. The resultant solution id centrifuged at 1800 rpm for 10 min. The precipitate collected from this process is then suspended in soulene-350 for 2 hours.

After this, the sample collected is centrifuged at 3000rpm for 5 minutes and the residue is observed under the microscope [35].

2.5 MICROWAVE DIGESTION

It is an effective, safe and novel extraction method used for the extraction of diatoms from postmortem samples. In this method, the sample is treated in the microwave after adding 6 ml of hno_3 and 2 ml of h_2o_2 in it. This treatment is done for about 5-10 minutes. After this, the sample is processed in vacuum filtration and then observed in SEM [36], [37].

2.6 COLLOIDAL SILICA GRADIENT CENTRIFUGATION METHOD

This technique homogenises soft tissues using Percoll solvents. Using a vortex mixer, 9 millilitres of Percoll solvent (colloidal silica particles, Pharmacia fine chemicals) and 2 millilitres of homogenate are combined. This solution is centrifuged in a refrigerator-cooled centrifuge set at 17,000 rpm for 60 minutes at 12°c. Once the supernatant has been removed, the solution is centrifuged once more for five minutes at 3000 rpm after being mixed with distilled water. Under a microscope, the pellets suspended at the base are gathered and examined [38].

3. MICROSCOPY TECHNIQUES FOR OBSERVATION OF DIATOMS

The permanent slide of diatom is observed under different types of microscopes for qualitative, quantitative, and morphological study. Some of these techniques are mentioned as:

3.1 LIGHT MICROSCOPY

This is one of the traditional methods which is based on the principle of optimising magnification, resolution and contrast. The compound light microscope with an oil immersion lens of 100X magnification is used to observe permanent diatom slides [39]. This microscope shows the morphological features (like shape, size, striae etc) of diatom. The use of a phase contrast condenser is recommended for the observation of minute morphological features [40].

The digital image is taken by using Zen Software with AxioCam 350 colour camera for further identification and study of these diatoms. The major drawback of this method is diffraction due to which fine details of diatoms are not visible [41].

3.2 TRANSMISSION ELECTRON MICROSCOPY

The better resolution of images is obtained by using Transmission electron microscopy. This technique provides two-dimensional images of diatom interior cell organelles (like sequence of protein molecules, cytoskeleton filaments etc). and outer frustules [42].

The basic principle of this method is that an enlarged fluorescent image is obtained when the electrons of the transmission beam interact with the atomic particles of the sample. The whole procedure takes place in a vacuum chamber as air molecules hinder the results. The sample preparation is done by using the traditional acid digestion method to remove all impurities [43].

3.3 SCANNING ELECTRON MICROSCOPY

The fine details of diatom frustules that were not visible by TEM and Optical microscopy, can be seen using SEM. It provides a three-dimensional view of the cell wall which helps in the identification of diatom species [42].

The basic principle of scanning electron microscopy is that the electron beam is projected to the surface of the sample which excites the atomic electrons of the sample. These atomic electrons scatter as secondary electrons, which interact with back-scattering electrons to form the crystalline image of the sample [44], [45].

The main drawback of this method is that the presence of organic and inorganic impurities hinders the results. Thus, different types of solvents (like H_2SO_4, and $KMNO_4$) were used for sample preparation. However, the treatment of these harsh chemicals damages the diatom cell. Therefore, in 2014 Jiang W et al. used ethanol as the main reagent. The sample obtained is mounted with dye and then dried at 90°C for the removal of ethanol. Aluminium foil is used instead of slide as it can carry large samples, is easily foldable and does not prevent the accumulation of static energy in the sample. Some other drawbacks of this technique are that it is highly expensive, requires vacuum conditioning, and cannot be used for analysis of live diatom cells [45], [46], [47],[77].

3.4 ATOMIC FORCE MICROSCOPY

This technique is considered more favourable than scanning electron microscopy as it allows the imaging of live and processed cell samples. This technique can be used in all environmental conditions which makes it more affordable and easier to use [48].

This method falls under the category of scanning probe microscopy. It involves moving a physical probe or cantilever with a sharp tip over the surface of a sample cell to create an image of the sample's morphology and height. The variations in frequency that the probe records are used to create these images. The resultant image has amplitude or height changes in addition to all the minute pored information [49], [50].

When it comes to treated samples, weak acids are preferred over strong ones because they don't break down the cell wall. Sample preparation is not done for live samples. This technique's main disadvantage is that it ages the probe through friction from frequent scanning use. Due to the several procedures involved, like contact and non-contact scanning, this process becomes time-consuming [49], [51], [52].

3.5 DIGITAL HOLOGRAPHIC MICROSCOPY

It is the method in which a laser beam is projected on the sample. This beam then divides into a sample beam having wavefronts that interfere with the reference beam to form a hologram. This holographic image is recorded as a numerical algorithm by a computer digital lens to form an image. This image contains minute structure details along with phase information. This allows us to understand the different physical properties of diatom. This technique helps in the qualitative analysis of diatoms by providing a 3-dimensional image of the surface and internal structure of diatoms [53], [54].

3.6 FLUORESCENT MICROSCOPY

Fluorescence microscopy is the method by which a substance or species is identified based on the colour it emits when exposed to electromagnetic radiation. This fluorescence of colour is observed because molecules absorb energy from photons of different wavelengths [55].

Diatoms are phytoplankton having fucoxanthin carotenoid and chlorophyll a & c. The electrons of these chemical pigments absorb photons of different wavelengths and get excited to higher energy levels. When these electrons return to the ground state a certain wavelength and colour of light is emitted which is a significant identification feature of diatoms [56], [57]. This technique along with digital holographic microscopy is used for better imaging of diatoms [58].

3.7 SLIDE SCANNING METHOD

It is the method used to develop the 3-dimensional images of diatoms. In this method total of 61 images (overlapping and individual images) of a slide are taken from different angles or depths. This is done by using a slice scanner (like BUM950A OR VS200) having an oil immersion objective lens. Later with the help of software (like Zerene

Stacker and Helicon Focus), all these images are compiled together to form a virtual 3-D image. This is called virtual slide scanning. This forms the basis of automated diatom identification and classification projects [59].

4. ARTIFICIAL INTELLIGENCE

In 1998, the automatic diatom classification and identification project was introduced in the hope of combining image processing and identification tools for the digital identification of diatoms. This approach required the imaging database and artificial intelligence for an automated diatom examination [60]. The identification of the diatom taxon was done manually after its imaging by studying morphological characteristics. However, due to the wide range of diatom species, this process was very time-consuming and there were a lot of human errors which questioned the credibility of the result in court. Thus, Artificial intelligence was introduced in this field with this project [61], [62].

Artificial intelligence can go through large numbers of data within a few seconds and the error rate with AI is negligible. This project emphasises the morphological identification of diatom species from the images obtained using different microscopy techniques with the help of artificial intelligence. The main focus in artificial intelligence was on deep learning which allows the quick classification and processing of large sets of data. In deep learning, the main emphasis was on the Convolutional Neural Networks as it allows the classification of species on the image itself. This model processes the pixels of the image and studies the frustule patterns and based on that identifies the cell [63], [64].

The steps taken for developing CNN software for the classification of the diatom images:

A. DIATOM DATABASE CREATION: The process involves the collection of all the images of diatoms that have been recorded using any microscopy mentioned above techniques in a database. The resolution of these images is adjusted along with proper labelling of these images. The details about the species in the image are also mentioned in the database for better classification of diatoms [64], [65],[76].

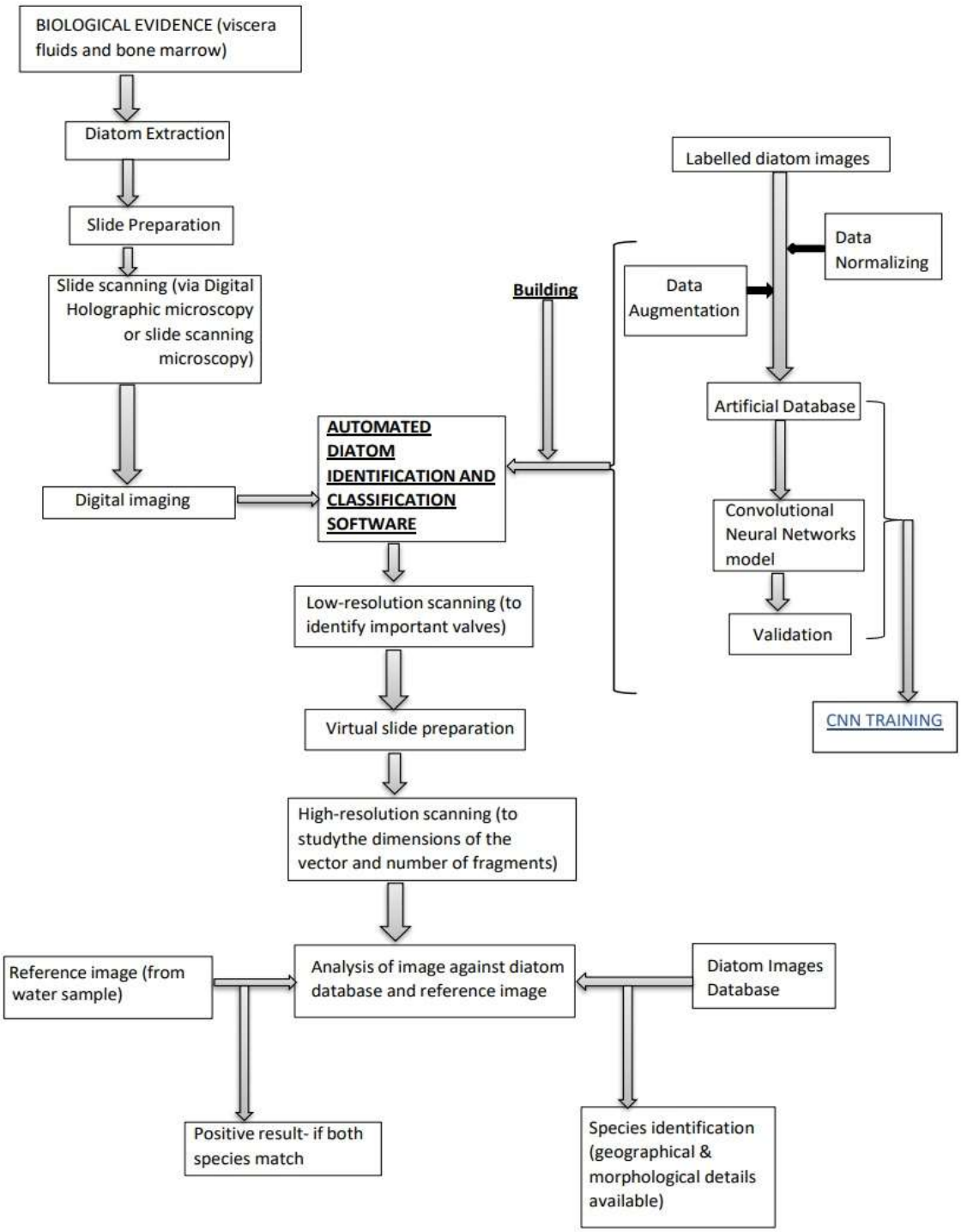

Fig 1. Schematic overview of working and manufacturing of automated diatom identification and classification software.

B. ARTIFICIAL DATABASE CREATION: An artificial database of images like the original database is created to feed the central neural system for training.

a. **Data augmentation**: The images from the original database are changed slightly by adjusting their colour, brightness, or angle to produce a large number of images.

b. Data normalising: The image quality of newly generated images is adjusted as per the original reference image. The properties like contrast or brightness are adjusted for precise training of the system [65].

C. TRAINING OF MODEL: The training of the CNN model is done by using the transfer learning framework. This means that the knowledge system will receive from the training process will

be used for the classification of species. In the training process, the images of the artificial database are fed to the model in forward propagation and all the results for those images are fed in backward propagation. This allows the model to understand the properties that have to be taken into consideration (like dimensions of vector, number of valves and fragments) for providing results [65], [66].

D. VALIDATION: this is the last step done for determining the accuracy of the model. In the beginning, a set of images from the database were kept aside for performing the validation test. In this test, only the output images are fed into the model and the result is observed. The frequency of positive results determines the accuracy of the model [67].

The working example of this model is SHERPA which works with slide scanning microscopy technique. Despite having multiple benefits, the major drawback of this approach is that the CNN model requires a large number of databases for training purposes and the present imaging database of diatoms is not developed yet. Thus, continuous research on diatoms is required to completely develop a diatom database.

5. DNA BARCODING:

Diatoms are small-sized phytoplankton having almost 200,000 species. These species have silica cell walls or frustules having minute differences which form the basis of species identification and classification based on morphological characteristics. However, the lack of advanced quick and simple extraction and imaging methods makes morphological identification less preferable. Thus, the application of microbiology is introduced in this field as DNA Barcoding. It is the process in which a specific gene locus or short DNA strand is used as a marker for species identification and classification.

The sequence of this genome is compared with the DNA database data to identify the taxon of the sample diatom. This comparative study of these genome sequences also helps in the identification of new species by analysing the similarities and dissimilarities in these markers [68], [69], [70].

The DNA barcoding of an unknown sample is done by following the steps:

A. DNA EXTRACTION: The organic content of the sample collected from the environment is digested using the acid digestion method. After this, the cells are lysed using an extraction buffer which separates the DNA. The concentration and quality of this DNA is checked using Agarose gel electrophoresis [71], [72].

B. AMPLIFICATION: The favourable strand of DNA is amplified using Polymerase Chain Reaction. The locus like 18s, 16s, *rbc*L and SSU are amplified using primers as these loci are proven to form the basis of species identification [72].

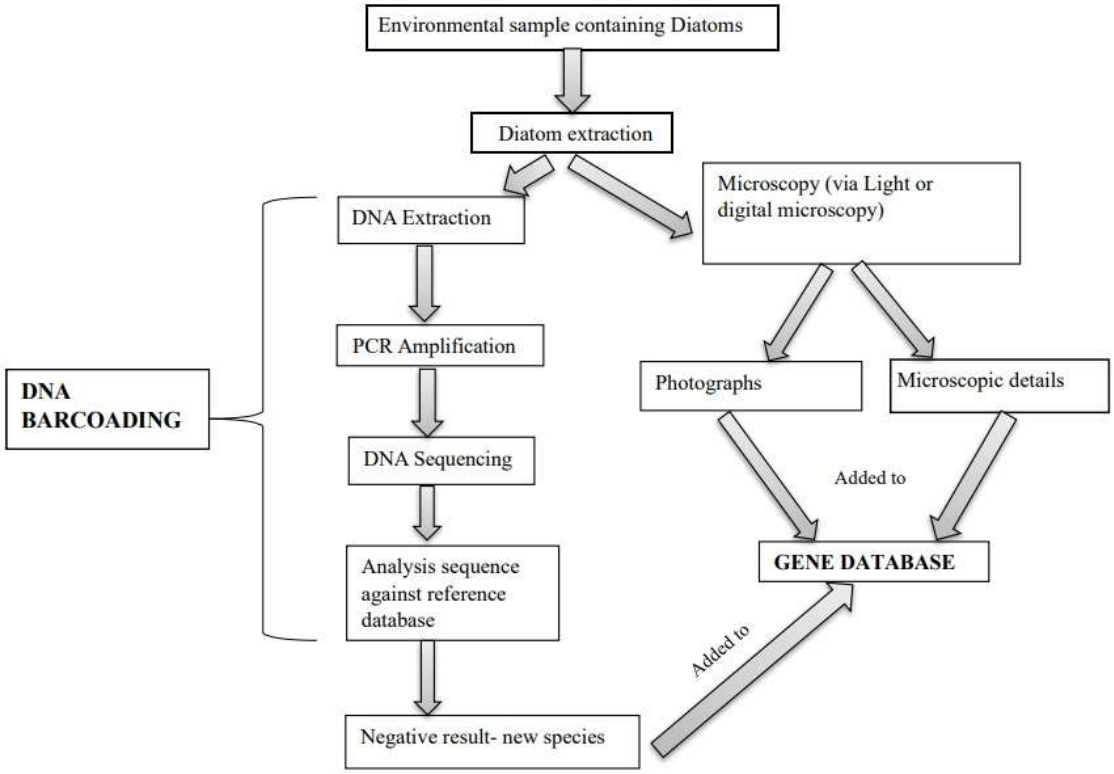

Fig 2. Schematic overview of DNA Barcoding

C. CLONING and SEQUENCING: The environment samples contain many species so to prevent contamination, cloning of amplified samples is done in the bacteria called E. Coli. This separates the targeted genome whose further sequencing is done without hindrance [72].

D. ANALYSING: The last step is analysing the genome of the environmental sample along with the database genome to determine the species of diatom [72].

The most common databases used for the comparative analysis are SILVIA, International Nucleotide Sequence Database Collaboration (INSDC) or GenBank. The data of genetic sequence along with the morphological features, images and geographical location is provided with the species in these databases. This plays an important role in the identification of the geographical location and nature of drowning death [73].

Despite being a quick and precise method, the major limitations of this process are:

A) The steps of DNA barcoding are not completely accurate as the extraction procedure used harms the cell DNA, while the genome amplifies does not provide information for some species. Thus, the specific genome which can be used in cases of all species is not yet determined.

B) There are almost 200,000 species of diatom and only 20,000-30,000 species information is present in gene databases. Thus, continuous research has to be done to maintain the high standard database for species identification [73], [74],[75].

6. CONCLUSION:

Diatoms are minute, stagnant microalgae with a silica-based hard outer membrane. Diatoms exist in a variety of sizes and forms, with over 10,000 species identified. Since drowning victims keep diatoms along with other microscopic water-borne organisms within their bodies, these aid in the identification of drowning deaths.

Using the previously described techniques, the viscera samples can be extracted and examined to analyse diatoms and establish drowning. When more than 20 diatoms per 100 µl of pellet are discovered from the sample, the analysis is considered positive. Deep learning has been introduced in this field via Automated Diatom Identification and Classification projects. This research produced SHERPA, a functional piece of software that can quickly identify a species and is a major asset in forensics investigations. Furthermore, DNA barcoding is a method that molecular science has developed for diatom species identification by genes. It verifies the species and helps us determine the crime's location. The underdevelopment of gene data and diatom images is currently the only prohibition in this sector. This limitation can be addressed with ongoing research.

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