

# Chapter 9

## Role of Diatoms in Forensics: A Molecular Approach



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**Abstract** Diatoms are the small, autotrophic, eukaryotic organisms found abundantly everywhere in nature. They are popularly called jewels of the sea, due to the beautiful ornamentations present on their frustule wall. They are regarded as golden standards in solving drowning-related crimes in the area of forensic science. Diatoms act as a supportive tool in deciphering the case investigations, due to the fact that their small size and diverse nature in the environment make their entry inside the body of drowned victim easy. While conducting autopsy of a drowned victim, the presence of diatoms in the lungs and other distant organs like the brain, liver, and kidneys as well as in femur reflects light on the cause of death. The presence of diatoms in various tissues of drowned victim reveals that the case is of antemortem drowning, whereas, in postmortem immersion cases, negligible number of diatoms will be present in the body of the victim. Various methods have been proposed by many scientists for the digestion of inorganic and organic material of diatoms for their identification purpose. However, with the advancement in research, molecular approach became more reliable and specific in the world of forensic limnology. In this chapter, we have discussed the efficiency of molecular tool; this approach helps in the making of database which in future will help the taxonomists to preserve the species and generate a record for study. Diatoms with the help of DNA barcoding can successfully help in solving drowning-related crimes with more accuracy and low chance of contamination.

**Keywords** Diatoms · Antemortem drowning · Postmortem immersion · Forensic limnology · Molecular approach

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## 9.1 Introduction

Diatoms (class Bacillariophyceae) are unicellular, photosynthetic eukaryotic microscopic algae widely present in every aquatic habitat. They are universally found in almost every moist substrate including soil, rocks, and aquatic plants either in the form of single cell or in colonies and sometimes as pseudo filaments (Bhardwaj et al. 2021). The colonies of the diatom cells are linked together with the help of mucus pads. In some circumstances, the mucus covers the entire diatom cell which gives them appearance of small seaweeds (Hendey 1973). Ehrenberg was the first scientist to study about diatoms in India (Gandhi 1957). Diatom cell is composed of silica ( $\text{SiO}_2$ ) cell wall popularly known as frustule which has soap box-like appearance. They are comprised of chlorophyll a,  $c_1$ , and  $c_2$  along with the carotenoid called fucoxanthin (Lee 2018). This fucoxanthin is responsible for communicating golden yellow color to the diatom cell; thus, they are also popularly known as golden brown algae. The presence of hard siliceous frustule in a diatom cell is the key identification feature of the class Bacillariophyceae. It comprises of beautiful geometric ornamentations which vary from cell to cell, thus helping in distinguishing particular type of diatom taxa. This ornamented frustule of the microalgae is not as stiff as calcite; rather, it is slightly flexible due to which they can undergo slight amount of deformation. The silica cell wall of diatoms has certain types of nanostructural impregnations such as areola, spikes, pores, stigma, and channels imparting unique patterns to the cellular surface (Almqvist et al. 2001).

Diatoms are capable of growing in various types of habitats such as freshwater, saltwater, terrestrial, damp places, ice, moist soil, etc. Generally, they are present as free-floating microscopic bodies in aquatic habitat, but some of them are attached to moist substrates like rocks with the help of stalks (Karthick et al. 2013). These microscopic algal species play huge role in the detection of water quality and paleoenvironmental reconstruction as their growth is very specific to certain parameters of their habitat such as pH, TDS, conductivity, temperature, light period, and other required nutrient availability. Studies conducted on diatom assemblages along with their species and relative abundance helps to denote the quality of water making them as an important indicator of environmental health (Round 1981; Reynolds 2006). They are regarded as sensitive agents toward organic matter, toxicants, and heavy metal contamination present in water bodies (Blanco and Bécares 2010; Morin et al. 2016). Due to this supreme quality, they are highly recommended as biomonitoring tool for the assessment of aquatic water bodies (Stevenson et al. 2012).

They show very close association with heterotrophic bacteria which mainly includes Proteobacteria, Bacteroidetes, and Actinobacteria (Gautam et al. 2017). Whenever a diatom cell dies, there frustule gets settled in the environment which is popularly termed as diatomaceous earth or diatomite (LeBeau and Robert 2003). There are more than 200,000 species of diatoms present in the environment making them diverse group of microorganisms (Saxena et al. 2022). Each diatom cell is composed of unique three-dimensional surface characteristic; there structure varies

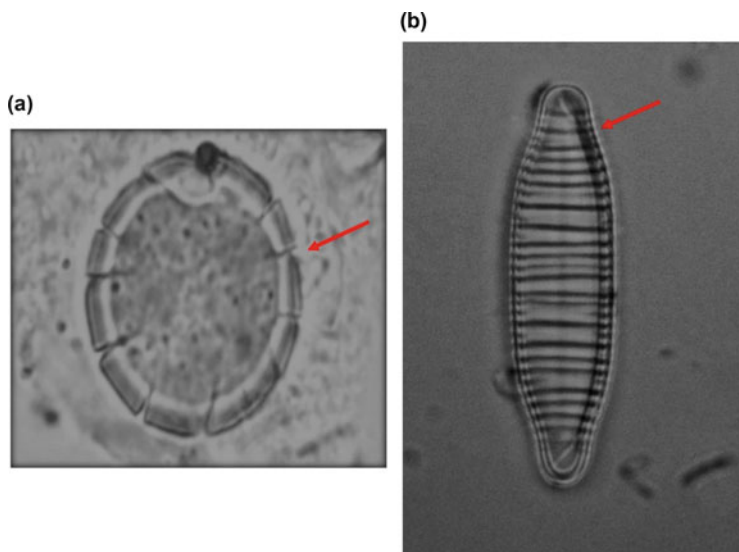
from rod-like, hexagonal, or circular as per the type of species (Amato 2010). Due to their diverse abundance, these microorganisms are responsible for 40% total marine primary productivity. Reproduction in diatoms occurs commonly by cell division; i.e., they undergo asexual reproduction. Cell division occurs mitotically along with transverse to the longitudinal axis of the individual cell. They are also capable of sexual reproduction by forming auxospores (Cupp 1943). Their population show increase in spring and autumn season as compared to winter and summer season (Trent 2004). The most common types of diatoms are pennate and centric. The centric diatoms are radially symmetrical, whereas pennate are elongated and bilaterally symmetrical in shape (Uthappa et al. 2018).

Diatoms play important role in global CO<sub>2</sub> fixation, making them suitable candidates in solving the problem of global warming (Aoyagi and Omokawa 1992; Chisti 2007). The outcome of the studies conducted by various researchers threw light on the relativity of diatom diversity residing in polar regions with severe cold condition in denoting global climatic changes undergoing in such regions (Bopp et al. 2005; Alvain et al. 2013). According to a report of geological survey, they are considered to produce 30% of gasoline which reflects their tendency for the production of biofuel also (Ramachandra et al. 2009). Their average tendency to form lipids in diatoms is more than any other microalgal species such as cyanobacteria, Ochrophyta, Chlorophyta, and other classes (Griffiths and Harrison 2009). According to the study conducted by Das et al. (2015), these microorganisms also serve as an important alternative source of aquaculture water remediation. Moreover, these microlagal species have been center of attraction for research in various fields of producing various forms of sustainable products like fine chemicals, medicinal drugs, biofuels, plastic, and food products (Lebeau and Robert 2003; Bozarth et al. 2009).

Apart from all the applications of diatoms in several fields like biotechnology, industrial, cosmetology, and medicinal, they have also shown their importance in solving drowning-related crimes in forensics. A branch named forensic limnology deals with deciphering of drowning investigations with the help of microalgae, i.e., diatoms. Their small size, siliceous cell wall, and wide abundance in and around waterbodies make them suitable evidence in forensic pathology (Pal et al. 2017).

## 9.2 Framework of Diatom Cell

Individual diatom cell has soap box-like structure; as discussed above, the outer wall is made up of silica known as frustule. This hard wall is resistant to environmental and chemical changes undergoing around it. The frustule has upper part and lower part called epitheca (epivalve/upper valve/mantle) and hypotheca (hypovalve/lower valve), respectively. These valves are interconnected with each other with the help of connecting bands called girdle bands. The upper valve is slightly larger than the lower, making their cell structure cell look like a box and lid. The frustule of diatom cell has beautiful three-dimensional patterns on it which are useful for the



**Fig. 9.1** Types of diatoms on the basis of their symmetry: (a) centric diatom, (b) pennate diatom

classification and identification of a particular diatom species (McLaughlin 2012). The process of biogenesis of both the valves and girdle bands are associated with the cell cycle. The valves are fabricated at the time of cell division, whereas the girdle bands are produced at the time of interphase. Both the valve and girdle bands are produced separately in a compartment known as silica deposition valves (SDV). Once their formation is complete, they get secreted out the cell through exocytosis and get accumulated on the cell surface at their respective positions (Heintze et al. 2020). On the basis of their symmetry, they are further classified as centric and pennate diatoms (Fig. 9.1). Pennate diatoms are bilateral in symmetry and have central spine-like structure called raphe which helps in their slight locomotion. Those pennate diatoms which contain raphe are termed as raphid diatoms. However, centric diatoms are radially symmetrical and lack the feature of raphe. Therefore, centric diatoms along with some pennate diatoms without raphe are called araphid diatoms (Williams and Kociolek 2007; Bhardwaj et al. 2021). According to reports, centric diatoms are abundantly found in marine water (Harwood and Nikolaev 1995).

### 9.3 Diatoms in Forensic

In the fields of forensic science, drowning-related crimes are very common and hard to solve. Forensic pathologist from the past had suffered a lot of problems as there are less or no relevant evidence to decipher drowning mysteries. Drowning is

defined as respiratory failure of a person who accidentally or forcibly gets submerged in liquid medium, commonly water. According to the recent reports of World Health Organization (2021), drowning is considered as third primary cause of deaths worldwide. Globally, about 236,000 deaths are occurring annually due to drowning. The diagnosis of cause and manner of death in such cases become difficult due to lack of evidence, or sometimes, due to prolonged overstay in water, the body gets putrefied. Forensic pathologists have to struggle in detecting the mode of death of victims recovered from water body. Experts in this field have to unravel all the possible circumstances to know the manner of death whether the case is of accidental or suicidal drowning or the body was dumped in water after homicide. It is not necessary that drowning only occurs in deep water bodies, but it can be possible in 5 cm–6 cm of fluid also. However, in such circumstances, other factors, such as narcotic influence, alcohol intoxication, epilepsy, head injury, cardiac arrest, etc., have to be considered carefully (Krstic et al. 2002). A special branch called forensic limnology has gained attention which comprises of application of microalgae especially diatoms in solving crimes related to drowning (Piette and Els 2006; Farrugia and Ludes 2011; Munro and Munro 2013). Diatom test is based on the principle that when a person is drowned in water body, he or she has the urge to respire; during this time of struggle, diatoms along with some other debris present in aquatic media enter inside the body of victims through body openings. The diatoms reach the lungs through respiration and get transported to several other organs through systemic circulation. These diatoms get penetrated in various organs such as the lungs, heart, spleen, kidneys, etc. However, their presence has been detected in distant organs like the brain and femur as well as in bone marrows. However, in cases where the victim was in an already dead condition and somehow his body was recovered from the water, the body will have a negligible number of diatoms. This implies the cause and site of death other than drowning and thus helps in deciphering the mystery (Krstic et al. 2002). The hard wall of diatom is resistant to the changing climatic conditions and chemical resistant and also remains intact in case of putrefaction. This makes them suitable candidates for corroborative evidence in drowning cases.

## 9.4 History of Diatoms in Forensic Science

Guy (1861) was the first scientist who elucidated about the entrance of aquatic debris inside the body of a drowned victim. In 1896, Hofmann was one of the scientists to detect diatoms from the lung fluid. Revenstorff (1904) solved the drowning mystery by using diatoms as corroborative evidence. Incze (1942) concluded the presence of diatoms in blood and parenchymatous organs and denoted that these microorganisms can enter travel to distant organs via the lungs. Thereafter, Tamaska (1949) showed the existence of diatoms in the bone marrow of the drowned victims as a sign of sure shot drowning. Thomas et al. (1961) suggested that diatoms act as reliable evidence to detect that victim has undergone a couple of breaths during submersion. Porawski (1966) denoted that these small microalgal species if present in the body organs or

bone marrow of drowned victim reflect the cause of death to be antemortem drowning. Timperman (1972) described the fate of diatoms inside the body of victim in a way that, whenever a person drowns inside the water body, water along with its contents including diatoms enters inside the lungs. Once the lung cavity gets congested with the water media, it starts imparting pressure on the alveolar walls. This leads to rupturing of peripheral alveoli and thus water and other contents pass into blood stream. Diatoms are one of the main components of water which due to their high abundance and small size enters and gets settled in distant organ till the person stops respiring. Those diatoms which are able to creep into the organs of drowned victims are called diatom-associated drowning (DAD). Numerous scientists conducted several experiments on various organs such as the lungs, liver, sternum, femur, kidney, stomach, and brain (Matsumoto and Fukui 1993; Pachar and Cameron 1993; Taylor, 1994; Pollanen et al. 1997; Ludes et al. 1999; Hürlimann et al. 2000), and their results supported the utility of diatoms in solving such cases. A criterion of concordance has been set for the applicability and validity of diatom test. This rule states that a significant number of diatoms must be present while performing diatom test before coming to any final conclusion (Pollanen 1998a, 1998b). Sidari et al. (1999) and Ludes et al. (1999) proposed that either 20 diatoms or 5 complete diatom frustules must be present per 100 l of pellet recovered from 10 g of sample from the body of drowned victim. Moreover, in case of diatoms present in the body of non-drowned victim or in post-mortem immersion cases, their quantity may be detected in the lungs but not in distant organs (Krstic et al. 2002; Kakizaki et al. 2018; Lunetta et al. 2013; Bortolotti et al. 2011). Overall, the quantitative (diatom density), qualitative (species), and morphological study of diatoms is a useful tool for making them supporting tool in drowning cases.

## 9.5 Why Only Diatoms as Supportive Evidence in Forensics

Earth's surface is full of microorganisms, but, apart from all of them, only diatoms are considered best supportive evidence by forensic experts in deciphering drowning cases. This is due to the major following reasons:

- There are diverse group of species with small size range which makes their entry inside the body organs feasible.
- The hard silica cell wall is resistant to the chemical changes while performing test in laboratory which helps in recovering of intact structure of diatoms from the respective body organ of drowned victim.
- In case of putrefied bodies or sometimes due to prolonged overstay in the water, many organs get blended, and only the bones are left behind. In such circumstances also, diatoms can be recovered and help in solving the case mystery.
- Their growth corresponds to certain specific parameters of the environment. Thus, by qualitative and quantitative analysis of diatoms from the body of

drowned victim and putative drowning site, the cause and site of death can be concluded successfully.

- A wide range of study has been done on the taxonomy of diatoms due to which these species are easy to identify and study.

## 9.6 Recovery of Diatoms from Postmortem Samples

While solving drowning cases, samples from the body of the deceased, most commonly soft tissues such as lungs, liver, heart, etc. or hard bones like femur and sternum, are sent for diatom test. These biological samples are analyzed in the laboratory, and various tests are performed for the recovery of the diatoms. For denoting the site of crime, water sample from the suspected drowning media is also used as reference material for the comparative study of diatoms. Several methodologies have been proposed for the efficient recovery of diatom frustule from the samples.

### 9.6.1 Acid/Chemical Digestion Method

The most common and oldest form of method is acid digestion or chemical digestion of samples. This method has been used globally and has proven successful in recovering intact diatom frustules from the samples (Singh et al., 2006). In this method, different strong chemicals, namely, nitric acid ( $\text{HNO}_3$ ), hydrochloric acid (HCl), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) or ( $\text{H}_2\text{SO}_4$ ) are used to digest the organic material present in the diatoms. This digestion helps in making the frustule view more elaborative for morphological study. Auer and Möttönen (1988) performed the study on 107 drowned victims. Thin strips of tissues, namely, lungs, liver, and kidney, along with the water sample were separated in a flask and allowed to boil by using distilled water. 10 ml of  $\text{HNO}_3$  with 30%  $\text{H}_2\text{O}_2$  was added to the samples, and the sample acid mixture was boiled carefully. Once the boiling was completed, this mixture was allowed to cool and washed with distilled water after frequent centrifugations at 3000 rpm. The final sediment was then analyzed microscopically. Ludes et al. (1994) conducted acid digestion test on 12 dead bodies in putrefied state. The putative drowning water sample and other soft tissues including lungs, liver, and kidneys were treated with  $\text{HNO}_3$ . This sample acid mixture was centrifuged at 2000–2500 rpm. After the completion of subsequent washings, the endmost residue was then used for microscopic observation. Pollanen (1998a, 1998b) solved cases of six homicidal drowning. The chemical digestion was performed by using  $\text{HNO}_3$  (50 ml) on the bone marrow and femur (50 g) of the drowned victims in a clean flask. This mixture was then boiled for 48 h and then kept undisturbed for cooling. Again, washing of this mixture was initiated in Oporto River with the help of repeated centrifugations by using distilled water. The final pellet was then observed under

phase contrast microscope. Gruspier and Pollanen (2000) solved the case mystery of five amputated legs recovered from the Lake Ontario, Lake Erie, and Niagara River. The digestion method was applied in the same manner as discussed above (Pollanen 1998a, 1998b). Krstic et al. (2002) executed chemical digestion by using  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{SO}_4$  along with saturated solution of permanganate on the tissue samples of laboratory rats, drowned corpse, and control samples to analyze the validity and utility of diatom test. Ago et al. (2011) studied nine cases of victims died in bathwater and one due to ischemic heart diseases in bathwater. The samples recovered from the body if the deceased was subjected to 10% formalin diluted with distilled water and kept undisturbed for few days. Thin sectioning of the tissues was done and washed with distilled water. Again, the samples were digested with  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$ , and qualitative and quantitative analyses of the diatoms recovered were analyzed under the microscope. Magrey and Raj (2014) suggested acid digestion as most relevant method to remove inorganic or organic material present inside the diatom cell. They solved 31 drowning cases happened in Jammu and Kashmir, India. They treated biological samples (sternum, femur, clavicle, and lungs) along with the suspected water sample with  $\text{HNO}_3$  for extraction of diatoms. The cases were successfully solved with outcome of diatom test. Lin et al. (2014) performed acid digestion to solve 100 drowning cases. Diatoms were successfully recovered from the fluid of sphenoid sinus and lower lobe of the lungs. They used  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$ :  $\text{HCl}$  in the ratio of 1:3 for sphenoid fluid and lower lobe of lungs, respectively. Coelho et al. (2016) studied drowning deaths that happened in Oporto River from November 2012 to March 2014. The water sample was treated with 96% (W/W) of 20 ml of  $\text{H}_2\text{SO}_4$  and tissue samples with 37% (W/W) of 20 ml  $\text{HCl}$ . The last residue left after repeated washing and centrifugations were place on clean slide and observed under microscope. Apart from utilizing acid digestion technique in biological samples recovered from humans, this method also became popular in extracting diatoms in veterinary context. For instance, Xu et al. (2011) conducted chemical digestion with  $\text{HNO}_3$  on body organs (lungs, liver, kidneys, and bone marrow) of 56 rats. The experiment was performed by submerging rats in water to mimic drowning. The outcome of the test remained successful in denoting the manner and site of drowning by comparative study of diatoms recovered. Fucci et al. (2017) threw light on the recovery of diatoms from the biological organs of 10 different wildlife animals and putative drowning site. Also, with the advancement of research in forensic limnology, many modifications were made in this technique and its applications. Scott et al. (2019) conducted experiment to extract diatoms from nine different types of natural and synthetic clothing types. Wang et al. (2015) executed experiment on 20 minced kidneys which were thoroughly mixed with water-rich in diatoms. An improved version of acid digestion technique was suggested by Pal et al. (2021). They conducted a study on lungs and bone marrow extracted from the bodies of 66 victims died due to drowning. They ran a comparative test by using reverse aqua regia solution and traditional acid digestion method. In reverse aqua regia,  $\text{HNO}_3$ :  $\text{HCl}$  in the ratio of 3:1 was added to the biological samples, and the mixture was heated at 60–70 °C for 2 h, whereas in other method 50 ml  $\text{HNO}_3$  was poured in sample and allowed to heat for 48 h at 60–70 °C on a hot



plate. The comparative study of diatoms recovered by using both techniques showed reverse aqua regia to be more efficient. This technique proved time saving more conventional and efficient in high yield of diatoms with clearer and intact morphological features.

### **9.6.2 Limitations of Acid Digestion Technique**

Being one of the most adopted methodologies, there were various limitations for the application of chemical digestion. For instance, working with strong acids such as HCl, HNO<sub>3</sub>, and H<sub>2</sub>SO<sub>4</sub> can lead to injuries or burns which can cause health hazards. Prolonged overstay of samples in such strong acid during the experiment can lead to destruction of diatom valves and thus can lead to false investigation. Additionally, repeated centrifugations at high rpm can also lead to contamination and loss of diatoms (Bhardwaj et al. 2021). In spite of all these drawbacks, this chemical digestion process with slight modification can give successful results suggested by Pal et al. (2021). Also, the chance of contamination can be avoided by using double distilled water during the washing of acid sample mixture and using clean and sterilized required equipment (Pollanen 1998a, 1998b).

### **9.6.3 Enzymatic Methods**

Many scientists conducted extraction of diatoms by enzymatic methods for forensic consideration (Ludes et al. 1994; Kakizaki and Yukawa 2015; Kakizaki et al. 2018). Ludes et al. (1994) conducted diatom analysis on 12 corpses recovered from several water bodies of France. The various tissues retrieved from the body organs of the deceased victims were treated with 500 µl of proteinase K (10 mg/ml), 100 ml of 0.01M of Tris-HCl, buffer solution (pH 7.5), and 2% of SDS. This mixture was incubated at 50 °C and left undisturbed. To this sample mixture the volume of solution was diluted by using 100 ml with distilled water. Then, centrifugations were performed at 3000 rpm for 15 min. The final residue was analyzed microscopically. Kakizaki and Yukawa (2015) performed an experiment on 20 lung samples recovered from 10 drowned victims. The digestion of the sample was done by using Qiagen Proteinase K, Qiagen Buffer ATL, and 5 NHCl. The tissue sample was minced properly and placed in polymethylpentene centrifuge tubes which consists of 6 ml buffer ATL and 1 ml of Qiagen Proteinase K. To check cross-contamination, negative control of 1 ml ultrapure water sample was used. Digestion of the sample mixture was done at 56 °C for 15–16 min, and then the sample was allowed to centrifuge. The final residue left was mixed with 13 ml of 5NHCl and heated at 75 °C. Once again, washing of the sample residue was done after centrifugation. At last ethanol was added to the final recovered residue, and further diatom analysis was done. Kakizaki et al. (2018) recovered diatoms from the body of 80 corpses retrieved

from various aquatic sites. The enzyme used for this experiment was papain extracted from *Carica papaya*. The outcome of the digestion method was found to be efficient at 50 °C for 1 h at the concentration of 0.5 mg/ml. With the help of this enzymatic method, the authors were successful in solving drowning cases of 80 victims. Enzymatic method was found out to be better than chemical digestion, but its major drawback was that it was costly.

#### **9.6.4 Microwave Digestion, Vacuum Filtration, and Scanning Electron Microscope**

Hu et al. (2013) conducted a study by merging microwave digestion and vacuum filtration preceded by scanning electron microscopy. For the experiment, 20 ml water along with 2 g of thin strips of lungs, liver, and kidneys were digested in microwave MW000 with the addition of 6 ml of HNO<sub>3</sub> and 2 ml of H<sub>2</sub>O<sub>2</sub> to the samples. The sample acid mixture was allowed to liquefy by rising the power for 5–10 min. This fluid was further subjected to vacuum filtration, and the results were automatically scanned by SEM. As compared to previously discussed methods of digestion, this method proved more efficient, rapid, and safer and has low chance of contamination. Zhao et al. (2017) solved the case mysteries of 128 drowned victims with the help of this method. From the comparative study of diatoms recovered from tissue samples and suspected drowning site's media, the case was successfully solved. Also, their findings threw light on the fact that, in case of antemortem drowning cases, the possibility of having diatoms in the lungs is 100% whereas 97% in other organs.

#### **9.6.5 Soluene 350 Digestion**

Matsumoto and Fukui (1993) performed an experiment which mimics the case of drowning. They took 5 g of various tissue samples from the rats and mixed them thoroughly with the water enriched in diatoms. The sample was allowed to centrifuge at 3000 rpm for few minutes. On the completion of centrifugation process, the pellet of the residue formed was transferred to 30 ml glass tube followed by the addition of Soluene 350. This sample mixture solution was set down in ultrasonic cleaner with full exposure to ultrasonic waves. To this sample solution, centrifugation at 3000 rpm for 5 min was initiated. The outcomes of the experiment conducted were better from the earlier traditional methods. Yoshimura et al. (1995) conducted study on the two victims whose bodies were recovered from the Yodo River, Japan. Thin sectioning of lungs, liver, and kidneys from the body of victims were taken for diatom test along with the water sample from the abovementioned river. Centrifugation of the sample was done by using Milli-Q water in order to avoid chance of

contamination. The supernatant was discarded after the centrifugation was complete, and the pellet left behind was incubated after the addition of Soluene 350 into it. The incubation of the sample solution was kept undisturbed overnight. Further after the incubation of the sample was complete, it was again centrifuged for 60 min. The final residue was observed under microscope, and the analysis of the diatoms recovered helped to solve the caseworks of drowned victims. Sidari et al. (1999) reported that this technique works better on freshwater samples as compared to seawater due to the reason that the diatoms recovered from seawater has less amount of silica as compared to those recovered from freshwater sources.

## 9.7 Preparation of Diatom Slides

The diatoms recovered after the digestion process were used for the qualitative, quantitative, and morphological study. The residue left after final centrifugation was placed on clean glass slides. The permanent slide of the sample was made by mounting the material with high refractive index mountant such as Naphrax (~1.74), DPX (~1.520), or Euparal (~1.483), and then coverslip is placed over the sample, and the slide was allowed to dry in order to fix the sample. After fixation of the sample, permanent slides are ready to be observed under the microscope.

## 9.8 Molecular Approach in Forensics

Diatoms are microorganisms diversely present worldwide. The correct identification and classification of these microalgal species is a laborious task for the experts beyond species level due to their multiple morphological patterns within a population. The frustule of diatoms has very minute changes in ornamentations which makes them different from other species. In the fields of taxonomy, the proper identification of a particular microorganism is an important task to achieve. Thus, many scientists have supported the application of molecular biology in determining the type of diatom to species levels which helps forensic experts who deals with solving drowning-related crimes via diatoms (Babanazarova et al. 2010). In forensics also, molecular techniques have been gaining vast popularity over traditional methods and are widely adopted in research also with new innovations and successful outcomes (He et al. 2008; Kakizaki et al. 2012; Kermarrec et al. 2013; Kakizaki et al. 2018). The fundamental and most important aspect of molecular approach is that they only detect live cells of diatom having DNA present in the body organs of drowned victims. They are unable to detect those diatoms which lack DNA and usually found during cross-contamination. However, apart from molecular approach, none other method used in this field, especially microscopic techniques, can distinguish whether the diatom has been recovered from the live cell or came from any sort of cross-contamination. This can have direct effect on misleading of

case investigation. DNA barcoding is the most suitable approach for the identification of diatom by using genetic sequence (Ratnasingham and Hebert 2007). It stands on the principle of standardizing short sequence of DNA which can be recovered and specified as distinctive identification marker for all the species on earth (Hebert et al. 2003). Hebert et al. (2003, 2004) was the first to formulate the term *barcoding* to assist identification. Various types of genes have been suggested and studied for the barcoding of diatoms like ribosomal RNA (16 s, 18 s), RUBISCO (ribulose 1,5-bisphosphate), cytochrome oxidase subunit I (COI), silicon transporter (SIT), and ribosomal internal transcribed spacer (ITS) (Evans et al. 2007; MacGillivray and Kaczmarek 2011; Zimmermann et al. 2011). Hamsher et al. (2011) according to their performed study stated *rbcl* to be the best suitable gene for identifying different diatom genera. They also reported COI-5P to be the best gene locus for differentiation of red and brown algae. Pollanen (1998a) suggested another important target for the amplification, i.e., frustulin protein. This protein is found in the hard silica cell wall called frustule. It has no role in wall formation and is just a constituent of the frustule (Bowler et al. 2008). A relevant DNA barcode must be short in order to get easily amplified. It should be flanked by conserved regions to make suitable universal primers. Moreover, it must contain the ability to identify diatoms to species level. To identify diatoms present in the human tissue with molecular approach, only those diatom-specific genes have to be amplified to get the correct results. A relevant reference database of diatoms helps in the screening of barcodes of diatoms. Quast et al. (2013) reported SILVA ribosomal RNA as the most substantial database which consists of 16S, 18S, and SSU sequences. Moreover, 16SrDNA helps to easily differentiate between human (eukaryotic 18SrDNA) and plant genome, thus helping in denoting the case study to be of antemortem or postmortem drowning (He et al. 2008). The 18SRNA gene has been broadly used as a barcoding marker. Zhao et al. (2017) used DNA barcodes from 18SrDNA at V7 region for differentiating 9 diatom species. The molecular approach of diatoms simply deals with designing of relevant primers, DNA extraction from the cells, PCR amplification, and sequencing. The primer for every species should be made carefully by focusing on conserved and hypervariable regions. The DNA extraction from the cell can be achieved by various methodologies such as  $2 \times$  CTAB (cetyltrimethylammonium bromide) method (Doyle 1990; Kumar et al. 2016). The PCR needs 10  $\mu$ l of solution which consists of 5  $\mu$ l of PCR reagent, 0.1  $\mu$ l of suitable primer, 20 ng of DNA, and double distilled water. A standard condition for the initiation of PCR has to be made which includes various cycles of different temperatures and variable durations, such as 94 °C for 10 min then 28 cycles of 30 s at 94 °C and then again 30 s at 60 °C and 30 s at 72 °C followed by final cycle of 10 min at 72 °C. For electrophoresis technique, 2% agarose gel has to be used (Li et al. 2019). Once the final products from PCR have been made and recovered, they are sent to Sanger sequencing for further analysis. However, the PCR technique varies according to the gene of interest and categories of primers (He et al. 2008). Other molecular techniques like FISH (fluorescent in situ hybridization) (Ishii et al. 2004), qPCR (Ahlgren and Rocap 2012), etc. have been used to trace the footprints of known taxa. DNA barcoding has been regarded as an

outstanding tool because of vast advantages. Some of them have been listed as follows:

- This method is time saving and more specific in their results.
- They promote recognition of new species.
- They are appropriate tool for large campaign such as Craig Venter's Global Ocean Sampling (Rusch et al. 2007; Desalle 2006; Rach et al. 2008).
- They aid in identification of complex morphological organisms which are unable to be identified by any other methods.

## 9.9 Application DNA from Diatoms Via Molecular Biology

Several limitations of various techniques used for extraction of diatoms have been discussed earlier in this chapter. Molecular approach due to its rapid high specificity tends to become more popular and efficient in the fields of forensic limnology for deciphering drowning case mysteries. Kane et al. (1996) solved a drowning case that happened in Japan by tracking picoplankton belonging to cyanobacteria with the help of 16SrRNA locus.

He et al. (2008) imitated the scenario of antemortem drowning by conducting an experiment of submerging 12 rabbits in Donghu Lake (China). DNA was extracted from the biological tissues of the drowned rabbits, and molecular approach was applied, and it was concluded that, by 16SrDNA gene locus amplification at 487 bp, the presence of plankton was there. This led to denoting the cause of death to be antemortem drowning. However, in postmortem category of rabbits, few numbers of planktons were detected only in two rabbits. This formed a strong base to detect the cause of death by using molecular tool with least number of errors. A suspicious case of drowning of 39 year-old lady had occurred in a well. The manner and cause of death became laborious to detect. The autopsy reports as well as diatom test showed no positive findings of diatoms. However, with the application of molecular technology, the amplification of 16SrDNA was observed, and the case was found out to be of antemortem drowning which helped efficiently to solve the case mystery. Reports of Suto et al. (2009) concluded the presence of specific bacteria, such as *Streptococcus salivarius*, *Streptococcus sanguinis* found in the throat, and *Aeromonas hydrophila*, in water samples can help to decipher the drowning mysteries with the help of molecular approach. Rutty et al. (2015) solved 20 cases of drowning-related casework with the help of molecular technology.

RÁCZ et al. (2016) suggested that detection of diatoms by using molecular tools would only brace up the autopsy diagnosis. Li et al. (2019) conducted study on 10 water sites in Nanjing section of Yangtze River for forensic application. They identified diatoms to genus level with the help of both optical and electron microscope and used 18SrDNA sequencing for the detection of species. The 18SrDNA has been used widely due to some major advantages such as that they are residing in all eukaryotic organisms. They contain numerous copies per genome and are very

expressive due to which the molecular study can be done at RNA level also. They are a mixture of conserved as well as variable nucleotide due to which they are suitable for phylogenetic reconstruction at various level of taxonomy (Vinayak and Gautam 2019).

Jiang et al. (2020) made a diatom array based on molecular approach and denoted 169 diatom species from a wide range of aquatic reservoirs of China. They also invented an auxiliary sample preparation method for the isolation of DNA which helps in identification of diatoms in the body tissues of drowned victims as well as in water samples. They conducted their study on samples taken from pure culture of various diatoms, tissues from six drowned victim's body, and eight from environmental water sites like lakes, rivers, or ponds. While extracting diatoms from the pure cell cultures, they generated a protocol based on the structural and composition diatom cell wall. The hard silica cell is resistant to all lysis agents in fact to guanidinium isothiocyanate, although they successfully removed the pectin and polysaccharide present in the composition of cell wall by  $0.5\times$  TE buffer. Once the cell wall was lysed, the DNA present inside the diatom cell gets exposed for further treatment. A 15 ml of diatom culture was centrifuged and then 1 ml of ddH<sub>2</sub>O was added to the pellet. After the completion of this step, the left residue was placed in 1.5 ml microtube and again centrifuged for 3 min at 12000 rpm. Later, the supernatant was discarded, and 50 mL of  $0.5\times$  TE (5 mM Tris-HCl and 0.5 mM EDTA pH 8.0) was added to the residue and kept for 30 min in water bath at 90 °C. Then, this sample was kept in an ice bath for few minutes to cool. Only 5  $\mu$ l of the supernatant was sufficient as genomic DNA template for PCR in order to amplify 18SDNA. For human tissues, samples placed at  $-80$  °C were first allowed to defrost at room temperature. Then 6 g of guanidinium isothiocyanate was added to 10 g of tissue sample. This mixture was homogenized properly and then transferred to 15 ml centrifuges tube. Incubation of the sample mixture was initiated at 65 °C for 1 h. Subsequent centrifugations were performed at 3000–4000 rpm for few minutes. Then, 4 ml of (0.1 M Tris-HCl (pH 8.0) + 2% SDS + 1 mM CaCl<sub>2</sub> + 0.1 M NaCl) was poured in the sample mixture, and again homogenization of the mixture was done. To this sample mixture, Proteinase K was added, and repeated centrifugations were initiated with the addition of ddH<sub>2</sub>O to the pellet. Finally, the mixture was transferred to a 1.5 ml microtube and again centrifuged for 14,000 rpm for 3 min. 500 mL 0.5 M Tris-HCl (pH 8.0), 10 mL 50 mg/mL 20 nm acid washed silica particles, 10 mL 10 mg/mL RNase, 30 mL 1500 U/mL DNase were added to the residue and vortexed. This mixture was administered for digestion at 50 °C for 1.5 h. Again, centrifugation was performed at 14000 rpm for 6 min, and then guanidinium isothiocyanate was added to the pellet. Repeated washing and centrifugation were done by using ddH<sub>2</sub>O. The final residue was mixed with 25 mL of 1X TE and placed in water bath at 90 °C for 30 min in order to extract the genomic DNA. Once the extraction was completed, the DNA was subjected PCR. The cycle of PCR was initiated in two rounds for the genomic DNA recovered from the human tissues, whereas a single round was sufficient for the DNA recovered from cultures. The amplification was based on 18srDNA by using specific primers and stable PCR conditions for 30 cycles. For the detection of the desired amplified products, 1%gel

electrophoresis was performed. The diatoms were identified on the bases of records present in NCBI database. The hypervariable regions of the diatom were detected and further used for making diatom arrays. Finally, diatom array produced were subjected to hybridization technique. Liu et al. (2020) studied casework of 23 victims; out of which, 19 were of known drowning cases in various water bodies.

## 9.10 Controversies in the Validation of Diatom Test in Forensics

Diatoms have been acting as key players to solve drowning-related crimes. However, a large extent of controversies was also there which were against their application and validity in forensics. We are familiar with the fact that the presence of diatoms in the body tissues of drowned victim makes the case of antemortem drowning. However, there have been many cases where the victim died due to other reason than antemortem drowning, but there were diatoms present in their body. This leads to several research studies which criticized the application of diatoms as supporting evidence in solving such crimes. Diatoms can be present already in the body via air inhaled or foodstuffs (Hendey 1980; Krstic et al. 2002; Gordon et al. 1988; Spitz and Fisher 1973; Yen and Jayaprakash 2007), and their presence will alter the results of diatom test during the autopsy of a victim, thus leading to false judgment. Hürlimann et al. (2000) postulated that the diatom density keeps on decreasing by 10–100 while entering from water to lungs and then 100–1000-folds while travelling to distant organs. Thus, the number of diatoms inside the victim's body depends upon their abundance in the aquatic habitat where drowning occurs. Langer et al. (1971) reported that the presence of diatoms inside the lungs of a person can be found if he smokes low-quality cigars. While smoking the diatoms, fragments of small size can penetrate inside the lungs easily. Lunetta et al. (2013) reported that diatoms can enter inside the body of non-drowned victims through wounds or injuries or via strong hydrostatic pressure of water. Lunetta et al. (2013) stated that diatoms can also occur in the body tissues of non-drowned victims due to contamination of equipment used while performing autopsy or diatom test. Apart from several controversies, this test is still in great use. The diatom test should be conducted by taking full measures in order to avoid contamination or cross-contamination. Moreover, while performing the autopsy, external and internal signs of drowning should be observed with full care and then related to the outcomes of study which will help to reach the appropriate judgment.

## 9.11 Conclusion

Diatoms act as golden standards for denoting the cause and site of death in drowning cases. From the past many years, these microalgal species remained very helpful for forensic pathologists in deciphering complex cases of drowning-related crimes, even though in such investigations where the only left evidence is the skeleton of the victim or sometimes the body recovered from water is in extreme putrefied stage. In such circumstance, application of diatoms in solving the crime remained successful. Due to their small size and hard silica cell wall, they get easily penetrated inside the body organs of a victim and settle down there till the person dies and all undergoing vital processes stops. The efficiency of diatom test depends upon quantity of diatoms, number of diatoms recovered from organs, and suspected drowning medium. Diatom tests are gold standard in drowning cases. Diatoms, on the other hand, cannot penetrate inside the body in already dead condition if thrown in water to alter the crime scene because there is no breathing or circulation due to which concentration of these species will remain nil in the distant organs. Certain parameters, such as contamination from various sources, the number of diatoms in the drowning media, the number of diatoms retrieved from organs, etc., have been found to influence the efficacy of a diatom test. Despite some above-discussed criticisms regarding their validity, diatom tests are commonly acknowledged as a useful tool in solving the mystery of drowning deaths. As a result, it is hard to argue that diatoms are not the gold standard in forensics. Under proper supervision, the diatom test has been shown to be accurate in the examination of drowning-related cases. DNA barcoding in analysis of diatoms remained a sensitive and specific reliable method to differentiate between diatom communities or finding new species. The nuclear, mitochondrial, as well as chloroplast genome act as strong genome markers in order to identify several diatoms present in water bodies and thus have direct effect on aquatic health also. However, DNA database do not contain sufficient DNA sequence to cover all diatom community. The focus should be on making plant or diatom specific PCR primers in order to maintain limited homology between human tissue and diatom cell. This technique not only helps to find out the cause of death in forensic drowning cases but also helps in setting up diatom database for reference, and a suitable diatom mapping can be generated. In future, diatom analysis data may benefit from the application of more advancement in molecular approaches such as PCR, diatom mapping, automatic diatom identification and classification (ADIAC), and artificial intelligence with neural networks. These techniques are more reliable and provide better results as compared to the old traditional methods.



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