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Extraction Of Diatoms From Teeth

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Abstract:
Diatoms, ubiquitous microalgae in aquatic environments, have long held forensic importance, particularly in diagnosing drowning cases. Conventionally, diatom analysis is performed on organs like lungs, liver, and bone marrow. This study explores an innovative, non-invasive method for extracting diatoms from dental surfaces, specifically the enamel, with the aim of determining time since submersion. Using extracted teeth submerged in water samples from different regions and analyzing diatom growth over 30 days, the study demonstrates a reliable correlation between diatom length and submersion duration. This technique not only conserves evidence but also provides rapid, accurate results crucial for forensic investigations.
Keywords: *forensic odontology, diatoms, enamel, postmortem interval, non-invasive, forensic science, regression analysis*

INTRODUCTION

Diatoms are unicellular, photosynthetic, eukaryotic organisms that are present in almost all the water bodies including springs, rivers, ponds, lakes ditches and also in freshwaters, brackish and marine waters[1] and also terrestrial habitats like wet rocks, mosses and soil[2.] Diatoms are a large group consisting of 200 genera and approximately 13000 species out of which 92 genera and about 569 species are reported in India. The diatoms are distinguished from other types of algae due to their structure and sculpturing of their walls. Diatoms analysis is a valuable tool in forensic science and is useful in diagnosis of drowning cases. In drowning cases, diatoms have an important role to play as it can determine the cause and manner of death. The presence of diatoms in microcirculation can assist in differentiating between ante-mortem and post-mortem drowning. It is important to understand that the diatoms extracted from the samples of the human body are matched to the diatoms present in the water at the site of drowning.

Morphology of Diatoms: The cell of the diatom consists of cell wall and protoplast. The cells are covered by a siliceous wall, called frustule. It consists of two overlapping halves, the theca. The upper one is epitheca and lower one is called hypotheca Both the theca consist of 2 portions a. Valve: the upper flattened top b. Connecting band or cingulum- incurved region.

The common region of the connecting bands, where both the theca are fitted together, is called the girdle. Depending on symmetry, the cells are divided into 2 orders:

1. Pennales (bilaterally symmetrical)
2. Centrales (radially symmetrical)

In some pinnate diatoms, an elongated slit is present on their valves, called raphe. The raphe is interrupted at its midpoint by thickening of the walled called the central nodule. Similar thickening is also present at the ends called polar nodules. Some members of the order Pennales, do not have raphe, called pseudo raphe. Based on electron microscopic studies, Hendey (1971) observed 4 basic secondary structures:

1. Punctae (small perforations on valve surface)
2. Canaliculi (tubelike narrow channels which run through the valve surface)
3. Areolae (large box like depressions)
4. Costae (riblike structures on the valve structures)

The cell wall is mainly made up of substances impregnated with silica. The content of silica varies from 1% to about 50% on the basis of dry weight of the cell.

Forensic Odontology : The term "Forensic Odontology" is defined as the application of dental knowledge to the criminal and civil laws in the judicial system. It is the combination of art of dentistry and science and science and law which is used to assist in the provision of justice in criminal investigation. A forensic odontologist are involved in the identification of whole or fragmented body parts (head/neck), to determine age, sex, race, occupation, socioeconomic status through comparison of ante-mortem and post-

mortem dental records. Personal identification is necessary for unknown deceased person in homicide, suicide, accident, mass disaster, etc., and for living individual who are missing or culprits hiding their identity. Forensic dentistry involves the analysis of dentition from living and deceased individual, including children, adolescent and adult. These analyses depend on morphological, radiological, chemical, histological etc. Apart from these the work of a forensic odontologist also includes assisting at the scene of mass disasters, age estimations, comparison of dentition with the record of a bite-mark left on a victim.

Diatoms and Forensics

When a body has been drowned diatoms go into the lungs and then are circulated to other internal organs through the individual's circulation. A forensic scientist will extract 100g of either a tissue sample or bone marrow from femur still attached to the victim or present the place of drowning, for crime scene investigation and post-mortem identification.

When using the diatom testing, scientist observe the large amount of diatoms present and determine a generalized time of death. If there are less than 20 different types of colonizing diatoms, then the organism's death is between 7 to 12 days. But if there are several types of colonizing diatoms the time of drowning shall be several weeks. Certain species of diatoms taxa grow after a specific interval of time under favorable conditions.

Necessity of diatoms

All the dead bodies recovered from the water medium are not necessarily died of drowning. They could have died either of a natural disease or of a trauma before entering, or inside the water[2]. Decomposition starts late in water than in air, most of the submersed dead body are detected only after its floatation to water surface, caused by decomposition. Even when the body is not decomposed, but is recovered from water, it undergoes rapid putrefaction, till it reaches morgue. Once decomposition sets in, almost all the features of ante-mortem drowning vanish. Ante-mortem injuries are evident during the process of drowning and the post-mortem injuries occur during the flow of the dead body in water or are inflicted by the aquatic animals which are a challenge to diagnose the cause and manner of drowning. Identification and comparison of diatom species further raise the hope of diagnosis of site of drowning. As the water never contains a specific species of diatoms, its applicability to the case becomes difficult and is now being challenged for its specificity to the place of drowning as they can gain access to the human tissues through various medium like through the lungs, by breathing of contaminated air or through foods with high diatoms content[10].

Estimating the time since death

Estimation of time since death is one of the most important aspect of forensic medicine. This estimation becomes difficult as the rate of decomposition advances and even when the corpses are immersed in water[11]. On estimation of an accurate time since death many conclusions can be narrowed down in a criminal investigation and also assist in determining the place of drowning, hence it serves as a useful piece of information. With advances in estimation the regression technique is usually used to determine the place of drowning by comparing the diatoms that have entered individual organs with the inhabitant diatoms in the water at the site of discovery[12-15]. Studies are done to attempt the time since death by applying forensic odontology techniques by analysis of the changes over time in the types and quantity of vegetative plankton (diatoms). Deposits on the enamel surface to order to determine its duration of underwater immersion as an attempt to determine the time since death[16].

The aim of the study is to be able to extract the diatoms from the surface of the teeth specifically form the enamel surface.

MATERIALS AND METHOD

Method for extraction of diatoms:

Teeth sampling

ample Collection and Preservation

One hundred freshly extracted teeth were collected from Surdeep Dental Clinic and charitable trusts in Vadodara. Teeth were categorized based on:

Gender: Male and Female

Condition: Decayed, periodontally compromised (with heavy calculus), stained, and fractured

All samples were labeled and stored at -4°C for 2-3 days before processing.

2.2 Submersion Protocol

Eighteen premolars and molars were immersed in 80 mL of Narmada river water (Ahmedabad main canal) in two glass beakers (8-10 teeth per beaker). Beakers were covered with petri dishes and kept at ~37°C under ambient sunlight in a forensic biology lab for 30 days.

2.3 Diatom Extraction Procedure

Teeth were retrieved every second day and brushed on all surfaces (buccal, lingual, mesial, distal, occlusal, furcation, apical) with hard-bristle toothbrushes.

Residue from brushes was rinsed into a conical flask with diatom-free distilled water.

Flask received 1N potassium permanganate ($KMnO_4$) and was left undisturbed for 24 hours.

After 24h, 4-5 mL of 36% hydrochloric acid (HCl) was added, and the solution was heated at 90°C until it turned colorless.

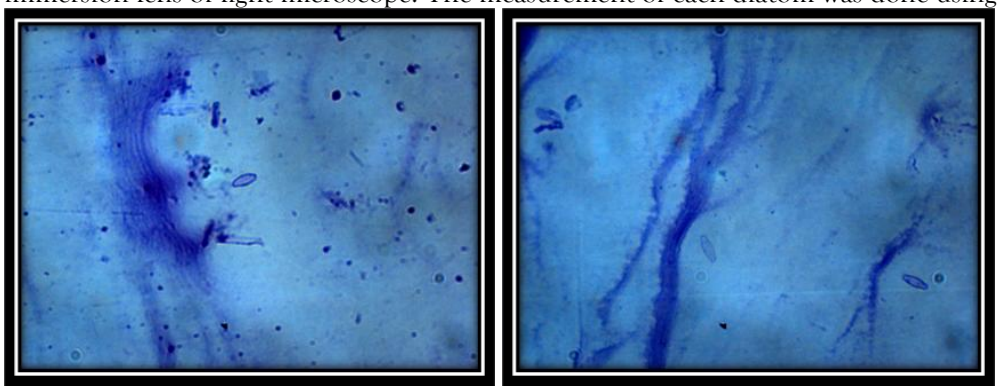
1 mL of hydrogen peroxide (H_2O_2) was added to complete oxidation.

Once cooled, 10 mL of the solution was centrifuged at 4000 rpm for 10 minutes.

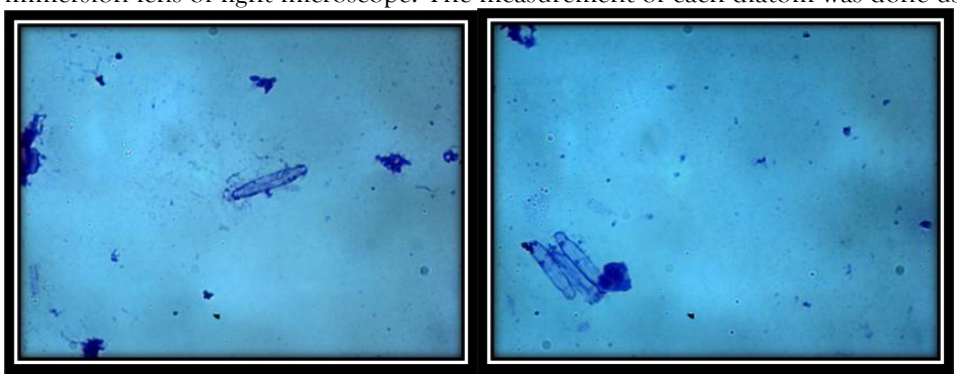
Supernatant was removed, and the pellet resuspended in distilled water.

A drop was heat-fixed on a slide, stained, and observed under 100X oil immersion.

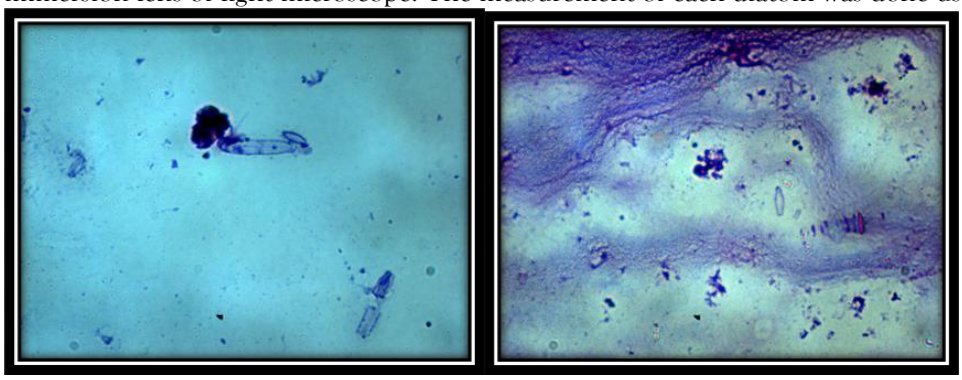
DAY 2:
For day 2 of the experiment a total of 16 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.



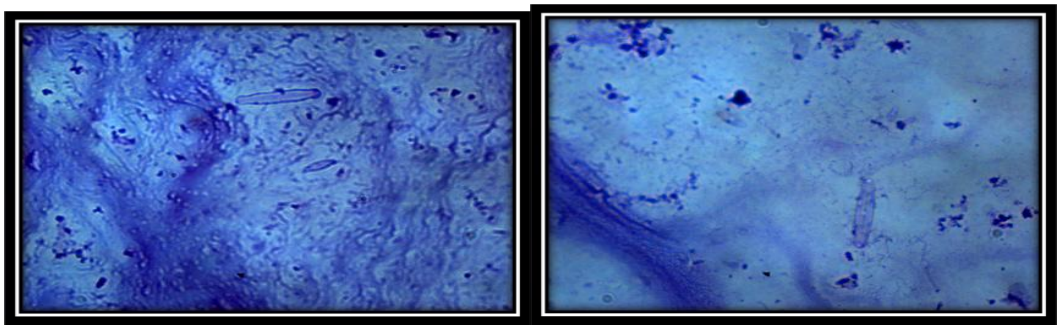
Length of diatoms was estimated to be between 23.793 µm to 31.972µm
DAY 4:
For day 4 of the experiment a total of 59 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.



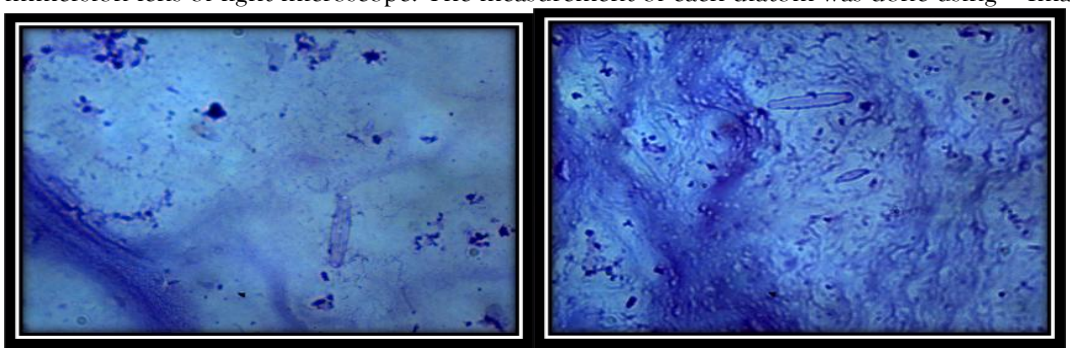
Length of diatoms was estimated to be in between 27.268µm to 79.057µm
Mean length of all the diatoms found in the teeth submerged in the water for 4 days: 38.86 µm.
DAY 6:
For day 6 of the experiment a total of 20 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.



Length of diatoms was estimated to be in between 31.185 µm to 84.619 µm
Mean length of all the diatoms found in the teeth submerged in the water for 6 days: 51.842 µm.
DAY 8:
For day 8 of the experiment a total of 36 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

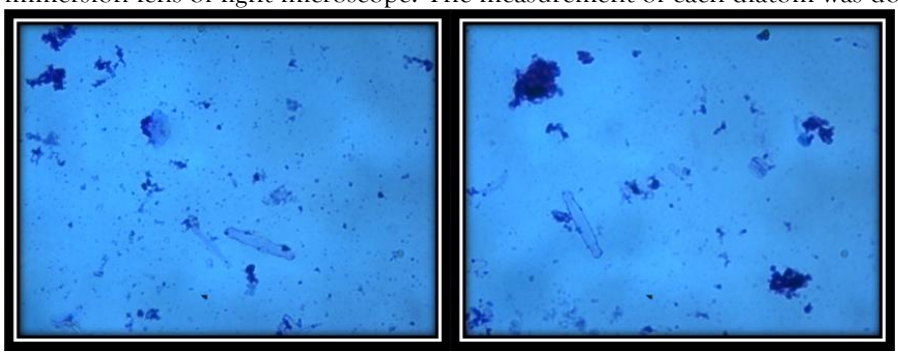


Length of diatoms was estimated to be in between 33.39 µm to 83.247 µm
Mean length of all the diatoms found in the teeth submerged in the water for 8 days: 33.34 µm.
DAY 8:
For day 8 of the experiment a total of 36 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.



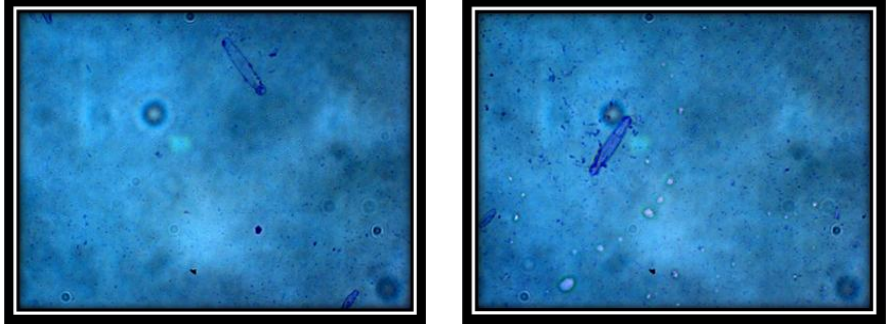
Length of diatoms was estimated to be in between 31.345 µm to 88.207 µm
Mean length of all the diatoms found in the teeth submerged in the water for 8 days: 33.34 µm.

DAY 10:
For day 10 of the experiment a total of 35 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

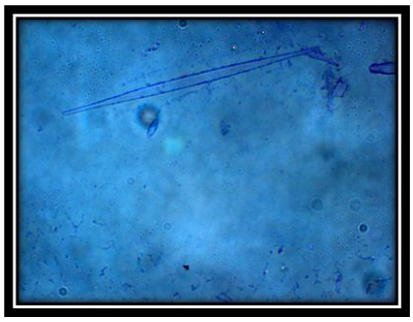


Length of diatoms was estimated to be in between 38.445 µm to 88.014 µm
Mean length of all the diatoms found in the teeth submerged in the water for 10 days: 44.99 µm.

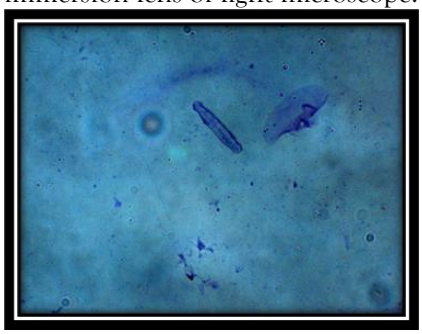
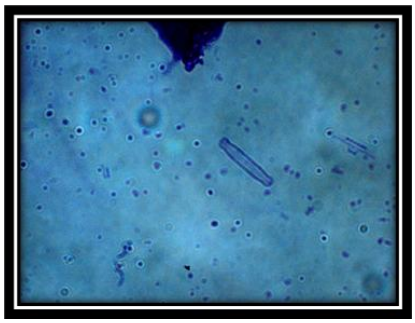
DAY 12:
For day 12 of the experiment a total of 15 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.



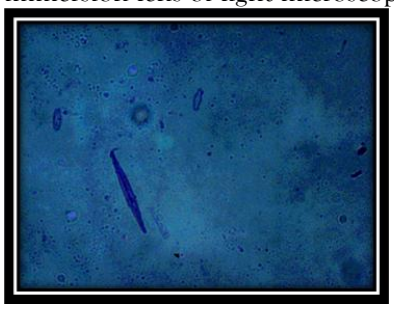
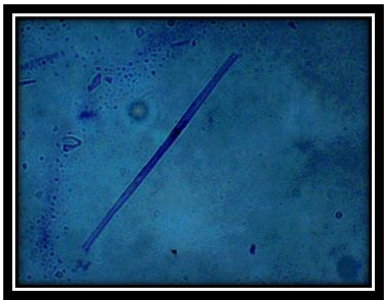
Length of diatoms was estimated to be in between 83.95 µm to 95.02 µm
Mean length of all the diatoms found in the teeth submerged in the water for 12 days: 62.01 µm.
DAY 14:
For day 14 of the experiment a total of 53 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

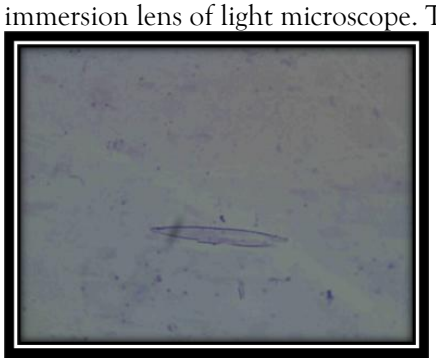
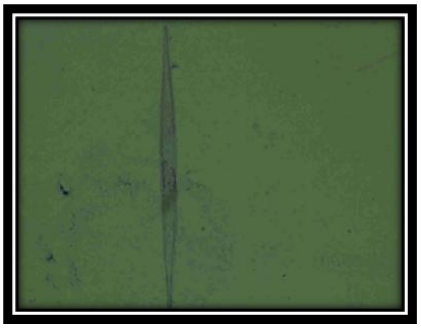
Length of diatoms was estimated to be in between 34.178 µm to 342.038 µm
Mean length of all the diatoms found in the teeth submerged in the water for 14 days: 52.82 µm.
DAY 16:
For day 16 of the experiment a total of 39 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

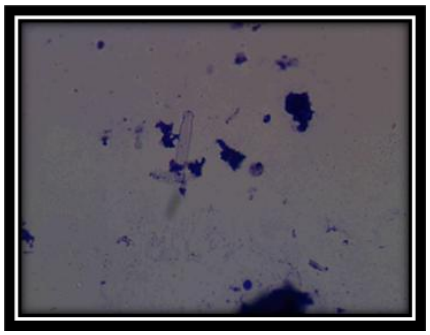
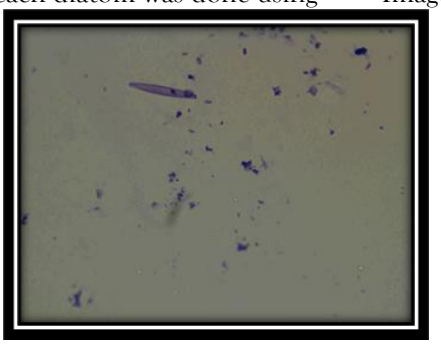
Length of diatoms was estimated to be in between : 54.54 µm to 90.836 µm
Mean length of all the diatoms found in the teeth submerged in the water for 16 days: 69.96 µm.
DAY 18:
For day 18 of the experiment a total of 18 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

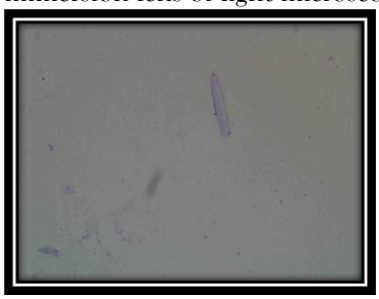
Length of diatoms was estimated to be in between : 88.855µm to 337.325µm
Mean length of all the diatoms found in the teeth submerged in the water for 18 days: 102.24 µm.
DAY 20:
For day 20 of the experiment a total of 14 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

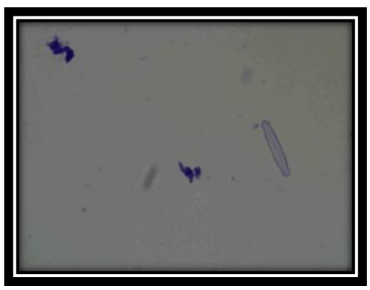
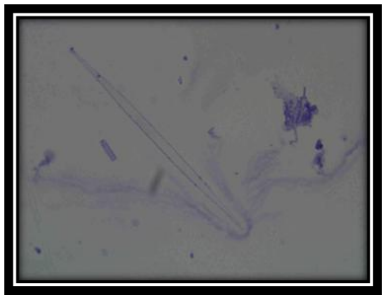
Length of diatoms was estimated to be in between : 82.752 µm to 299.221 µm
Mean length of all the diatoms found in the teeth submerged in the water for 20 days: 126.24 µm.
DAY 22:
For day 22 of the experiment a total of 12 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

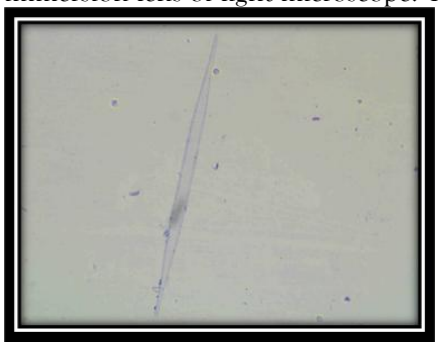
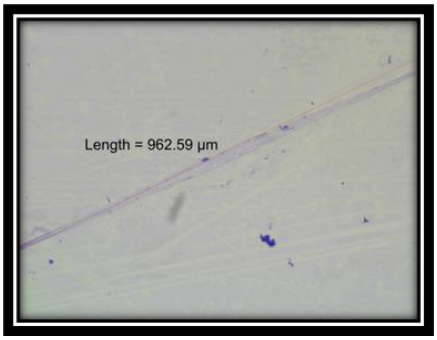
Length of diatoms was estimated to be in between : 69.522 µm to 263.168 µm
Mean length of all the diatoms found in the teeth submerged in the water for 22 days: 139.521 µm.
DAY 24:
For day 24 of the experiment a total of 42 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.




Length of diatoms was estimated to be in between : 61.937 µm to 161.592 µm
Mean length of all the diatoms found in the teeth submerged in the water for 24 days: 138.002µm.
DAY 26:
For day 26 of the experiment a total of 31 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

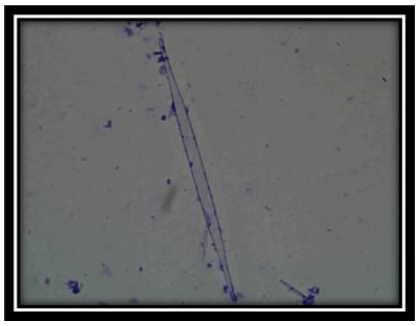
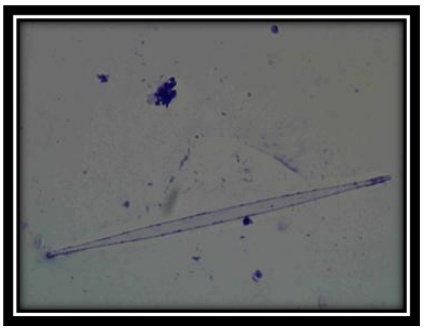



Length of diatoms was estimated to be in between : 158.342 µm to 636.961µm
Mean length of all the diatoms found in the teeth submerged in the water for 26 days:199.207µm.
DAY 28:
For day 28 of the experiment a total of 29 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

Length of diatoms was estimated to be in between : 302.54 µm to 968.38 µm
Mean length of all the diatoms found in the teeth submerged in the water for 28 days:358.163 µm.
DAY 30:

For day 30 of the experiment a total of 45 diatoms were analyzed. Pictures were taken using 100X oil immersion lens of light microscope. The measurement of each diatom was done using Image J.

Length of diatoms was estimated to be in between : 441.67 µm to 812.496µm
Mean length of all the diatoms found in the teeth submerged in the water for 30 days: 496.461µm.

Statistical analysis:

To analyze the data gathered through the experiment, statistical techniques like day wise average, time series graph, correlation and regression have been applied. A brief of each of these techniques and their methodology is mentioned below.

1. Day wise Average

The size of the diatoms was measured on each alternate day for a period of 30 days, in order to understand the trend in the change in the length of the diatoms, average has been calculation for all the 15 days. The number of diatoms varied from the observation of one day to the other day and hence average can provide meaningful comparison instead of the size of each diatom. The following table depicts the same.

NO. OF DAYS	AVERAGE LENGTH OF DIATOM
2	53.26
4	38.86
6	51.842
8	33.34
10	44.99
12	62.01
14	52.82
16	69.96
18	102.24
20	126.24
22	139.521
24	138
26	199.207
28	358.163
30	496.461

Table 1: MEAN AVERAGE OF LENGTH AND DAYS

From the above table we can infer that the size of diatom is mainly increasing from one observation to the other except for a few occasions due to some uncontrollable factors mentioned in the previous section.

2. Time series graph:

A time series chart, also called a times series graph or time series plot, is a data visualization tool that illustrates data points at successive intervals of time. Each point on the chart corresponds to both a time and a quantity that is being measured. The X-axis represents the time points and the graph represents the change in the measured quantity from one point of time to another. It provides pictorial representation and aids in understanding the direction as well as the magnitude of the change

The following is the time series graph representing the change in the length of the diatom over the period of time taking into consideration the data reported in the table above.

The time series graph above clearly represents the overall increase in the length of the diatoms from day 2 to day 30. The rate of increase in the beginning is comparatively slower and even decreases a little on day 8 and day 14. After day 14 we observe a constant and higher increase in the length of the diatoms till day 30. The data points in the graph above are representing the length of the diatom in microns till two places of decimals. The trend line indicates the existence of a relationship of the length of the diatoms with the time period, this relationship is investigated further with the help of correlation and regression.

3. Correlation of length of diatoms with the number of days of submerged samples

The correlation represents the strength of relationship between two or more variables, one of the most standard measures of correlation, the Karl Pearson correlation coefficient represented by 'r'. the value of correlation coefficient varies from -1 to +1. The sign of the value represents the direction of the relationship and the magnitude of the value represents the strength of the relationship. The negative value indicates an inverse relationship, that is, with the increase in the value of one variable the value of other variable decreases and vice-versa. The positive value indicates a direct relationship that is with the increase in value of one variable, the value of the other variable also increases and vice versa. The closer the value is to '1', higher is the strength of the relationship between the variables. The correlation coefficient ($r< 0.5$) represents weak relationship, ($0.5<r<0.8$) represents moderate relationship and ($r>0.8$) represents strong relationship. The following table represents the Correlation of length of diatoms with the number of days of submerged samples

	NO. OF DAYS	LENGTH OF DIATOMS
NO. OF DAYS	1	
LENGTH OF DIATOM	0.804196	1

TABLE 2: Correlation of length of diatoms with the number of days of submerged samples

The correlation between the length of diatoms and number of days is found to be moderately strong and positive in direction. The degree of association between the two variables can be further explained by the coefficient of determination Coefficient of determination is the squared value of coefficient of correlation, represented by 'r^2'. The coefficient of determination represents the amount of variation in one variable that can be explained with the help of other variables. The coefficient of determination for the length of diatoms and numbers of days the samples were submerged is represented below:

Multiple R	0.80419626
R Square	**0.646731625**
Adjusted R Square	0.619557135
Standard Error	**5.516831448**
Observations	15

TABLE 3: The coefficient of determination for the length of diatoms and numbers of days the samples were submerged.

The r^2 value of 0.6467 represents that around 65% of the variation in the length of diatom can be

explained with the help of the number of days for which the samples were submerged in water. The remaining 35% variation in the length of the diatom can be attributed to other reasons not included as a part of this study. The coefficient of determination indicates that time period has a significant contribution in the change of length of diatom. The cause effect relationship is explored further through regression analysis.

4. Regression analysis

The regression techniques helps to establish the cause effect relationship between two or more variables. The regression equation predicts the value of dependent variable based on the values independent variable. The standard regression equation is represented by

Y=a+bx

y- Represents dependent variable

x- Represents independent variable

b- Represent slope of the regression line

a- Represents intercept of the regression line

The following scatter Plot and table represents the result of the regression analysis between the length of diatom and the number of days the samples were submerged in water. The table output has been generated in Microsoft excel.

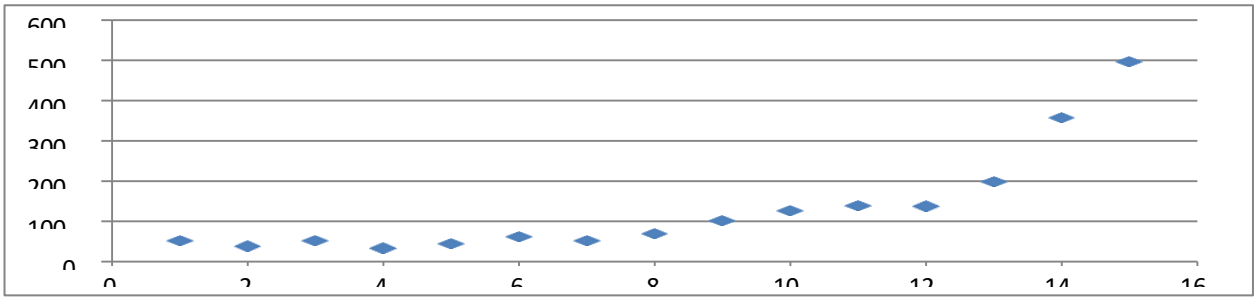
ANOVA								
	df	SS	MS	F	Significance F			
Regression	1	724.3394201	724.3394201	23.79922	**0.000301**			
Residual	13	395.6605799	30.43542923		**REGRESSION IS SIGNIFICANT**			
Total	14	1120						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	8.842610669	2.044882791	4.324262841	**0.000825**	4.42491	13.26031	4.42491	13.26031
LENGTH OF DIATOM	0.054583393	0.011188689	4.878444236	**0.000301**	0.030412	0.078755	0.030412	0.078755

For the above analysis the length of the diatom has been considered as independent variable (x) and the number of days has been considered as dependent variable (y). the regression analysis has been undertaken at 95% confidence level or 5% significance level (α = 0.05). The 'p-value' for the intercept and the slope are 0.000825 and 0.000301 respectively, indicating that the regression coefficients are significant. Also the f-value (0.000301) which is much less than the significance level represents the overall significance of the regression equation obtained.

The regression equation can be represented as follows:

EQUATION: NO. OF DAYS = 8.842610669+ (0.054583393*MEAN LENGTH OF DIATOM)

DISCUSSION



The results of the study mentioned above section were done using a limited number of teeth samples and hence the equation obtained did not have an appropriate relevancy. The types of diatoms observed from

the same river that is Narmada varied from city to city. Hence the environmental conditions play a very important role in the type of diatom found in the waters of the same river:
The variations seen in the type included:

1. Narmada river at origin (Amarkantak): Hyalosynedra lacvigata, Synedra Ulna, Synedra tabulata, Nitzschia,
2. Narmada river flowing through the city of Jabalpur: Navicula, Fregilaria capunica, Fragillaria intermedia, Grammatophora,
3. Narmada flowing in Ahmedabad: Nitzschia, Nitzchia Sicula, Nitzchia Acidonata, Fragillaria intermedia, Navicula
4. Narmada flowing in Bharuch: Navicula Hamiltoni, Navicula, Caloneis, Nitzchia
5. Narmada meeting the Arabian sea in the Gulf of Khambhat: Navicula, Caloneis, Nitzschia.

The second part of the study which deals with establishing a correlation between the length of diatom and the number of days of immersion of the teeth samples into the Narmada river water flowing through Ahmedabad gave us appropriate results after day 12. Various factors mentioned in the above section affected the growth of diatoms and the probability of the increase in growth of diatoms with increase in the number of days the samples were immersed in water is 64.69% according to the equation derived above.
The equation derived at the end of study is:

Equation 1: NO. OF DAYS = 8.842610669+ (0.054583393*MEAN LENGTH OF DIATOM)
As the graph suggests a linear regression after day 12 to day 30 the equation showed greater accuracy and better results. Hence the equation derived from day 12 to day 30 is:
EQUATION 2 : NO. Of DAYS=12.3293958+ (0.043910783*MEAN LENGTH OF DIATOM)

CONCLUSION:

This study introduces an innovation in the field of forensic odontology by serving to extract the types of diatoms from the surfaces of the teeth immersed in water. This study also determines that the type and species of diatoms change by change in the environmental conditions. The study also serves to approximate the number of days of immersion of teeth samples into the water by measuring the length of the diatom. The equation could have been more appropriate if the samples could be emerged for a longer time in the waters of Narmada River. Hence initiative shall be made to increase the number of days since the samples could be immersed in water in order to get an accurate calculation. This equation when appropriate can help to estimate the number days since the body or samples could have been present in the water by measuring the length of the diatom. By mixing the waters of all the places from where the water had been collected, it can be predicted that the environmental conditions and other uncontrollable factors affect the type and species of diatoms. It can be estimated that certain type of diatoms requires certain favorable conditions in order to grow and maintain its existence. The study serves to reduce the time to extract the diatoms and in order to come to a conclusion of cause of death and time since drowning of the sample. The extraction diatoms through this technique requires 3-4 hours to come to a conclusion which is very less when compared to other techniques used in forensic science. The technique used does not involve any damage to the evidence which is important as the diatoms are brushed from the surface of the diatom and no invasive techniques are used.

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