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Digestion of Concentrated Water Samples of Diatoms for Environmental Purpose by a Newly Designed Method

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ABSTRACT

Diatoms analysis in water samples is laborious and time-consuming. Therefore, speedy digestion and clear diatoms frustules are the foremost criteria for the detection of diatoms in concentrated water samples. Here, we have described a novel method for diatoms analysis from water samples containing a large amount of debris/sediments. The samples were digested using nitric acid and hydrochloric acid in a ratio of 1:1:1 on a ceramic hot plate under a fume hood for two hour. Then concentrated solution formed was again diluted with deionized water and again heated then allowed to stand undisturbed for two hours for sedimentation. The processed samples were mounted, dried, and then visualized under a light microscope at 1000X magnification. The processed samples were fixed for SEM analysis. The method is reproducible, offers fast, resulted in complete digestion of sediments retained diatoms frustules, and maintains structural arrangements of areolae and striae as well able to recover diversity of diatoms. The method is fast, economical, time-saving sensitive, labor-saving, and accurate for qualitative and quantitative diatom analysis. This method is suitable both for ecological, taxonomic descriptions, forensic and paleontological studies where a large sample quantity is required for the study of the diversity of diatoms.

Keywords: Diatoms; oxidizing agents; digestion; methods; microscope; frustule

INTRODUCTION

Studies on diatoms in the recent past have increased tremendously due to their role as an indicator in forensic sciences, oil exploration, assessment of climate changes, and in nanotechnology (Hu et al., 2013; Jamali et al., 2012; Kireta et al 2012). Even though reported for their worldwide distribution, certain species of diatoms are restricted to particular

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environmental conditions. Therefore, their presence can predicate particular environmental conditions. Some of the diatom's species are reported for their ability to resist highly alkaline and saline and temperature fluctuations (Yu et al., 2019).

Diatoms samples must be collected from the hard surfaces of rivers and streams to produced representative samples. The initial samples collected for diatoms isolation may contain a large amount of organic matter. Diatom's surface possesses siliceous cell walls and organic material. The organic material must be properly removed without the destruction of frustules for the proper identification of diatoms (Seaborn and Wolny, 2000). For the analysis of diatoms from water samples, complete digestion of sediment is performed to remove debris which otherwise can interfere with microscopy characterization. Therefore, strong suitable oxidizing agents are commonly used to specifically retrieve the clear suspension containing diatoms (European Standard 2003). Several methods have been described for the preparation of diatoms from different sources (Quantin et al., 1995; Singh et al., 2006; Hu et al., 2013). All of these methods have certain pros and cons such as either time consuming, expensive, destruct the diatom valve hence are not suitable for light microscopy (LM), and Scanning electron microscopy (SEM) analysis (Trobajo and Mann, 2019).

Since, the current methods for diatoms extraction and quantitative microscopy are laborious and often prevent accurate compilation of diatoms records (Abranthes et al., 2005). For the taxonomic and ecological studies, accurate characterizations of diatoms from benthic communities are required. Diatoms also play an important role in diagnosing of drowning deaths (Rana and Manhas, 2018; Singh and Verma, 2019). Such studies can only be done by avoiding any damage to frustules loss or break so that the better and precise prediction of species composition and number could be done (Zoto et al., 1973). The extraction of biomineralized components from sediments is time-consuming and also involves steps to remove siliceous or calcareous parts besides organic matter (Tsutsui 2018). The slide containing diatoms to be used for microscopy should be free of organic and inorganic residues for the detailed morphological studies. In such cases, if incomplete digested samples would hinder the microscopic analysis and then prevents the proper identification of diatoms (Blanco et al., 2008).

The purpose of this study is to develop a method that helps in speedy digestion, clear diatoms frustules with a high reclaiming ratio for LM, and SEM examination from concentrated diatoms samples.

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Materials and Methods

Sample collection

Water samples were collected from various sites located in three major water reservoirs (Pong dam, Bhakra dam and Chamera dam) of Himachal Pradesh, India. Samples were collected from benthic stones and surface sediments having biofilms by scrapings their uppermost surface with a toothbrush and then the concentrated samples were lifted with the help of dropper and taken into 50 ml plastic containers and fixed with ethanol.

Processing of samples

The digestion of samples (10ml) was done with a solution of nitric acid (10ml) and hydrochloric acid (10ml) in a ratio of 1:1:1 on a ceramic hot plate at 150°C for two hours in a fume hood. Then the concentrated solution was again diluted with 40 ml of deionized water and heated. The final prepared samples were again suspended in 40 ml of deionized water and kept undisturbed for two hours for sedimentation. A white or grey color residue at the bottom of the container indicated complete digestion of the material. Then 10-100 µl was sample was mounted on coverslip and allowed to dry at room temperature. The dried samples on a coverslip were treated with absolute alcohol and then air-dried for five minutes in hot plate and dipped in xylene carefully. Then mount the coverslip containing sample on glass slide with the help of DPX and again dried. After that samples were visualized using a Leica DM 3000 LED microscope at 1000X by using immersion oil and photographs were captured.

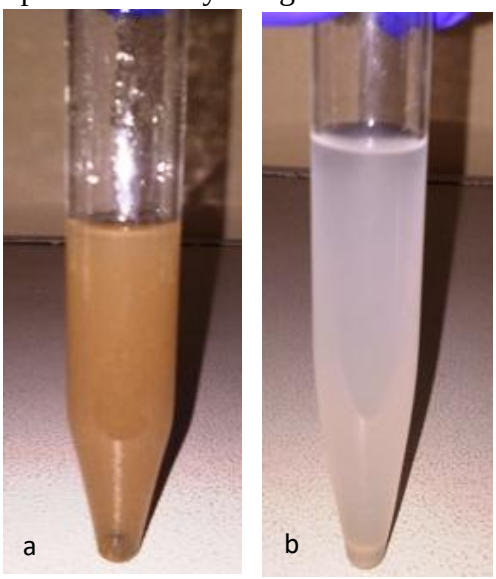

Fig. 1: Digestion of sample using nitric acid and hydrochloric acid in a ratio of 1:1:1

a- Concentrated water sample before digestion **b-** sample after digestion with new method.

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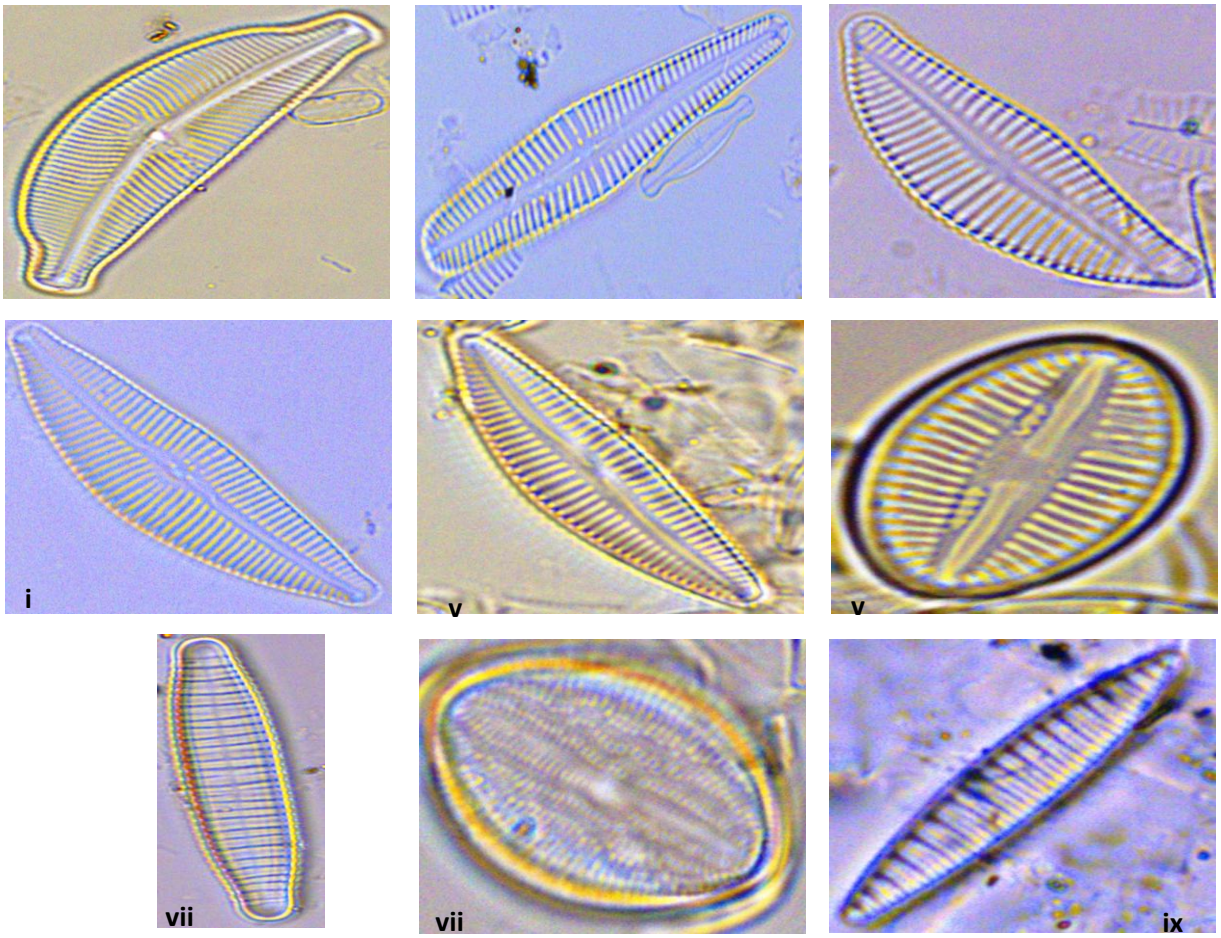

Fig.2A: Diatoms observed under Light Microscope **i**-*Cymbella tumida* ii-*Gomphoneis species* **iii**-*Encyonema species* **iv**-*Cymbella neocistula* **v**-*Cymbella neocistula* **vi**-*Diploneis species* **vii**-*Diatoma vulgaris* **viii**-*Cocconeis placentula* **ix**-*Nitszhia species*

Scanning electron microscopy analysis of Diatom

For the analysis of diatoms under SEM a small amount of digested sample was fixed using 3% glutaraldehyde and 1% aqueous solution of osmium tetroxide OsO4) method (Salwan et al., 2020). Ten microliters of fixed samples were mounted on double adhesive tape and air-dried for one hour at room temperature. The air-dried samples were gold coated for one minute using DII-29030SCTR Smart Coater (JEOL). For SEM analysis, images were captured using JEOL IT 500 under high vacuum conditions, at a voltage of 15kV, probe current of 15-30µA, and working distance (WD) of 10-12 mm.

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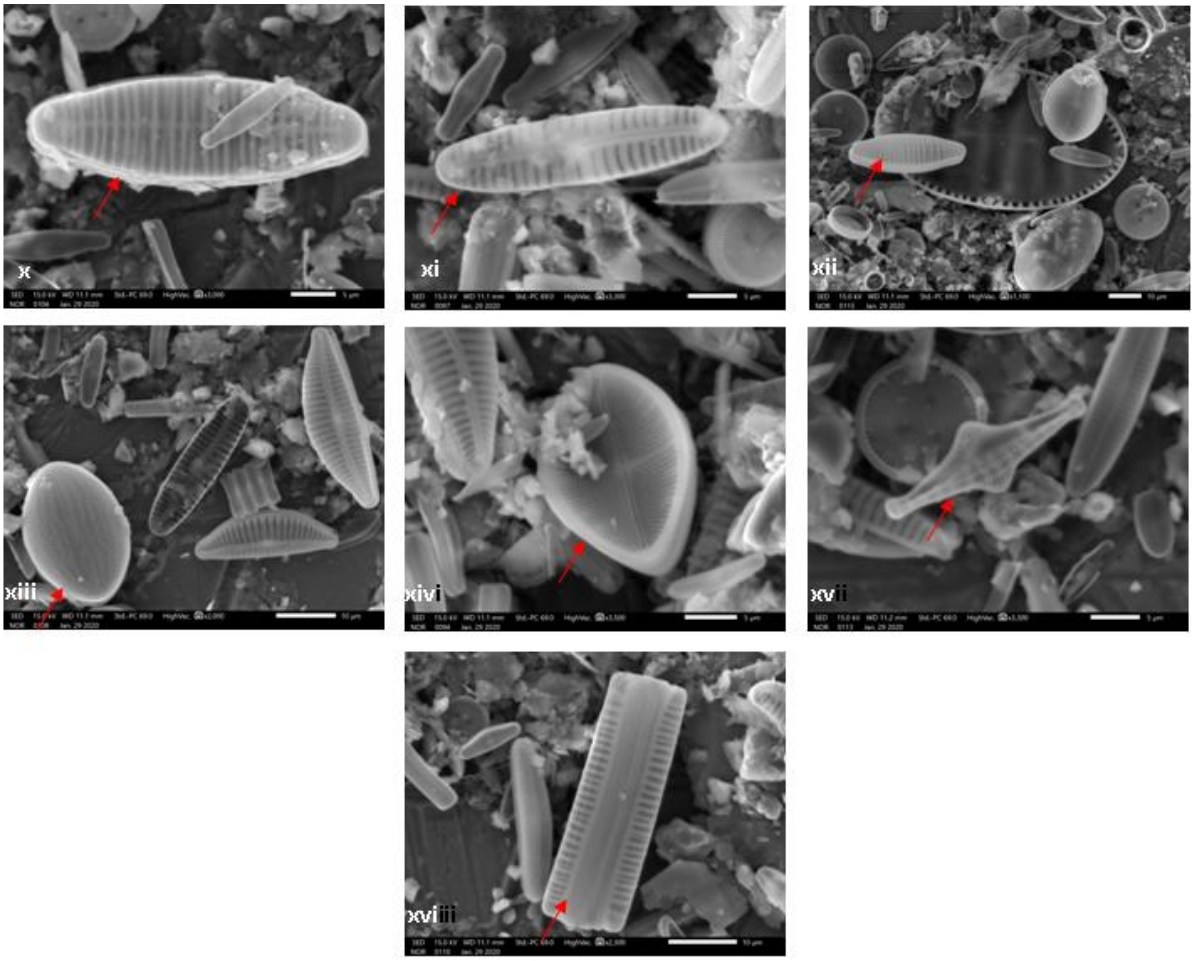

Fig. 2B: SEM images of diatoms ***x****-Diatoma vulgaris* ***xi****-Staurosira species* ***xii****-Cymatopleura species* ***xiii****-Diatoms assemblages of Cocconeis placentula, Staurosira species, Cymbella species* ***xiv****-Cocconeis placentula* ***xv****-Grunowia tabellaria* ***xvi****-Diatoma vulgaris*

Results and Discussions

The diversity of digested diatoms samples obtained using the Leica DM 3000 LED microscope is shown (Figure 2A) whereas for SEM images are shown (Figure 2B). The results of our study revealed that digestion of sample using a solution of nitric acid (10ml) and hydrochloric acid (10ml) in a ratio of 1:1:1 resulted from a clear suspension with less or no organic matter (Figure-1). Moreover, the samples digested with nitric acid and hydrochloric acid method showed clear girdle structure, and since most of the frustules were intact and did not show much destruction. The samples prepared also showed that the two girdles were closely associated with each other and thus can be useful in the identification and classification. The clear suspension resulted in a higher recovery rate from the

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concentrated water samples. The choice of oxidizing agents for the digestion of water samples depends on the amount of organic and inorganic debris in the water samples. The literature cited on the time consumption for oxidizing of water samples showed that there is great variability in processing time which varied from 1 hour (Winter and Duthie 2000), 3-4 hour (Carr*et al* 1986), 24 hours (Schmid and Schulz 1979, Zampella *et al* 2007) and 48 hours (Bergey, 1995) (Table 1).

Oxidizing agents like nitric acid and hydrochloric acid used in our study are most effective in the production of clear suspension and even found capable of digesting all inorganic components. One of the advantages of our method is that it digested the samples in less time and does not require labor. Microscopic observations of the digested samples were clear as shown in the images and also showed better preservations. We have used hydrochloric acid and nitric acid as strong oxidizing agents for the digestions of water samples in a ratio of 1:1:1 v/v. Since the collected samples contained a large amount of organic matter and thus their complete digestion by use of strong acids treatment followed by heating produced clear suspension. Strong heating of water samples at 150 $^{\circ}$C is recommended in our newly designed method for speedy digestion of samples which reduces the processing time as well as the production of clear suspension for a microscopic image. Moreover, the treated samples of diatoms do not cause in the separation of girdle bands i.e. epitheca and hypotheca. Earlier reported methods such as nitric acid/potassium dichromate and bleach method are found to produce little differences in the extent of joined frustules (Carr et al., 1986). Literature cited on type of oxidizing agents utilized for diatoms processing showed that various oxidizing agents in various combinations were utilized for the digestion of water samples like hydrogen peroxide (European standard 2003), sodium hypochlorite (Carr*et al* 1986), sodium dodecyl sulfate (Schmid and Schulz 1979) and nitric acid (Mcbride 1988) (Table 1).

The methods described here are best suitable when the samples are concentrated and contain a large amount of debris. One of the disadvantages of our method was that some of the centric diatoms get dissociated from epitheca and hypotheca while all pinnate and some centric diatoms remain closely associated and thus their structure was visible in LM and SEM observations. Sometimes non-random distribution of diatoms in permanent slides causes unbiased estimation of cell density and thus processing techniques must complement counting protocols to obtain better results (Alverson *et al* 2003). Trobajo and Mann in their study showed that their newly designed method was not suitable for ecological and

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palaeoecological studies due to the small amount of representative subsample (Trobajo and Mann 2019).

Table-1: Represents the various methods for diatoms samples processing

S. No.	Name of Method	Reagents used	Processing Time	Reference
1.	Hot hydrogen peroxide	30 % (100 volume) hydrogen peroxide solution, Dilute (e.g. 1mol/l) Hydrochloric acid	1h to 3h on a hot plate at 90 $\pm$5 $^{\circ}$C	European Standard 2003
2.	Cold hydrogen peroxide	30 % (100 volume) hydrogen peroxide solution, Dilute (e.g. 1mol/l) Hydrochloric acid	At least four days at normal temp.	European Standard 2003
3.	Hot hydrogen peroxide with potassium dichromate	30 % (100 volume) hydrogen peroxide solution, Dilute (e.g. 1mol/l) Hydrochloric acid Potassium dichromate	0.5 h to 3h on a hot plate at 90 $^{\circ}$C	European Standard 2003
4.	Cold Acid (permanganate) method	Dilute (e.g. 1mol/l) Hydrochloric acid conc. sulphuric acid potassium permanganate oxalic acid	Samples processed immediately without heating or leaving to oxidised	European Standard 2003
5.	Sodium hypochlorite method	5.25% Sodium hypochlorite solution	Treated sample was allowed to stand 1-2 h at normal temp.	Carr et al., 1986
6.	$K_2S_2O_8$ method	$K_2S_2O_8$ solution (0.2-0.4µg/5ml)	The samples were heated to 60 -90 $^{\circ}$C for 6 to 8 h	Ma & Jeffrey 1978
7.	Sodium dodecyl sulphate (SDS) method	5-6% Sodium Dodecyl sulphate solution	The samples were treated with 5-6% sodium dodecyl sulphate solution for 24 h at room temperature	Schmid & Schulz 1979
8.	Nitric acid method	Sample should be 10 times of its own volume of nitric acid	The samples were kept overnight then heat in boiling point for 5 minutes	Mcbride 1988
9.	Dry method	Sample taken in muffle furnance	The samples were heated for 15-20 min at 560 $^{\circ}$C	Zoto et al., 1973

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The time allowed for the digestion of inorganic and organic samples is one of the critical steps for the digestion of water samples. In our study, two hours digestion by using HCl and HNO_3 produces clear suspension in concentrated samples when used in the ratio of 1:1:1. The choice of ratio usually depends on the amount of organic debris in the samples and the digestive capabilities of oxidizing agents used for the study. Blanco et al used hydrogen peroxide in a ratio of 1:1 for six hours for the digestion of samples (Blanco 2008). Literature cited on diatoms samples digestion showed that pre-treatment may also be required for marine water samples as salt may hinder in settling of diatoms in the slides. Certain pre-treatment like HCL (Trobajo *et al* 2004), HNO_3 (Nagy 2011), and deionized water (Zoto *et al* 1973) were utilized for the removal of salts. Since in our study we have tested only freshwater concentrated samples so we have not done any pre-treatment. However, deionized water was added in successions after the digestion of concentrated samples to remove acid as well as salts.

In our method, we have utilized the decantation method for the settling of diatoms. Earlier reports showed that settling of diatoms frustules can be done either by centrifugation or decantation. Sometimes, centrifugation is found to cause the separation of diatoms valves which hinders in their proper identification. Battarbee *et al* in their study showed that vigorous stirring may also destroy the spine processes of fragile diatoms (Battarbee *et al* 2001). In our study, we have allowed the cover slips to dry at room temperature rather than heating. Drying of slides at room temperature would help in proper distribution of diatoms valves and thus the quality of diatom slide help in proper identification. Alverson *et al* (2003) reported that heating of diatoms in cover slips causes uneven distribution of diatoms in slides. Moreover, unbiased sample distribution would also hinder proper interpretations of results. More experimental designs are required in diatoms processing (Blanco, 2008) so that random fields can sufficiently cover the diatoms for counting and their proper distribution (Alverson *et al* 2003). The material on the cover slips should also be densely distributed rather than significant clumps and edge effects. Dehydration of samples by using various concentrations of alcohol is required for the clarity of diatoms frustules.

Conclusions:

The analysis of diatoms samples is based on the clarity of the samples. Sometimes concentrated samples having silt and large amount of organic material posed a challenge for the type of oxidizing agent utilized for their digestion. The present method reported in this

study is well suited to counter such types of samples. A combination of nitric acid and hydrochloric acid helps in speedy and clear digestion of diatoms samples which is helpful in capturing of clear image for microscopic studies. This method is also suitable when a large amount of sample is required for ecological and paleontological studies especially where a representative sample is required. The damage caused by centrifugation due to the separation of diatoms valves and spines is avoided by decantation. Although this method for processing of diatoms may not be a replacement of the earlier reported method, but it is definitely fast, reproducible and results a clear suspension.

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