The Comparison of Different Diatom Digestive Method using HCl-H_2O_2 and HCl -$KMnO_4$ in Telaga Pengilon Dieng

Christopher Hardian Widyanta [a], Tri Retnaningsih Soeprobowati [a,b,c,*], Jumari [a], Riche Hariyati [a]

[a]Department Biology, Faculty Sciences, and Mathematics Diponegoro University

[b]School of Postgraduate Studies, Diponegoro University,

[c]Center for Paleolimnoloy (CPalim), Universitas Diponegoro, Indonesia;
*trsoeprobowati@live.undip.ac.id

Received 01 December 2020 Accepted 13 December 2020 Published 17 December 2020

Abstract

Identification diatom has been applied as an important key to tracing paleoenvironmental conditions. The knowledge for diatom extraction from sediment is continuing, but the diatom digestive methods in the tropical area is still limited. Telaga Pengilon, located in Dieng Indonesia, is rich in organic material in the sediment, the sample also associates with epiphytic and benthic diatom. Therefore, extracting pure diatom frustules free of organic matter from the sediments is essential for this application. To find the appropriate method for extracting diatoms in Telaga Pengilon, this research compares HCl with H_2O_2 and HCl with $KMNO_4$ as the digestive reagent. Based on the statistical result (HCl, $KMnO_4$) less time consumed than (HCl, H_2O_2) during the digestive process ($p<0.05$) and produced the same amount of residue ($p> 0.05$). For Pinnularia and Frustulia, the majority of diatoms could be extracted using both methods. Under microscopy, the structure of diatoms remained almost perfect after digestion with H_20_2, the striae clearly visible and the residue digested correctly. Another method using HCl, $KMnO_4$ fragmentation was found for some Pinnularia, and for Frustulia, the striae are clearly visible. This study demonstrated that different diatoms have different resistance for reagents and some of the locations need different reagents because different materials are contained in the sediment and need help to remove material organically. As far as the HCl and H_2O_2 are appropriate digestion methods in a tropical area such as Telaga Pengilon, HCl, $KMnO_4$ can be a substitute for the less time-consuming process.

Keywords: Diatom, digest, methods, HCl, $KMnO_4$, H_2O_2

INTRODUCTION

Diatoms are microscopic aquatic organisms that spread out in many different aquatic environments. Diatom has an essential role as a primary oxygen producer, contributing 20% of oxygen (De Tommasi, 2016). Diatoms are preserved in the sediment because of their special cell wall made from silica (Round *et al.*, 2007). Their silica cell called frustule has specific surface ornamentation which made diatom easier for identification process Taylor, 2016; Soeprobowati *et al.*, 2012, 2016). Diatoms are well known as bioindicators because of their sensitivity to respond to environmental changes. (Antoinades, 2008; Hobbs *et al.*, 2009; Griffiths, 2015).

Diatom is unicellular, photosynthetic, and autotrophic organisms. Its frustule is comprised of two thecas. Each theca is composed of the valve face (epivalve and hypovalve) and the valve mantle. The two valves are smaller than the other and fit one inside another. Two kinds of frustules depend on their shape making the diatoms subdivide into two major order Centrales and Pennales (Hasle & Syvertsen, 1997; Ajay & Sakshi, 2018). Diatom generally ranges in size from 2-200 µm. The siliceous wall can be highly patterned with various pores, its pattern is a tool to

* Corresponding Author
E-mail trsoeprobowati@live.undip.ac.id

identify the genera and species (Rohn & Frade, 2006).

Diatom analysis consists of three steps i.e., digestion (separation diatom from sediment particles), preparation (mounting diatom residue to the coverslip), and identification-enumeration (Soeprobowati *et al.,* 2016). There are many diatom digestion methods, such as using nitric acid (HNO_3) and H_2O_2 with potassium dichromate (KCr_2O_7) as a catalyst. Through repeated washing until the pH is neutral (Julius & Theriot, 2010), in the other research digestion by adding 37% H_2O_2 then heated until 80°C for about one hour, after that add $KMnO_4$ solution until precipitate occurred, then the precipitate was removed by adding a little HCl which dissolved in H (Van der Werff, 1955). The method by Setty dried the samples overnight at 125°C, then digest with 15% H_2O_2, heated in a water bath for 20 minutes, then wash the sample with distilled water and remove the supernatant, by using HCL 25%, then rewash it with distilled water (Setty, 1966).

In this study, we investigated the difference of two digestive methods based on Battarbee (1937;1986) Hydrochloric acid plus hydrogen peroxide) and based on Taylor (2007) using (Hydrochloric acid and Potassium permanganate). This is a methodological study with applying them to a paleoenvironmental study in Telaga Pengilon. Telaga Pengilon is a crater lake in Dieng with clear water and the pH tent to alkali around 6-7 Soeprobowati *et al.,* (2016). The land around Telaga Pengilon is facing environmental degradation due to intensive agricultural use, mostly potatoes (Soeprobowati *et al.,* 2018). Previous research in Telaga Pengilon used diatom to trace past environmental changes (Soeprobowati *et al.*, 2018). This research aims to compare 2 digestive methods for diatom analysis, which were HCl-H_2O_2 and HCl-$KMnO_4$ to digest the sediment from Telaga Pengilon Dieng.

METHODS

Sampling site

The research was conducted in Telaga Pengilon, Dieng Indonesia altitude of 2,096 meters above sea level, and the samples were taken from different sites, especially from surface sediment across four different areas during the dry season (April-May). Collection samples across the region reflect the differentiation of sampling site area, and diatom were sampled and combined from approximately 0-3 cm surface sediment from the edge at water depth around 30 cm, using a scalpel and preserved in the refrigerator -4°C.

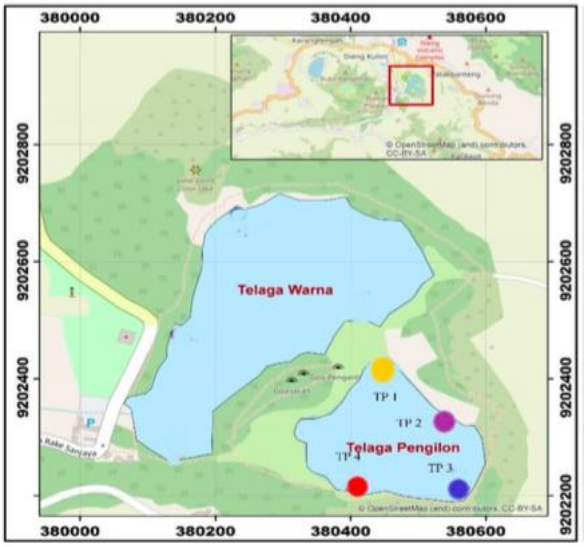

Figure 1. Research site in Telaga Pengilon showing the location of the four study areas outlet connected to Telaga Warna (yellow), near anthropogenic activity tourism (red and pink), near riparian vegetation (blue)

Digestive method using HCl and H_2O_2

These digestion methods using Battarbee (1937; 1986); Soeprobowati *et al.* (2012) modified from Battarbee the sample was either cleansed acid digestion. Each sample was sliced and weighed about 1 gr, then digested with 50ml HCl 10% heat on a hotplate about 90°C a fume cupboard until all organic material had been oxidized for about 2 hours. Wait for 24 hours, then remove the acid and wash in 80 ml distilled water. These processes repeat five times until the pH reaches neutral. After the repeated rinse, add the sample with 50 ml H_2O_2 heat again for 2 hours long, decant off supernatant and repeat the washing process at least five times to clean diatom frustules and remove $CaCO_3$. The morphology of

each taxon is identified based on the morphological similarity between the taxa, the terminology and identification used Krammer & Lange-bertalot (1991a, 1991b; 1991c;1991d; 2004).

Digestive method using HCl and $KMnO_4$

In this digestion method using Taylor *et al.* (2007), 10 ml $KMnO_4$ solution was added to each sample, mixed, then left for 24 hours. In a fume cabinet, 5-10 ml concentrated HCl (32%) was added to the samples, then heated on a hotplate 90°C for 1 to 2 hours until the solution became clear or turned on into yellow color. After the oxidation has completed, the samples are allowed to cool and then transferred to a 15 ml centrifuge tube. The samples were rinsed by centrifuging with distilled water at 2500 rpm for 10 minutes repeated the centrifugation process four times. After centrifugation, decants off the supernatant and filled with distilled water. The morphology of each taxon is identified based on a morphological similarity between the taxa, the terminology and identification used Krammer & Lange-bertalot (1991a, 1991b; 1991c; 1991d; 2004).

Data Analysis

Differences in time and residue between each method were evaluated statistically by comparing means using one way ANOVA; the analysis were carried out with SPSS.

RESULTS

Based on the analysis, 69 taxa were identified belonging to 21 genera, Gomphonema, Navicula, Eunotia, Pinnularia (Table 1) considered abundant species, regarding the generic level. Methods from Battarbee showed that more species could be identified than Taylor methods, the different result of identification process caused by the undigested residue that covered some diatom striae disturbed identification process. Some of the sediment contains different material that needs different time to digest.

Table 1. List of species using different methods

No	Species	Method	
		HCl, H_2O_2	HCl, $KMnO_4$
1	*Achnanthes clavei* (Grunow)	v	
2	*Achnanthes delicatula* (Kutzing)	v	
3	*Achnanthidium atomus* (Hust)		v
4	*Achnanthidium saprophilum* (Kobayasi & Mayama)	v	
5	*Aulacoseira tenella* (Nygaard)	v	
6	*Brachysira brebissonii* (Grunow)	v	
7	*Craticula halophila* (Grunow)	v	
8	*Cymbopleura heteropleura* (Ehrenberg)		v
9	*Cymbopleura inaequalis* (Ehrenberg)	v	
10	*Cymbopleura perprocera* (Krammer)		v

Table **1**. List of species using different methods (Cont.)

No	Species	Method	
		HCl, H_2O_2	HCl, $KMnO_4$
11	*Cymbopleura sublanceolata* (Krammer)		v
12	*Denticula elegans* (Kutzing)	v	v
13	*Denticula valida* (Pedicino)	v	
14	*Diploneis ovalis* (Hilse)	v	v
15	*Diploneis smithii* (Brebisson)	v	
16	*Encyonema gracile* (Rabenhorst)	v	
17	*Encyonema latum* (Krammer)	v	
18	*Encyonema neogracile* (Krammer)		v
19	*Encyonema obscurum* (Krasske)	v	
20	*Encyonema silesciacum* (Bleisch)		v
21	*Eunotia arcus* (Ehrenberg)		v
22	*Eunotia bilunaris* (Ehrenberg)	v	v
23	*Eunotia incisa* (Smith)	v	v
24	*Eunotia minor* (Kutzing)	v	v
25	*Eunotia formica* (Ehrenberg)	v	
26	*Eunotia pirla* (Carter & Flower)	v	
27	*Eunotia praerupta var. bidens* (Smith)	v	
28	*Eunotia subarcuatoides* (Alles, Norpel & Lange-bertalot)	v	
29	*Fragilaria brevistriata* (Grunow)	v	
30	*Fragilaria perminuta* (Gunrow)	v	
31	*Fragilaria pinnata* (Ehrenberg)		v
32	*Fragilaria synegotesca*	v	
33	*Frustulia saxonica* (Rabenhorst)	v	
34	*Frustulia crassinervia* (Brebisson)	v	
35	*Frustulia rombhoides* (Ehrenberg)		v
36	*Gomophonema septa* (Moghadam)		v
37	*Gomphonema demersum* (Reichardt)		v
38	*Gomphonema insularum* (Kociolek)	v	v
39	*Gomphonema laticollum* (E. Richardt)	v	
40	*Gomphonema pantropicum* Reichardt)	v	
41	*Gomphonema parvulum* (Kutzing)	v	
42	*Gomphonema olivaceoides* (Patrick)	v	
43	*Gomphonema subclavatum* (Grunow)	v	
44	*Gomphonema subtile* (Ehrenberg)	v	
45	*Gomphonema vibrio* (Ehrenberg)		v
46	*Hantzchia amphioxys* (Ehrenberg)	v	
47	*Luticola muticopsis* (Van Heurck)	v	
48	*Luticola saxophilla* (W.Bock ex Hustedt)	v	
49	*Navicula americana* (Ehrenberg)		v
50	*Navicula bottnica* (Grunow)		v
51	*Navicula inelegans* (Grove & Sturt)		v

Table **1**. List of species using different methods (Cont.)

No	Species	Method	
		HCl, H_2O_2	HCl, $KMnO_4$
52	*Navicula leptostriata* (E.G.Jørgensen)	v	v
53	*Navicula linearis* (Grunow)	v	
54	*Navicula perminuta* (Grunow)		v
55	*Navicula longicephala* (Hudstedt)	v	
56	*Navicula angusta* (Grunow)	v	
57	*Neidium iridis* (Ehrenberg)		v
58	*Nitzschia lacuum* (Lange-bertalot)		v
59	*Nitzschia palea* (Kutzing)		v
60	*Pinnularia divergentissima* (Smith)	v	
61	*Pinnularia gibba* (Ehrenberg)	v	
62	*Pinnularia macilenta* (Ehrenberg)		v
63	*Pinnularia microstauron* (Ehrenberg)		v
64	*Pinnularia rhenohassiaca* (Krammer & Lange bertalot)	v	
65	*Pinnularia sinistra* (Krammer)		v
66	*Pinnularia subcapitata var. Elongata* (Krammer)	v	
67	*Pinnularia viridis* (Nitzsch)	v	
68	*Stauroneis javanica* (Grunow)		v
69	*Staurosira brevistriata* (Grunow)	v	

Table 2. The different time and residue methods

Site	Time (minutes)		Residu (gr)	
	HCl. H_2O_2	HCl. $KMnO_4$	HCl. H_2O_2	HCl. $KMnO_4$
Pengilon 1	4584	1608	13.43	13.58
Pengilon 2	4580	1607	13.58	13.94
Pengilon 3	4582	1608	14.08	12.9
Pengilon 4	4580	1607	13.53	13.57

The time required for complete digestion of sediment with two different diatom digestion methods were shown in Table 2. the statistical result showed differences among two methods ($p<0.05$) digestion methods using HCl and H_2O_2 needed the most extended time around ± 4580 minutes. in contrast, digestion methods using $KMnO_4$ needed around ± 1608 minutes, the different time caused by different step during the rinsed process using aquadest. The sample was oxidized with Hydrochloric acid (HCl) and Hydrogen peroxide (H_2O_2) need allowed sample for overnight to separate the natant and supernatant, to remove the supernatant, samples were rinsed repeatedly with deionized water, these processes were repeated three times every 8-10 hours. While the sample was oxidized with Potassium permanganate ($KMnO_4$) were rinsed using centrifuge 2500 rpm for 4 minutes repeated 4 times.

Digestion methods by Battarbee (1973; 1986) using Hydrochloric acid (HCl) and Hydrogen peroxide (H_2O_2) particularly suitable for preparing bulky samples such as epiphyton, hot hydrogen peroxide is rapidly oxidizing samples with high organic content, the purpose for adding 10% HCl is to eliminate excess $CaCO_3$ present in sediment samples. Sediment will effervesce (fizz) if $CaCO_3$ is present (Ruhland *et al.*, 1999), Battarbee procedure with water bath method is efficient for large numbers of sediment sample (Renberg, 1990) also helps to break up chain diatoms and separate single frustules, and often

gives a better distribution for observation using a light microscope (Serieyssol *et al.*, 2010).

The amount of residue for two different digestion methods was compared by the weight of the sample after digestion; based on statistical results, there is no difference between the amount of residue ($p>0.05$). As demonstrated in Table 2, Hydrochloric acid (HCl) and potassium permanganate ($KMnO_4$) methods had the same residue with Hydrochloric acid (HCl) and Hydrogen peroxide (H_2O_2) method, it means there are no differences on digestion capability aspect, but potassium permanganate ($KMnO_4$) is suitable as a complement combined with Hydrochloric acid (HCl) 32%, this method is more efficient because of the less of time-consuming.

Diatoms may be destroyed by different function and concentration of the digestive reagent. Decalcification is important if the sample treats with nitric or sulphuric acid (HNO_3/H_2SO_4) later, because this acid combines with calcium will cause the formation of an insoluble formation, in this case, HCl-$KMnO_4$ is the recommended technique if the sample does not contain calcium, however, HCl-$KMnO_4$ is recommended for samples with high organic material. Besides H_2O_2 method is less corrosive than acid, good for the sample that needs a little cleaning and for research where corrosion should be limited such as SEM studies (Taylor, 2007).

Digestion became less chemically abrasive since the diatoms extracted from the samples were preserved better than those extracted using 10% hydrochloric acid and potassium permanganate. The lower degree of destruction of the diatoms allowed more genera to be identified in the sample (Fucci *et al.*, 2015). Our data confirm previous suggestions that the use of long H_2O_2 oxidation times for cleaning external organic material from fresh diatom frustules has a problematic impact on the integrity of non-crystalline silica (Galbraith, 2006).

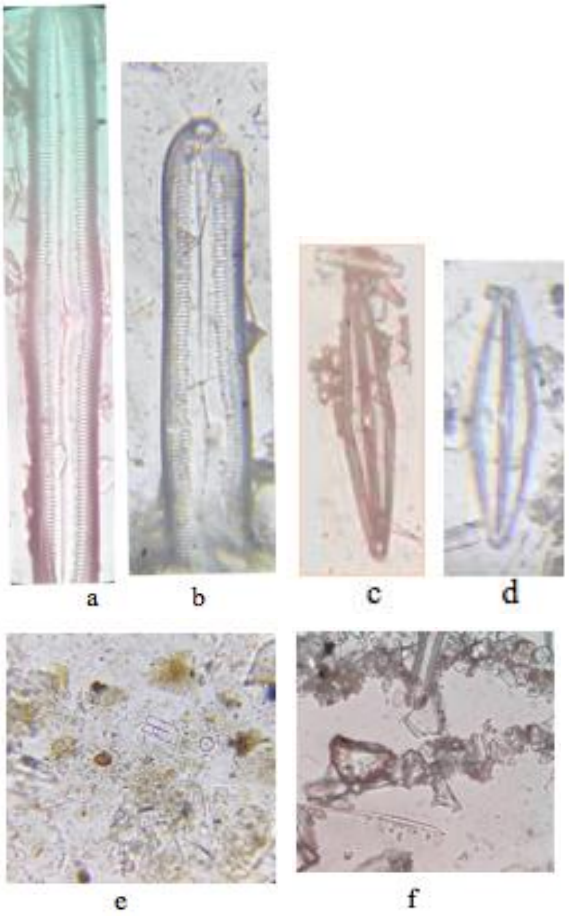

Figure 2. The difference between *Pinnularia* using HCl.H_2O_2 methods (a) and *Pinnularia* using HCl.$KMnO_4$ (b) the difference of *frustulia* using HCl. H_2O_2 methods (c) and *frustulia* using HCl.$KMnO_4$ (d), the difference of broken *frustulia* using HCl. $KMnO_4$ methods (e), and broken *frustulia* using HCl.H_2O_2 (f)

The second method used for the South Atablefrican digestion procedure, the common use of acid with $KMnO_4$ improves the removal of organic material as the hydrogen ions contributed by the sulfuric acid aid the oxidative action of $KMnO_4$ on organic matter, adding oxidizing power. Once the color change was obtained, samples were centrifuged (Morales *et al.*, 2013). Under the light microscope, the frustule of Pinnularia looks intact, striae looks clearly visible, and the background looks clear with a little junk which consists of broken frustules after digestion by HCl-H_2O_2, while the digestion using HCl-$KMnO_4$. The frustule of Pinnularia remains only

half, the striae look clearly visible, and the background is also transparent. Therefore, it contains a broken frustule, which is a little bit more than the HCl-H_2O_2 method. After digestion using HCl-H_2O_2, the frustule seems intact, the central area and raphe also look almost clearly visible, and the background is more transparent than the HCl-$KMnO_4$ method. *Frustulia* represented in figure 2 uses HCl-$KMnO_4$ to extract the frustule from the sediment. The frustule seems intact, the central area and raphe look clearly visible. There are broken frustules in the background, the amount of the broken frustules is more significant than digestion using HCl-H_2O_2.

CONCLUSION

This research compares HCl with H_2O_2 and HCl with $KMnO_4$ as the digestive reagent to find the appropriate method for extracting diatoms from Telaga Pengilon. The structure of diatoms was relatively more clear after digestion with HCl-H_2O_2, compared to HCl-$KMnO_4$ as the striae were more clear to make it easier in the identification process.

ACKNOWLEDGEMENTS

This research was supported by the Indonesian government through Research Grant for university research (PTUPT), PNBP DIPA Undip Number 101-120/UN7.4.3/PP/2019. The heartfelt thanks to Alam, and Geyga for helping in the fieldwork and lab work.

REFERENCE

Antoniades D, Hamilton PB, Douglas MSV, Smol JP. 2008. Diatom of North America: The freshwater floras of Prince Petrcik, Ellef Ringnes, and Northern Ellesmere Island from Canadian Arctic Archipelago. *Iconographia Diatomologica*, 17.

Ajay R & Sakshi M. 2018. Significance of Diatoms in Diagnosis of Drowning Deaths: A Review, *Journal of Forensic & Genetic Science.*

Battarbee RW. 1973. A new method for estimating absolute microfossil numbers, with special reference to diatoms. *Limnology and Oceanography*, 18, 647–653.

Battarbee RW.1986. Diatom analysis. In: Berglund, B.E. (ed.) *Handbook of Holocene Paleoecology and Paleohydrology*, Wiley & Sons, London, 527–570.

De Tommasi E. 2016. Light manipulation by single cells: the case of diatoms. *Journal of Spectroscopy.*

Fucci N, Pascali V, Puccinelli C, Marcheggiani S, Mancini L, and Marchetti D. 2015. Evaluation of two methods for the use of diatoms in drowning cases. *Forensic Science, Medicine, and Pathology*, 11(4), 601-605.

Galbraith ED. 2006. Interactions between climate and the marine nitrogen cycle on glacial-interglacial timescales. *Ph.D. Thesis*, Univ. of British Columbia.

Griffiths K. 2015. *High-latitude responses of diatom and chironomid assemblages to regional climate changes and other environmental stressors*. Canada: Queen's University Kingston, Ontario, Canada.

Hasle GR & Syvertsen EE. 1997. Marine diatoms, in Tomas CR (Ed.) *Identifying Marine Phytoplankton*, 5-385.

Hobbs WE, Wolfe P, Inskeep WP, Amskold A, and Konhuser KO. 2009. Epipelic diatoms from an extreme acid environment: Beowulf Spring, Yellowstone National Park, USA. *Nova Hedwigia*, 135, 71-83.

Krammer K & Lange-Bertalot H. 1991b. Süsswasserflora von Mitteleuropa. Bacillariophyceae, Teil 3: Centrales, Fragilariaceae, Eunotiaceae. Jena. *Gustav Fischer Verlag*, p 576.

Krammer K & Lange-Bertalot H. 2004. Bacillariophyceae 4. Teil: Achnanthaceae, Kritische Erganzungen zu Navicula (Lineolatae), Gomphonema Gesamtliteraturverzeichnis Teil 1-4 [2nd revised edition] [With "Ergänzungen und Revisionen" by H. Lange Bertalot]. In: H. Ettl *et al.*, Suesswasserflora von Mitteleuropa. *Spektrum Akademischer Verlad Heidelberg*, 2(4), 1-468.

Krammer K & Lange-Bertalot H. 1991c. Bacillariophyceae. 3. Teil: Centrales, Fragilariaceae, Eunotiaceae. - In: ETtl H, Gerloff J, Heynig H, M Ollenhauer D. (Eds): *Süsswasserflora Von Mitteleropa Fischer Verlag, Stuttgart*, vol. 2/3, pp 1-576. ISBN 978-3-8274-1987-3

Krammer K & Lange-Bertalot H. 1991d. Süsswasserflora von Mitteleuropa. Bacillariophyceae, Teil 3: Centrales, Fragilariaceae, Eunotiaceae. Jena. *Gustav Fischer Verlag*, pp 1-576. ISBN: 978-93-83083-77-0

Krammer K & Lange-Bertalot H. 1991a. Bacillariophyceae. 4. Teil: Achnanthaceae, Kristische Ergänzungen Zu Navicula (Lineolate) Und Gomphonema. In: ETtl H, Gerloff J, Heynig H, M Ollenhauer D. (Eds)*: Süsswasserflora Von Mitteleropa Fischer Verlag, Stuttgart*, vol 2/4, p 437.

Levin EA, Morgan RM, Scott KR, and Jones VJ. 2017. The transfer of diatoms from freshwater to footwear materials: An experimental study assessing transfer, persistence, and extraction methods for forensic reconstruction. *Science & Justice*.

Morales LV, Sigman DM, Horn MG, and Robinson RS. 2011. Cleaning methods for the isotopic determination of diatom-bound nitrogen in non-fossil diatom frustules. *Limnology and Oceanography: Methods*, 11,101-112.

Nagy SS. 2011 Collecting, Cleaning, Mounting, and Photographing Diatoms. In: Joesph Seckbach PK, editor. *The Diatom World*. Springer, New York, pp 5-11.

Renberg I. 1990. A procedure for preparing a large set of diatom slides from sediment cores. *Journal of Paleolimnology*, 4, 87-90.

Rohn EJ & Frade PD. 2006. The Role of Diatoms in Medicolegal Investigations I: The History, Contemporary Science, and Application of the Diatom Test for Drowning. *Forensic Examiner*, 15(3).

Round FE, Mann DG, and Crawford RM. 2007. *Diatoms: Biology and Morphology of the Genera*. Cambridge, p 747.

Ruhland K, Karst T, Paterson A, Eaves GR, Smol JP, and Cumming BF. 1999. *Standard Sediment Sample Preparation Methods for Siliceous Microfossils (Diatoms and Chrysophyte Scales and Cysts)*. Paleoecological Environmental Assessment and Research Laboratory (PEARL), Department of Biology Queen's University.

Schowe KA. 2012. Diatom communities across a gradient of acid mine drainage on the West Coast, South Island, New Zealand. *Thesis*. Master of Science in Environmental Science, University of Canterbury.

Serieyssol K, Chatelard S, and Cubizolle H. 2010. Diatom fossils in mires: a protocol for extraction, preparation, and analysis in palaeoenvironmental studies. *International Mire Conservation Group and International Peat Society*, 12, 1-11.

Soeprobowati TR, Hadisusanto S, and Gell P. 2012. The diatom stratigraphy of Rawapening Lake, Implying Eutrophication History. *Am J Env Sci* 2012, 8(3), 334-344.

Soeprobowati TR, Suedy SWA, and Hadiyanto. 2016. Diatom and water quality of Telaga Warna Dieng, Java, Indonesia. *IOP Proceeding. Earth and Environmental Sciences*, 55, 1-6.

Soeprobowati TR & Suedy SWA. 2018. Find the future from the past: Paleolimnology in Indonesia. *E3S Web of Conferences*, 31, 08002. EDP Sciences.

Taylor JC & Cocquyt C. 2016. Diatom from the Congo and Zambesi Basins. *Methodologies and identification of the Genera*. ISSN 1784-1291.

Taylor JC, Harding WR, Archibald CGM. 2007. An Illustrated Guide to Some Common Diatom species from South Africa. *Water Research Commission*, pp 178. ISBN 1-77005-484-7.