Digestion diatom using Battarbee and Ruhland methods for Pengilon Lake, Dieng, Wonosobo, Central Java, Indonesia

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Abstract. Diatoms have been used as a bioindicator of water quality since 1990s. Digestion methods are a fundamental process prior to paleolimnological analysis with the aim to remove organic material from diatom cells which makes it easier for the identification process. This research objective was to evaluate different methods for digestion diatoms that suitable for samples from tropical sediment, especially from Pengilon Lake Dieng, Central Java, Indonesia. Pengilon Lake was formed by eruption of Mount Prahu with clear water and used as an irrigation source. This research was compared Battarbee and Ruhland methods. In the Battarbee method, the samples were freeze-dried, and approximately 1 gram of each sample was measured and used HCl-H₂O₂ for extraction of the diatom. In the Ruhland method, the samples were freeze-dried, and approximately 1 gram was digested in 10 ml of concentrated HNO₃-H₂SO₄. Under the Battarbee method, at least 21 diatom species were identified, and under the Ruhland method, 23 diatom species were identified. Ruhland method is less time consuming around 33 hours, where the Battarbee method around 73 hours. From the visibility Battarbee method, striae are clear, but some undigested material disturbing identification process. The different results of diatom caused by the imperfect digestion of the organic material, which is influenced by chemical components for extracts organic. As far as the Ruhland method are an appropriate digestion method in a tropical area such as Pengilon Lake.

1. Introduction
Indonesia has many tropical lakes by which the diatom composition and assemblages are affected by the complex interaction of physical, chemical, and biological factors. Dieng is a volcanic territory was formed by the primordial mountain. Pengilon Lake is one of the various ecosystems around Dieng Plateau, the lake was formed by volcanic activity [1,2]. Pengilon Lake is well known for its tourist attraction and potential as a water source for agricultural irrigation. Pengilon Lake has a pH of around 2.52-7.9. Pengion Lake is determined as a conservation area based on Regulation of Ministry of Agricultural No. 740/Ktps/Um/11/1978 30 November 1978 that the total conservation area about 39.6 ha [3,4]

Diatoms are unique and specific microscopic algae; the body comprises two valves, specifically epitheca and hypotheca, forming frustule, with silica cell walls (SiO₃). Therefore, diatoms are easily collected and stored and record changes in the short and long term [5]. Diatoms are used as environmental bio-indicators. This is because diatoms greatly affect aquatic life. They an important role
as a food source in the food chain for various marine organisms and play a role in the transfer of carbon, nitrogen, and phosphate [6].

The result of diatom analyses from lake sediments are usually presented in the form of percentage diagrams in which the variation in the percentage frequency of individual taxa or groups of taxa are shown for successive levels in the sediment [7,8]. There are currently over 260 known living diatom genus with more than 100,000 species [5]. The diversity and species composition of diatom has studied by diatonic for a century, but the diatom composition in the tropical area also been unclear, also research about diatom communities in the tropical mountain area is still limited [1,2]

To get a correct interpretation result of using diatom for water quality and paleolimnological assessment, there are lab work activities that have to be performed precisely consists of three stages: digestion-to separator diatom from materials, preparation-to mount diatoms on the slide, and identification of enumeration to determine the species name [3]. There is various method for diatom digestion, that is Ruhland method such as using HNO_3 and H_2SO_4 at the Paleocological Environmental Assessment and Research Laboratory (PEARL), Queen’s University, approximately 1 gram of sediment was digested in 10 ml of concentrated nitric acid (HNO_3) and sulfuric acid (H_2SO_4) using a water bath digestion [9], and Battarbee (1937;1986); Soeprobowati et al. (2012) such as using HCl and H_2O_2 modified from Battarbee the sample was either cleansed acid digestion. The step of the Battarbee method is each sample was cut every inch, then digested using 50 ml of 10% HCl and heating it on an electric stove at 90 C for 2 hours carried out in the acid chamber. Leave it for 24 hours, then take the supernatant and wash it with 80 ml of distilled water, washing it 5 times with the aim of removing CaCO_3. Heat using 50 ml of H_2O_2 and reheat for 2 hours, then wash again with distilled water 5 times [10]. The morphology of each taxon was identified using the Krammer & Lange-bertalot book, 1991a, 1991b; 1991c; 1991d; 2004 [11-15]

In this research, we compared two different digestion methods from Battarbee (Hydrogen chloride (HCl), Hydrogen peroxide (H_2O_2) with Ruhland method Nitric acid (HNO_3), Sulfuric acid (H_2SO_4) to digest and applying them to paleoenvironmental study in Pengilon Lake. Pengilon Lake has a function for agricultural products such as potatoes, cabbage, carica, and carrot [16]. Previous research in Pengilon Lake used diatom to trace past environmental changes [17]. This study aim to examine two digestive methods for diatom analysis, which were Battarbee method and Ruhland method to find effective methods based on identification result, time-consuming and weight residue.

2. Methods
The study was carried out in four different sites located in Pengilon Lake, Dieng. Sediment samples were collected in April 2019, the sample sites are located in a location near Warna Lake (site 1) around the channel of Pengilon Lake, site 2 represents the area near with agriculture, site 3 is located in the forest around the lake that dominated with grass and site 4 is an area with highest sediment deposition.

Figure 1. Research sites in Pengilon Lake, Dieng, Wonosobo, Central Java
Digestive Method using HCl – H₂O₂. This digestion using the method of Battarbee (1937; 1986); Soeprobowati et al. (2016). A sediment sample of 1 gram was put into a beaker and given 50 ml of 10% HCl. Sediment samples were heated on a water bath for 2 hours at a temperature of 80-90°C. Sediment samples were deposited for 6-12 hours. The supernatant removed and then added as much as 80 ml of distilled water. The 4th treatment was repeated three times; hence the pH was neutral. The supernatant was taken only 50-70%. Sediment samples were added with 50 ml of 10% H₂O₂. Sediment samples were heated for 2 hours at 80-90°C. Once heated, and then cooled for 6-12 hours. The supernatant removed and then added 80 ml of distilled water. The treatment of the ten repeated three times. Diatom residue dripped over 1 400 ml coverslip, and then placed on a hotplate and heated for 20 minutes and let the adhesive Naphrax. Identification was achieved by distinguishing morphological characteristics similar to taxa, terminology, and identification using Krammer & Lange-Bertalot.

Digestive Method using H₂SO₄ – HNO₃. This digestion using the method of Ruhland et al. (1999). Glass beaker prepared. HNO₃ 5.3 ml put in a glass beaker advance slowly. 4.7 ml H₂SO₄ was added to the beaker slowly and while stirring using a stirrer. The acid mixture is gradually poured into the sediment in each glass and then stirred (2-5 ml, 5-7 ml, and 7-10 ml). Then the digestion process is the same as the Battarbee method.

3. Results and discussion

The study compared two different digestion methods for the extraction of diatoms. The extraction method used is the modification method of Battarbee using 10% Hydrochloride acid (HCl) and 10% Hydrogen peroxide (H₂O₂) and other methods using Sulfuric Acid (H₂SO₄) and Nitric Acid (HNO₃) used in Canada, PEARL. Both of these methods were tested to sediment or soil samples from the Pengilon Lake, Dieng.

| Location | Time (Second) | Residue (gram) |
|----------|---------------|----------------|
|          | HCl-H₂O₂      | HNO₃-H₂SO₄     | HCl-H₂O₂ | HNO₃-H₂SO₄ |
| Site 1   | 4584          | 2094           | 13.43    | 12.98      |
| Site 2   | 4580          | 2238           | 13.58    | 13.48      |
| Site 3   | 4582          | 2006           | 14.08    | 14.35      |
| Site 4   | 4580          | 1879           | 13.53    | 13.18      |
| Average  | 4581.50       | 2029.25        | 13.6550  | 13.4875    |

The difference in digestion time was calculated based on the accumulated amount of time (preparation until the completion of the digestion method) with units of minutes, then data entered into SPSS application for T-Test. Based on the data obtained, the Ruhland method required a shorter time than the Battarbee method; the difference obtained was very significant between the two methods; this was due to the more extended accumulation of the Battarbee method by 4581.50 minutes (73 hours 21 minutes) while the strong acid just need 2029.25 minutes (33 hours 49 minutes) (Table 1); which caused by the length of time during the deposition in the Ruhland method which was around 6-12 hours by four times with the centrifugation stage for 20 minutes, while in the Battarbee method which was about 6-12 hours by four times, the supernatant was discarded and aquades were added three times, without a centrifugation step.

The residual weight difference was calculated based on the accumulation amount of time (preparation until the completion of the digestion method) in units of minutes, then data entered into SPSS application. The residual results obtained from the two methods based on calculations did not significantly different. This was due to the accumulated weight of the Battarbee method, which was 13.6550 grams, while the Ruhland method obtained an average residual weight of 13.4975 grams (Table 1); this was given that the samples used were the same and had the same variance.
Table 2. List of species using different methods

| No. | Species                                                                 | Site 1 | Site 2 | Site 3 | Site 4 |
|-----|-------------------------------------------------------------------------|--------|--------|--------|--------|
| A   | B                                                                       | A      | B      | A      | B      |
|-----|-------------------------------------------------------------------------|--------|--------|--------|--------|
| 1.  | *Achantidium minutissimum* (Lange-Bertalot)                              | √      | √      |        |        |
| 2.  | *Brachysira brebi sono i. thermalis* (Grunow)                            |        | √      |        |        |
| 3.  | *Chamaepinnularia mediocris* (Lange-Bertalot)                           |        |        |        | √      |
| 4.  | *Cymbella tropica* (Krammer)                                            |        |        |        | √      |
| 5.  | *Cymbopleura naviculiformis* (Auerswald ex Heiberg Krammer)             |        |        |        | √      |
| 6.  | *Diploneis elliptica* (Kützing)                                         |        |        |        |        |
| 7.  | *Diploneis ovalis* (Hilse)                                              |        |        |        | √      |
| 8.  | *Encyonema mesiana* (Chilnoky) D.G. Mann                                |        |        | √      |        |
| 9.  | *Encyonema silesiacum* (Bleisch) D. G. Mann                             |        |        |        | √      |
| 10. | *Eunotia circumborealis* (Lange-Bertalot & Nörpel)                      |        |        | √      | √      |
| 11. | *Eunotia flexuosa* (Kützing)                                            |        |        |        | √      |
| 12. | *Eunotia formica* (Ehrenberg)                                           |        |        |        | √      |
| 13. | *Eunotia minor* (Kützing)                                               |        |        |        | √      |
| 14. | *Eunotia hamsherae* (Kützing)                                           |        |        |        |        |
| 15. | *Eunotia tropica* (Hustedt)                                             |        |        |        |        |
| 16. | *Flagilaria symrgrotesca* (Grunow)                                      |        |        |        |        |
| 17. | *Frustularia crasinervia var. saxonica* (Rabenhorst & Westendorp)      |        |        |        | √      |
| 18. | *Gomphonema affine* (Kützing)                                           |        |        |        | √      |
| 19. | *Gomphonema angustatum var. productum* (Grunow)                         |        |        |        | √      |
| 20. | *Gomphonema guaraniarum* (Metzeltin & Lange-Bertalot)                   |        |        |        | √      |
| 21. | *Gomphonema gracialis* (Ehrenberg)                                     |        |        |        | √      |
| 22. | *Gomphonema hawaiiense* (E. Reichardt)                                 |        |        |        | √      |
| 23. | *Gomphonema parvulum* (Kützing)                                         |        |        |        | √      |
| 24. | *Hantzschia amphioxys* (Ehrenberg)                                     |        |        |        | √      |
| 25. | *Luticula acidoclina* (Lange-Bertalot)                                  |        |        |        | √      |
| 26. | *Navicula exilis* (Kützing)                                             |        |        |        | √      |
| 27. | *Navicula cryptonella* (Lange-Bertalot)                                 |        |        |        | √      |
| 28. |                                                                         |        |        |        | √      |
Navicula hemasoides (Kützing)  
Navicula radiosa (Kützing)  
Navicula veneta (Kützing)  
Nitzchia palea (Skvortsov)  
Nitzchia perminuta (Grunow)  
Pinnularia viridis (Ehrenberg)  
Pinnularia microstauron  
Navicula veneta (Kützing)  
Navicula radiosa (Kützing)  
Navicula hemasoides (Kützing)  
Navicula veneta (Kützing)  
Navicula radiosa (Kützing)  
Navicula veneta (Kützing)  
Navicula radiosa (Kützing)  
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Navicula veneta (Kützing)

In the Battarbee method, 21 species from 16 genera were found, namely Achnantidium, Brachysira, Cymbella, Cymbopleura, Diploneis, Encyonema, Eunotia, Flagilaria, Gomphonema, Hantzchia, Luticola, Navicula, Nitzchia, Pinnularia, Pulchella, and Sellaphora. Simultaneously, the robust Ruhland method discovered as many as 23 species from 11 genera comprising Achnantidium, Chamaepinnularia, Encyonema, Eunotia, Frustulia, Gomphonema, Navicula, Nitzchia, Pinnularia, Planothidium, and Straurosira. The same species found on the second method were Achnantidium minutissimum, Encyonema silesiacum, and Navicula cryptotenella.

Figure 2. The different of Achnantidium minutissimum (a) Battarbee (b) Ruhland
Based on comparing the *Achantidium minutissimum* species in the Battarbee and Ruhland methods, the results obtained from the strong acid method's observation are more frustule than the Battarbee method. However, the striae in the Battarbee method seem clearer—comparison of *Encyonema silesiacum* species using the strong acid and battarbee methods. The results obtained from the Ruhland method's observation that the striae were more clearly visible than using the Battarbee method and frustule, which was longer, and the nodule area was more visible in this method. Frustule of diatom are basically composed by porous, hydrated, amorphous silica provided with several surface defects such as Si-OH and Si-H groups [18]. The results obtained from observations on the Battarbee striae method were noticed more clearly, whereas by using the Ruhland method, the structure of the diatoms was not visible. However, the frustule was more likely an imperfect digestion process causing this.
Figure 5. The different organic materials (a) Battarbee (b) Strong Acid Magnification 100x

Based on the difference between the two methods, the results obtained were the most organic material contained in the Battarbee method (Figure 4); this was allegedly due to the deposition time and digestion time were less than the specified time to make the digestion process less than perfect. Ideal diatom test should be simple at the digestive process, safe and time saving; instruments and reagents required are cheaper and free of diatom; the damage of digestive reagents to diatom is mild; the organics residue is minimal and hardly interfere with microscopic observation [19]. The number of species in a microscope screen is higher in the Ruhland method; this suggests that the Ruhland method digests organic material perfectly, combine about using centrifuge than setting down the supernatant an alternative for less consuming time.

4. Conclusion
This research compares Battarbee method and Ruhland method as the digestive reagent to find an appropriate method for extracting diatom from Pengilon Lake. The structure of diatoms by the digestion method using HCl and H$_2$O$_2$ (Battarbee) obtained the results of striae in the observed species, specifically Achantidium minutissimum, Encyonema silesiacum, and Navicula cryptonella were seen more clearly and more organic matter compared to the Ruhland method. However, in the process of digestion, it takes longer. In Ruhland method, frustule looks longer, nodules are clear, and striae are not apparent. From these observations, it can be noticed that Ruhland method are a reasonably appropriate method for the digestion process in sediment samples located in the tropics.

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