EU4Environment in Eastern Partner Countries:
Water Resources and Environmental Data (ENI/2021/425-550)

MANUAL FOR TREATMENT OF BENTHIC DIATOM SAMPLES AND PREPARATION OF PERMANENT DIATOM SLIDES

following instructions of European standard EN 13946:2014

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ABOUT THIS MANUAL

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ABOUT EU4ENVIRONMENT – WATER RESOURCES AND ENVIRONMENTAL DATA

This Programme aims at improving people's wellbeing in EU's Eastern Partner Countries and enabling their green transformation in line with the European Green Deal and the Sustainable Development Goals (SDGs). The programme's activities are clustered around two specific objectives: 1) support a more sustainable use of water resources and 2) improve the use of sound environmental data and their availability for policy-makers and citizens. It ensures continuity of the Shared Environmental Information System Phase II and the EU Water Initiative Plus for Eastern Partnership programmes.

The Programme is implemented by five Partner organisations: Environment Agency Austria (UBA), Austrian Development Agency (ADA), International Office for Water (OiEau) (France), Organisation for Economic Co-operation and Development (OECD), United Nations Economic Commission for Europe (UNECE). The action is co-funded by the European Union, the Austrian Development Cooperation and the French Artois-Picardie Water Agency based on a budget of EUR 12,75 million (EUR 12 million EU contribution). The implementation period is 2021-2024.

https://eu4waterdata.eu

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1. Introduction

This Manual aims to provide both conceptual and practical recommendations for the laboratory treatment of benthic diatom samples. It describes most common methods for cleaning diatom samples and preparation of permanent slides for microscopic examination. The term 'cleaning' refers to removal of the organic content of diatoms and all the organic material in diatom samples to allow observation of the ornamentation of diatom frustules. Diatom identification is based on fine structures of diatom cell walls, which can be well visible only when the organic content of the cell is removed. After the 'cleaning' procedure, the diatom sample contains only clean silicious diatom frustules and inorganic particles.

There are several different methods available and specified in the literature. The selection of the method depends on the practitioner preferences, but also on the type of the sample and the amount of organic content. Details of methods that are given in this manual are the most common and described in the EN 13946 (CEN, 2014), but other methods may be equally suitable.

Diatom samples treated following these instructions are suitable for microscopic analysis for purposes of ecological status assessment according to Water Framework Directive (2000/60/EC).

2. Methods for cleaning diatoms for microscopic examination

Below we present the two most common treatment methods; more methods can be found at www.diatoms.org web (Spaulding et al. 2021) for example.

Samples rich in organic matter in addition to diatoms need stronger oxidation than clean ones and the optimum ratio of sample to oxidants may need to be tested first. Quantities of reagents can be adapted, so long as ratios between reagents are maintained, in order to suit local conditions and the samples. In general, it is recommended to start the treatment with less aggressive methods and if this fails, then use more aggressive oxidizing agents. Solid carbonates can cause problems and can influence the type of treatment and the sequence of oxidants used, e.g., in carbonate-rich waters the carbonates shall be removed by HCl (see 2.3.1.) before using strong sulfuric acid in order to prevent formation of gypsum.

Important note: It is always necessary to keep the sample well mixed or shaken when homogenization is required. The community counts can be significantly altered and unreliable if sample is not well homogenized.

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2.1. Equipment

Safety: All methods require a laboratory and safety equipment. Make sure you use gloves, glasses and laboratory coat.

Take care to ensure that all apparatus are as clean as possible, in order to minimize the risk of contamination between samples. Stirring rods and Pasteur pipettes shall not be used for more than one sample so that diatoms are not passed from one to another.

Apparatus:

- fume cupboard or equivalent system
- hot plate, sand bath or water bath
- beakers or boiling tubes (one per sample)
- clean Pasteur pipettes
- centrifuge (optional) - if a centrifuge is not available, samples can be allowed to stand overnight whilst solid material settles, after, which the supernatant should be poured off carefully. This prolongs the procedure depending on the volume of the suspension, settling of 1 cm of solid material in suspension takes approximately 1 hour.
- centrifuge tube (optional)

2.2. Hot hydrogen peroxide method

Safety: Hydrogen peroxide is an OXIDISING agent. Do not use this chemical outside of a fume cabinet if heat is to be used to speed the reaction. When handling H2O2 wear acid resistant gloves, goggles and a lab coat.

This treatment procedure is mild, less aggressive, but sufficient for common benthic samples. Diatom frustules often remain connected after the treatment, which may be advantage in case of heterovalval diatoms.

2.2.1. Reagents

- Oxidizing agent, e.g., hydrogen peroxide solution, H2O2, 30 % (volume fraction) or 60%.
- Diluted hydrochloric acid, e.g., c(HCl) = 1 mol/l.
- Distilled water

2.2.2. Procedure steps

1. Homogenize the sample by shaking.
2. If the sample is not sufficiently concentrated, then transfer a subsample of 10-15ml to centrifugation tube and use the centrifuge (max RPM 2000 r/min, max time 3 minutes) to concentrate it or let the diatoms settle in a boiling tube or beaker (column of 1 cm of diatom suspension settle approximately in 1 hour). Remove the supernatant leaving an amount of 2 ml (in tube) of 5ml (in beaker) of concentrated sample.
3. Transfer the concentrated subsample either into a boiling tube or beaker or leave it in the centrifugation tube, if it is boiling resistant. An amount of 2-5 ml is sufficient depending on the sample concentration.
4. Add twice the amount of hydrogen peroxide and more (if subsample volume is 2ml then add at least 4 ml of hydrogen peroxide) and heat on a hotplate, sand bath or water bath at about (90 ± 5) °C until all organic material has been oxidized (typically 1 h to 3 h). Any visible plant material in macrophyte samples may be removed after 30 min. Caution is needed whilst pouring cold concentrated hydrogen peroxide onto rich organic material and aquatic plants, and also during the heating process. Well oxidized (‘burned’) diatom sample appears white or beige in colour. If

the colour remains brown and the bleaching is not sufficient or the reaction does not continue (no visible 'sparkling') despite of organic content, add more hydrogen- peroxide and continue heating the sample until the oxidation is complete. This may take up to 3-4 hours, but usually 1 hour is sufficient if correct amount of hydrogen peroxide is used.

5. Remove the beaker or boiling tube from the heat. Add a few drops of hydrochloric acid to remove remaining hydrogen peroxide and any calcareous precipitates. Allow to cool down in the fume hood for at least 45 minutes.
6. Rince the sample with distilled water to remove any reagents or oxidising agents with distilled water using a centrifuge or via sedimentation. The rinsing process should be repeated at least three times.
7. When all traces of hydrogen peroxide and acid have been removed, mix the cleaned diatom suspension with small amount of distilled or demineralized water and transfer to a clean vial for storage.
8. Add a few drops of 4 % formalin to prevent fungal growth for long-term storage. The sample can then be stored indefinitely.

2.3. Cold acid (permanganate) method of cleaning (sulfuric acid + potassium permanganate)

Safety: Sulfuric acid is a strong corrosive agent and both with potassium permanganate are oxidative agents. Do not perform any activities using these chemicals outside of a fume cabinet. When handling HCl wear acid resistant gloves, goggles and a lab coat.

Samples with moderate organic content can be oxidized using sulfuric acid and potassium permanganate. This is more aggressive treatment leading to separation of most of the diatom frustules in the sample.

2.3.1. Reagents

- diluted hydrochloric acid, e.g., c(HCl) = 1 mol/l
- concentrated sulfuric acid, H_2SO_4
- potassium permanganate, $KMnO_4$, as crystals (approximately 0,1 g to 0,5 g per sample) or a saturated solution of potassium permanganate (1 ml to 2 ml per sample).
- saturated oxalic acid, $C_2H_2O_4$.

Dissolve approximately 10 g oxalic acid crystals in 100 ml distilled or demineralized water over gentle heat whilst stirring. Allow to cool. Crystals of oxalic acid should precipitate out. If not, add some more oxalic acid and repeat the heating and cooling stages.

2.3.2. Procedure steps

If calcareous material is present (or suspected) in the sample, this should be removed first. Add diluted hydrochloric acid drop wise until effervescence, indicating carbon dioxide release has ceased. Rince the sample at least three times with distilled water using a centrifuge (max RPM 2000 r/min, max time 3 minutes) to remove the hydrochloric acid.

1. Homogenize the sample by shaking.

2. If sample is not sufficiently concentrated, then transfer a subsample of 10-15ml to centrifugation tube and use the centrifuge (max RPM 1500, max time 3 minutes) to concentrate it or let the diatoms settle in a boiling tube or beaker (column of 1 cm of diatom suspension settle approximately in 1 hour). Remove the supernatant leaving an amount of 2 ml (in tube) of 5ml (in beaker) of concentrated sample.
3. Transfer the concentrated subsample either into a tube or beaker or leave it in the centrifugation tube, if it is boiling resistant. An amount of 2-5 ml is sufficient depending on the sample concentration.
4. If calcareous material is present (or suspected) in the sample, this should be removed first by adding few drops of hydrochloric acid until the reaction stops. Afterwards, sample must be rinsed with distilled water using a centrifuge (or sedimentation) to remove the reagent.
5. Carefully add equal volume of concentrated sulfuric acid (e.g. add 2 ml of sulfuric acid to 2 ml of diatom suspension).
6. Add approximately 0,1 g of solid potassium permanganate (or a few drops of saturated potassium permanganate solution) and agitate gently to allow the crystals to dissolve. The suspension will turn purple after this stage. If using potassium permanganate crystals, it is important that these have dissolved completely before proceeding to the next step.
7. Slowly add 10 ml of saturated oxalic acid to the sample, which will result in strong effervescence. The end-result should be a suspension of bleached particles (mainly diatom valves).
8. Add distilled or demineralized water and centrifuge (max RPM 2 000 r/min for 5 min) and decant and discard the supernatant. Add distilled or demineralized water again and stir. Repeat the centrifugation stage at least three times to remove all traces of acidity from the suspension. The pH of the supernatant can be easily checked with indicator paper.
9. When the sample supernatant is neutral, mix the diatom pellet in a small amount of distilled or demineralized water and transfer to a clean. Add a few drops of 4 % formalin to prevent fungal growth. The sample can then be stored indefinitely.

3. Preparation of diatom slides

Care shall be taken to ensure that all equipment is as clean as possible, in order to minimize the risk of contamination between samples. Pasteur pipettes shall not be used for not more than one sample so that diatoms are not passed from one sample to another. Cover slips may be cleaned with detergent soap and stored in ethanol until needed, this allows even distribution of diatom valves.

3.1. Equipment

- fume cupboard or equivalent system
- hot plate
- clean Pasteur pipettes
- clean glass slides
- round cover slips

3.2. Reagents

- a diatom mountant with a refractive index > 1,6 (e.g. Naphrax, Pleurax).
- distilled water

3.2.1. Diatom mounting media for preparation of permanent diatom slides

Many important ultra-structural details of diatoms occur at the limit of resolution of light and all commonly used mounting media used in cytology have a refractive index similar to that of diatom valves. Amorphous diatom silica has a RI of 1.43. The higher the difference between RI of diatoms and mounting medium, the better the contrast. Mounting media with a RI substantially lower than 1.7 might work for strongly silicified diatoms but are not optimal for weakly silicified ones. In result, slides with diatoms mounted in the common mounting media have not sufficient contrast to allow investigation of all important details. For this reason, diatoms must be mounted using a medium of higher refractive index than that of the diatom valves. Two mounting media have currently refractive index of > 1,6: "NAPHRAX" r.i. 1.69 and "PLEURAX", r.i. 1.73. Others, such as "Hyrax" r.i. (refractive index) 1.71 and "ZRAX", r.i. 1.7 are no longer available on the market, preparation procedure of them is unknown.

NOTE: If a mounting medium with convenient refractive index is not available, do not experiment with others with lower RI. These do not provide sufficient contrast, although use of DIC or phase contrast may help to investigate the fine details, try to get maximum quality slides and make effort to get appropriate mountant.

1. Naphrax®

Internationally, Naphrax® can be exported only toluene-free due to current transport regulations, which do not permit the carriage of toluene by air. It can be supplied worldwide as a hard toluene-free resin and the hard resin must be diluted with toluene on receipt before use. Please follow suppliers' instructions. The mounting medium can be ordered from Brunel Microscopes Ltd. There is also a detailed preparation procedure described by Dr. G. Rosenfeldt (High-refractive mounting media (PLEURAX / NAPHRAX / ZRAX), see http://www.mikrohamburg.de/Tips/TE_Mountingmedia.html for details.

Orders:
Web: https://www.brunelmicroscopessecure.co.uk/acatalog/Diatom_Mountants.html
Item: Naphrax Mountant Export: 200mls

Brunel Microscopes Ltd
Unit 2 Vincients Road
Bumpers Farm Industrial Estate
Chippenham
Wiltshire
SN14 6NQ
United Kingdom
Chippenham, SN14 6QA.

2. Pleurax

Pleurax is a substance prepared by the reaction of phenol and sulfur in the presence of anhydrous sodium carbonate (Figure 1). The resulting Pleurax resin has even higher refractive index than Naphrax, if well prepared, and provides very good contrast for diatom observation in light microscope.

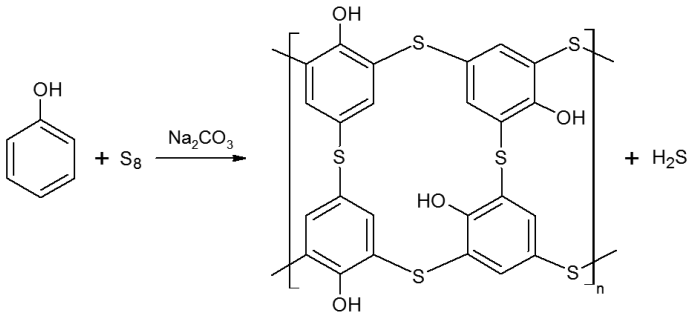

Figure 1: Reaction scheme of Pleurax preparation (from Vojíř, 2017)

PREPARATION OF PLEURAX

The procedure described below was compiled from information published on the web by Dr. G. Rosenfeld (http://www.mikrohamburg.de/Tips/TE_Mountingmedia.html) and from publication of Vojíř (2017). For more details see the cited sources.

! Safety warning: *During the reaction, hydrogen sulphide (H_2S)– **highly toxic gas** with an irritating and suffocating effect – releases in large quantities. At the same time, part of the phenol evaporates.* ***It is therefore necessary to carry out the entire preparation in a well-functioning hood.*** *Synthesis without an appropriately equipped laboratory with an effective fume hood is a **lethal risk**. Due to the high toxicity of H2S an effective fume hood is required!*

Reagents:

- crystalline phenol
- powdered sulphur
- anhydrous sodium carbonate

Procedure steps:

1. Melt 110 grams of crystalline phenol in 250 ml beaker on a magnetic stirrer on a gentle temperature (t_t = 40,5 °C).
2. Then in a rounded bottom flask, add 40 grams of powdered sulphur to the melted phenol and slowly heat the mixture up to 150 °C (internal thermometer!) in an oil bath. Oil bath must be in a non-magnetic pot.
3. Add a spatula tip (approximately 100 mg) of anhydrous (!) sodium carbonate (catalyst) and raise the temperature to 170 °C, stirring continuously. Beware to not exceed the temperature of 181,7°C, which is the boiling point of phenol. At 160°C the reaction starts with gentle foaming and H2S is set free. Cover the beaker with a piece of cardboard to avoid the evaporation of the phenol. Check the production of H2S with lead acetate paper from time to time.
4. Due to the toxicity of hydrogen sulphide, Vojíř (2017) recommends placing an attachment piece on the flask to attach a rubber tubing. The end of the rubber tubing can be placed directly to the

fume hood suction point so that all toxic fumes of hydrogen sulphide are redirected to the suction point of the hood. Photo of the apparatus from Vojíř (2017) is shown on Figure 2, Figures 3 and 4 show the apparatus scheme of Vojíř (2017).

5. Stir for 4 hours. From time to time take a small sample with a glass rod and dissolve the product in a test tube containing about 5 ml of isopropanol. The sample must be solved completely, otherwise sulphur will still be present. Note: Already after about two hours the reaction is nearly complete resulting in a dark brown resin, which is yellow in thin layers. But continue heating for four hours and check the production on H2S with wet lead acetate paper.

Figure 2: Photo of apparatus for Pleurax preparation (after Vojíř, 2017)

6. If even then some sulphur is left, add some more phenol and sodium carbonate and keep heating. The reaction temperature is not critical, but should not exceed 170 °C, otherwise too much phenol will evaporate, and sulphur will spoil the product.

7. When the reaction is finished remove the cardboard and heat for another hour to remove phenol. The volume will decrease significantly. The resulting product still has the smell of H2S, but this odour disappears with time. Some phenol in excess is no problem but will decrease the refractive index.

8. Allow the resin to cool down to 100 °C and continue stirring, then add 50 ml of isopropanol.

9. After a homogeneous solution has formed, pour it into 50 ml vials, filling them to two-thirds. If the viscosity of the cold mixture is too high, add some more isopropanol and heat in a laboratory oven to obtain a homogeneous solution. Now the mounting medium is ready to use.

10. If it medium becomes harder during storage, use isopropanol for dilution.

Yield of pure resin: about 60 grams.

Comments on Pleurax (after Dr. G. Rosenfeld (2023, November 16)

- Phenol is usually coloured slightly red by oxidation products. These impurities do not hamper the synthesis, they even act as a catalyst. Freshly distilled in vacuum phenol is colourless, but in this particular case not reactive enough.
- PLEURAX is soluble in isopropanol and acetone, but not in toluene or xylene.
- When preparing diatoms, put a small sample on an object slide, let it dry completely, cover it with one drop of isopropanol to remove the air from the frustules, then cover with

PLEURAX, add a cover slip and remove the solvent by heating. Only dry PLEURAX has the high refractive index wanted. The yellow colour is no drawback.

- The long-term stability of PLEURAX is unknown.
- The synthesis of PLEURAX is preferable in comparison to NAPHRAX.

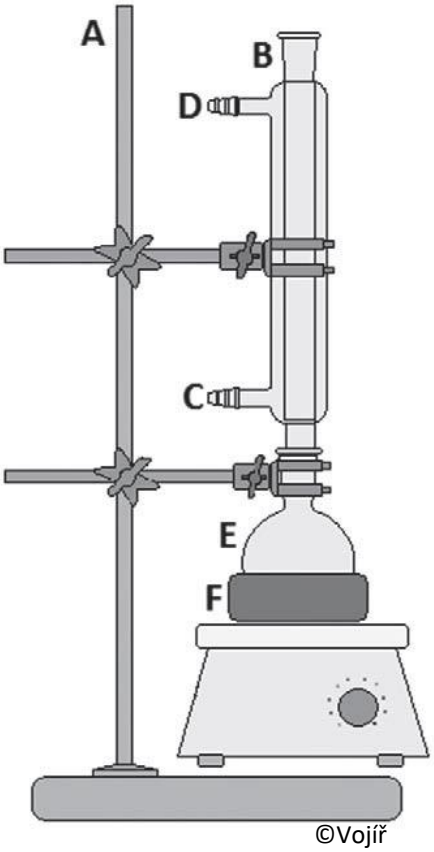

Figure 3: Alternative apparatus for Pleurax preparation A – holder, B – Liebig's cooler, C – water inlet, D – water outlet, E – round bottom flask, F – heating mantle (picture from Vojíř, 2017)

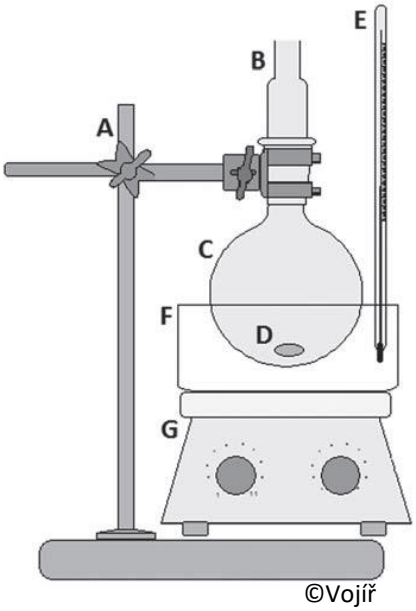

Figure 4: Apparatus for Pleurax preparation A – holder, B – rubber tubing adapter, C – round bottom flask, D – magnetic stirring, E – thermometer, F – heating oil bath, G – magnetic stirrer hot plate (picture from Vojíř, 2017)

3.3. Procedure – mounting diatoms on slides

1. Clean slide and cover slip and dry.
2. Dilute the cleaned diatom suspension to a suitable concentration with distilled water in a vial. When held up to the light, fine particles should be just visible in the suspension. If the suspension appears distinctly milky or turbid, then the suspension is too dense. The density of valves can also be checked quickly by evaporating a drop of the suspension on a cover slip and examining this under medium power objective (i.e. 400 ×). If the suspension is very dilute, either centrifuge the sample again to concentrate those diatoms or allow the diatoms to settle and decant the excess supernatant. Ethanol may also be added to dilute the suspension. This also helps the diatoms to spread evenly on the cover slip.
3. Homogenize the suspension by shaking the vial. Use a clean Pasteur pipette to take part of the suspension from the central part of the vial.
4. Apply the material on a round cover slip forming a medium-high meniscus.
5. Apply the material from the same vial on a second cover slip forming a higher meniscus than on the previous slip to reach higher diatom density on the cover slip when diatoms settle. Another option is to increase the concentration of the diluted suspension by adding few more drops of the original clean concentrated diatom suspension to the vial and apply the homogenized suspension on the cover slip forming similarly high meniscus.
6. Allow the liquid to evaporate, preferably by leaving the cover slip in a warm, dust-free environment, not disturbing the slips. Avoid using a hotplate, diatoms would be forming clumps with strong 'side effect'. This usually takes 12 hours. The result should be a thin grey film on the cover slip.
7. Apply a small drop of mountant (Naphrax/Pleurax) on the slide (not cover slip).
8. Turn over the cover slip and place it on the mountant droplet.
9. Gently heat until all bubbles have evaporated and let cool.
10. Check the slide under the microscope. Ideally, there should be 10-25 diatom valves per field under 1000x objective. If the slide has too many valves or too few, repeat the procedure and adapt the dilution.
11. Now your slide is ready for microscopic analysis.

In principle, the slides preparation procedure must meet the following criteria:

- Use round cover-slips – only that allows random distribution of diatoms and enables regulation of diatoms' density.
- The slide and the coverslip must be clean. To test: a drop of water must spread evenly.
- The distribution of valves on the cover slip should not be significantly clumped, but be evenly dense, without significant edge effects, over the whole area of the coverslip.
- Ideally, there should be 10-25 valves, but not less than 1 per microscopic field of view when viewed at 1000 x magnification.

- The mountant should be properly cured, with no air bubbles, and should spread right to the edge of the coverslip.
- Never make a preparation on the slide, always on the coverslip. Preparation on the slide results in serious deterioration of image quality.
- Mount two cover slips on each slide with different diatoms' density.

4. References

CEN (2014). Water quality – Guidance for the identification and enumeration of benthic diatom samples from rivers and lakes. EN 14407: 2014. Comité European de Normalisation, Geneva.

Rosenfeld, G. (2023, November 16). High-refractive mounting media (PLEURAX / NAPHRAX / ZRAX). http://www.mikrohamburg.de/Tips/TE_Mountingmedia.html.

Spaulding et al. (2021). Diatoms.org: supporting taxonomists, connecting communities. Diatom Research 36(4): 291-304. doi:10.1080/0269249X.2021.2006790.

Vojíř, K. (2017). Příprava pleuraxu pro tvorbu trvalých rozsivkových preparátů. Biologie. Chemie. Zeměpis. 26. 23-28. 10.14712/25337556.2017.1.2.