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Water quality — Guidance for the routine sampling and preparation of benthic diatoms from rivers and lakes

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee EH/3/5, Biological Methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Foreword

This document (EN 13946:2014) has been prepared by Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by September 2014, and conflicting national standards shall be withdrawn at the latest by September 2014.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 13946:2003.

This document contains the following technical changes in comparison with the previous edition:

— this European Standard is now also applicable for the sampling of benthic diatoms in lakes.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

Diatoms are an important component of aquatic ecosystems and constitute a water quality monitoring tool where the primary objective is either a measure of ecological status based on diatoms as one compartment of the ecosystem or the impact of specific components of water quality (e.g. eutrophication, acidification). The requirement for the monitoring of such processes is inherent in the Water Framework Directive (2000/60/EC) [7] and Urban Waste Water Treatment Directive (91/271/EEC) [8] in addition to other EU Directives and international agreements. This European Standard covers aspects of sampling and preparation relevant to assessment of water quality and ecological status using benthic diatoms. These sampling instructions will result in samples suitable for quantifying relative numbers of benthic diatom taxa present. If it is necessary to quantify absolute numbers of taxa, or fresh weight per unit area, modifications to the method are required, which are not within the scope of this European Standard.

The use of diatoms as indicators of river and lake quality is widely accepted both in Europe and the USA. The methodology is based on the fact that all diatom species have tolerance limits and optima with respect to their preference for environmental conditions such as nutrients, organic pollution and acidity. Polluted waters will tend to support an increased abundance of those species whose optima correspond with the levels of the pollutant in question. Conversely, certain species are intolerant of elevated levels of one or more pollutants, whilst others may occur in a wide range of water qualities.

Methods using diatoms to assess water quality have been developed in several European countries (recent work is summarized in the proceedings of three symposia [1] to [3]. The methodologies for evaluating the diatom data vary but the sampling and preparation processes are similar [5, 6].

According to the precise usage to which this European Standard is to be put it is essential for specifiers and users to mutually agree on any necessary variations or optional procedural details prior to use.

All numerical values given in this standard are approximate.

WARNING — Persons using this European Standard should be familiar with usual laboratory practice. This European Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

1 Scope

This European Standard specifies a method for the sampling and laboratory preparation of benthic diatoms for ecological status and water quality assessments. Data produced by this method are suitable for production of water quality indices based on the relative abundance of taxa.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
artificial substratum
introduced substratum
substratum introduced into river or lake by operator specifically for colonization by diatoms

2.2
benthic diatoms
diatoms living on natural or artificial substrata, rather than suspended in the water column

2.3
boulder
mineral substratum with a diameter > 256 mm

2.4
cobble
mineral substratum with a diameter > 64 mm and ≤ 256 mm

2.5
ecological status
measure of the structure and functioning of aquatic communities

2.6
euphotic zone
part of the water column in which there is sufficient light for photosynthesis

2.7
frustule
cell wall of diatoms, composed of silica and consisting of two valves linked by two or more girdle bands

2.8
habitat
specific environment in which an organism lives

2.9
pebble
mineral substratum with a diameter > 16 mm and ≤ 64 mm

2.10
riffle
shallow part of a stream with swift flow, usually with a broken surface

2.11
substratum
natural or non-natural material from which benthic diatoms are sampled

2.12
taxa
taxonomic units, for example families, genera or species

2.13
valve
structural component of the diatom frustule

3 Principle

Benthic diatoms from submerged hard surfaces or submerged macrophytes in rivers, streams or littoral zones of lakes are sampled in order to produce representative collections of the diatom assemblage indicative of water quality. Samples are cleaned using strong oxidizing agents in order to prepare diatoms for identification and enumeration.

The data obtained from the microscopic analysis of these samples are suitable for the production of diatom based water quality indices (see [1], [2], and [3]).

4 Equipment

4.1 Equipment for field sampling

4.1.1 Appropriate water safety equipment

4.1.2 Waders

4.1.3 Stiff toothbrush (or other similar instrument) or **knife** (or other suitable blade).

4.1.4 Plastic tray (approximately 30 cm × 20 cm or larger).

4.1.5 Sample bottles with a tight fitting lid.

4.1.6 Indelible marker pen (or other means of labelling samples).

If labels are used, these shall be capable of withstanding wet conditions.

4.1.7 Hoe, with a fine-meshed net attached, attached to long handle (if vertical hard surfaces are to be sampled).

4.1.8 Glass-bottomed box or bucket (“Aquascope”) used for finding suitable substrata under some circumstances.

4.1.9 Plastic bags

4.2 Laboratory equipment

See Annex A.

5 Reagents

Reagents used in the preparation of the diatom frustules need not be of analytical grade but should be of a quality appropriate for the digestion process.

5.1 Preservatives

Preservatives are required to stop cell division of diatoms and decomposition of organic matter. No preservative is necessary if the sample is to be processed within a few hours of collection, as long as steps are taken to minimize cell division (i.e. by storage in cool, dark place). Lugol's iodine can be used for short-term storage; however, it is not suitable for long-term storage, due to problems caused by sublimation. Buffered formaldehyde or ethanol are recommended for long-term storage of samples. Samples may also be deep-frozen.

5.1.1 Buffered formaldehyde solution, HCHO, volume fraction at least 4 %.

Dilute a stock solution of formaldehyde to a volume fraction of 4 % in a solution buffered to pH 7. Suitable buffers include HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), borate and hexamethylene-tetramine.

A final solution with a volume fraction of 1 % to 4 % in the sample is recommended. The quantity required will depend upon the amount of organic matter present in the sample.

NOTE The buffer is necessary to prevent dissolution of the silica frustules. This is only necessary in alkaline waters, as diatoms dissolve in alkaline water.

5.1.2 Lugol's iodine

Dissolve 2 g potassium iodide and 1 g iodine crystals in 300 ml distilled or demineralized water. The resultant liquid should be dark brown coloured. It should be stored in an air-tight and light-proof container to minimize sublimation.

Add 1 to 5 drops of Lugol's iodine per 100 ml sample to give a final dark brown colour. More may be necessary if samples are rich in organic matter.

5.1.3 Ethanol, C_2H_5OH, volume fraction 70 %.

A final concentration of 20 % is recommended for medium-term storage.

5.1.4 Diluted hydrochloric acid, HCl, volume fraction 10 %.

10 % hydrochloric acid can also be used for medium-term storage. By adding acid, the diatoms detach from their substrata and iron- and calcium-complexes will dissolve.

5.2 Reagents for cleaning diatoms

See Annex A.

5.3 Reagents for preparing permanent slides

A diatom mountant with a refractive index > 1,6 is required (e.g. Naphrax[1)]).

1) Naphrax is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

6 Procedure

6.1 Choice of substratum

Diatoms can be found growing on most submerged surfaces. However, the composition of the assemblage varies depending upon the substratum chosen. Ideally, a single substratum should be used at all sites included in a survey.

Areas of the river bed or lake littoral zone with naturally occurring moveable hard surfaces (large pebbles, cobbles and boulders) are recommended wherever possible. If such hard surfaces do not occur naturally, then it is also possible to sample vertical faces of man-made structures such as quays and bridge supports (as long as these are not made from wood). Other man-made hard surfaces, such as bricks may also be sampled, if these have been in the river or lake for long enough to ensure that assemblages are in equilibrium with their environment. At least four weeks is recommended but the period depends upon environmental conditions. See also 6.3.3.

Samples of diatoms may also be collected from submerged macrophytes. Where possible, comparative studies in rivers or lakes should be based on samples collected from the same macrophyte species (or group of morphologically similar species).

There will also be situations where other substrata (e.g. sand, finer silts) are characteristic of the water body. These can also be sampled; however, there is no widespread agreement on appropriate methods. Users should consult the technical literature and conduct preliminary experiments before deciding on their approach. Consideration should also be given to the introduction of artificial substrata within the euphotic zone.

6.2 Sample site selection

A segment of river or lake shore that has substrata suitable for sampling should be selected. As a general rule, this should be about 10 m in length, but longer segments can be appropriate, depending upon the physical uniformity of the sampling site and the availability of substrata. In rivers “riffles” are preferred, as these tend to have a good variety of natural hard surfaces (see 6.1) but “runs” and “glides” are also suitable.

A detailed description of the site (location, width, depth, substratum type, percent cover of macrophytes, shade, etc.) is required on the first occasion that a sample is collected. A photographic record is also recommended. This information serves as an aid to data interpretation and to help future samplers locate the site. On subsequent visits, notes may be limited to major changes that have occurred since the previous visit, and any variations in sampling protocol employed.

6.3 Sampling methods

6.3.1 Moveable natural hard surfaces

In general, cobbles are the preferred substratum for sampling, as these balance substratum stability (allowing diatom communities to develop) with manoeuvrability. Pebbles and boulders may also be used. At least five cobbles should be sampled. However, if cobbles are unavailable, then either five small boulders or ten pebbles should be sampled. An area of approximately 10 cm^2 or more should be scraped or brushed. If fewer suitable substrata are available, then a note of this should be made.

The following microhabitat conditions should be fulfilled:

a) Areas of heavy shade should be avoided. If it may not be avoided, then a note should be made to this effect. Areas very close to the bank should also be avoided.

b) The substrata shall be submerged for long enough to ensure that assemblages are in equilibrium with their environment. At least four weeks is recommended but the period depends upon environmental conditions. It shall be ensured that the surfaces are submersed in water at all times and exposure to air is

avoided. All depths that may be easily sampled wearing waders are usually suitable, as long as these remain in the euphotic zone.

c) In general, samples should be collected from within the main flow of the river at the sample site. Zones of very slow current (approximately ≤ 20 cm s^{-1}) should be avoided as these allow the build-up of loosely attached diatoms, silt and other debris.

d) Sampling locations in lakes should be well away from inflow streams or obvious human influences and in locations where free exchange of water with the main basin is possible (enclosed bays should be avoided).

Collect a selection of substrata from a variety of locations within the sample site, which fulfil the microhabitat requirements listed above. Where suitable substrata are very abundant, random or stratified sampling strategies may be appropriate within a defined sample site.

Remove any loosely attached surface contamination (e.g. organic debris) by washing the substratum briefly in the river or lake water. Place the substrata in a tray, along with approximately 50 ml of river or lake water.

Wash a stiff toothbrush (4.1.3) in clean river or lake water and rub it on a clean surface in order to minimize any diatom contamination from previous samples. Brush the upper surface of the substratum vigorously to remove the diatom film, rinsing the toothbrush periodically in the water.

A knife or other sharp instrument (4.1.3) may also be used to remove the diatom film. This will be more effective at removing firmly-attached diatoms, but will be less efficient at penetrating crevices on rough surfaces, may cause more damage to frustules and may lead to more rock particles being transferred to the sample. However, it is unlikely that there will be any quantitative difference in results. The knife should also be rinsed in river or lake water and cleaned before use.

Alternatively, the diatom film may be removed using a toothbrush or knife (4.1.3) and washed directly from the surface of the substratum into a sample bottle (4.1.5). The toothbrush or knife may also be used to remove diatoms from the substratum and then rinsed directly into a sample bottle (4.1.5), if this is preferred.

If > 75 % of substrata are smothered with filamentous algae, these should be sampled in preference to substrata lacking such growths. Remove as many of the filaments as possible prior to brushing or scraping, as above.

Replace the substratum in the stream or lake, and repeat the process for the other replicate substrata. Transfer the water, which should now be brown and turbid due to the presence of diatoms, from the tray into the sample jar.

Label the sample bottle (4.1.5) with details relevant to the sample. Transfer the sample to the laboratory in a cool, dark place. If samples are brought to the laboratory within 24 h and these precautions are followed, it is not necessary to add preservatives in the field. If preservatives are necessary, then these should be added immediately after collection, unless there are other reasons (e.g. health and safety) why preservatives cannot be used in the field. All future handlers of preserved samples shall be informed of the nature of any preservatives present.

6.3.2 Method for sampling vertical man-made surfaces *in situ*

The criteria listed above for microhabitat selection should be followed as far as possible.

As this type of sampling is often necessary in lowland, navigable rivers and lakes a sample depth of about 30 cm is recommended to allow for fluctuating water levels and wave action.

The hoe (4.1.7) should be agitated in the water in front of the area to be sampled to dislodge any loosely attached materials.

Scrape the surface with the sharpened blade of the hoe (4.1.7) to remove the attached diatoms. An area of approximately 10 cm^2 should be scraped. The diatom film adhering to the hoe blade should then be removed directly into a sample bottle (4.1.5) into which some river or lake water has been placed.

NOTE 1 Specialized apparatus can also be useful under some circumstances. More details are given in reference [4].

This process should be repeated at least three times and the replicates pooled. For details regarding labelling and transfer to the laboratory see 6.3.1.

NOTE 2 It is also possible to scrape the outer rim of a polyethylene sample bottle (50 ml – 200 ml) vigorously upwards along the vertical surface. The biofilm is collected directly into the bottle.

6.3.3 Use of artificial substrata

Substrata with heterogeneous surfaces (e.g. rough tiles, frayed polypropylene rope) are preferred over substrata with smooth surfaces (e.g. glass slides). These should be left in the river or lake for long enough to ensure that assemblages are in equilibrium with their environment. At least four weeks is recommended, but the period depends upon environmental conditions and longer periods of exposure may be appropriate under some circumstances (i.e. very oligotrophic conditions, low temperatures, heavy shade).

Detailed methods will depend upon the type of substratum chosen. If rough tiles are used, then samples may be collected as described in 6.3.1. For frayed ropes, the final 5 cm is removed with a pair of scissors or brushed with a toothbrush and placed in a sample bottle (4.1.5). Full details of methods are necessary if results from introduced substrata are to be interpreted correctly.

Care should be taken with the design and deployment of introduced substrata to ensure that they do not interfere with the activities of legitimate river or lake users and to minimize risks of vandalism. Extra, replicate substrata should be deployed, to allow for potential losses due to spates or vandalism.

Where introduced substrata are to be used for comparative studies in the same water body, it is important that all substrata are exposed to identical conditions. Both the length of exposure and the start date shall be the same (to allow for the impact of hydrological events upon the developing diatom assemblage).

For details regarding labelling and transfer to the laboratory see 6.3.1.

6.3.4 Sample collection from submerged macrophytes and macroalgae

Sample the entire plant (five replicates) and place into a plastic bag (4.1.9) for transfer to the laboratory. Stir or agitate the plants vigorously in some distilled or demineralized water in a large beaker to dislodge attached diatoms. Remove the macrophytes from the beaker, allow the diatoms to settle and pour off the supernatant.

Alternatively, cut some lengths at random from submerged plants using a knife or scissors and put the sections into a sampling bottle (4.1.5). These may be fractionated further in the laboratory, if required, and the macrophyte sections plus attached diatoms placed directly in a flask for cleaning (6.4.2).

In the case of filamentous macroalgae, it is also possible to gently squeeze a handful of material and to collect the expressed suspension (which will contain epiphytic diatoms) in a sample bottle (4.1.5).

Methods described in 6.3.5 may also be appropriate under some circumstances.

6.3.5 Sample collection from emergent macrophytes

In general, samples should be collected from emergent macrophytes only if there are portions that remain permanently submerged but which are not contaminated by the bottom sediments.

Cut stems at water level and put a plastic sampling bottle (4.1.5) or glass jar upside down on the underwater stem. Cut off the stem below the mouth of the bottle, then turn bottle plus stem upside down and close. In the lab remove the diatoms from the stem by stirring, scraping or gentle brushing.

If the diatom film is not delicate, then it may be possible to cut 5 to 6 stems and transfer these directly to a sampling container without the need to invert bottles over the stem.

6.4 Preparation prior to microscopic examination

6.4.1 Preservation and preliminary laboratory treatment

Samples should be placed in a cool, dark place on return to the laboratory and allowed to stand for at least 24 h, after which suspended material has settled to the bottom of the jar and the clear supernatant can be poured off carefully. Alternatively, the sample may be centrifuged. Any large substrata or coarse plant material should first be removed by passing the sample through a kitchen sieve. The speed and time required to effect complete sedimentation of all diatoms (including very small taxa) will depend upon the characteristics of the individual centrifuge used. Preliminary tests should be performed to ensure that no diatoms are left in the supernatant at the selected speed and time.

If preservatives are necessary, but were not added in the field, then these should be added now. All future handlers of preserved samples shall be informed of the nature of any preservatives present.

A preliminary microscopic examination of the sample is recommended. Unusual features (e.g. large numbers of empty frustules) should be noted.

A portion of the sample shall always be retained in case problems are encountered during the preparation process.

6.4.2 Methods for cleaning diatoms

For accurate identification of diatoms, it is necessary to remove all the cell contents and mount the diatoms using a mountant with a high refractive index (5.3). This can be effected by exposure to strong oxidizing agents. Hydrogen peroxide is the most commonly used oxidizing agent and is recommended here, but other methods will also give satisfactory results. Some suitable protocols are described in Annex A.

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. 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 (5.1.4) before using strong sulfuric acid in order to prevent formation of gypsum. Treatment with dilute HCl (5.1.4) will also be necessary for samples which are rich in iron.

6.4.3 Preparation of permanent slides

Dilute the cleaned diatom suspension to a suitable concentration. When held up to the light, fine particles should be just visible in the suspension. If the suspension appears milky or turbid, distilled or demineralized water should be added to reduce their concentration. 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. If this fails, repeat the preparation procedure.

Ethanol may also be added to dilute the suspension. This also helps the diatoms to spread evenly on the cover slip.

Shake the vial containing the suspension of cleaned material. Using a clean Pasteur pipette, remove some of the liquid from the central part of the tube (which should appear to be turbid). Place a drop onto a new cover slip. Allow the liquid to evaporate, either by leaving the cover slip in a warm, dust-free environment (e.g. in a

desiccator) or by heating gently on a hotplate. The result should be a thin grey film over about two-thirds of the cover slip.

Follow manufacturer's guidelines on use of the mountant. Ensure that the mountant spreads right to the edge of the cover slip. Allow to cool and check under microscope. Ideally, the preparation should have between 10 to 15 valves per field at a magnification of 1 000 ×. If the slide has too many valves per field, repeat the procedure with a more dilute sample of digested diatoms.

Label the slide with, as a minimum, details of location and sampling date plus any codes necessary to cross-reference the slide to other information.

Annex A
(informative)

Methods for cleaning diatoms for microscopic examination

A.1 General

Many different methods for cleaning diatoms have been specified in the literature and are suitable for use for studies of water quality. Details of methods are given here, but other methods may be equally suitable. Quantities can be adapted, so long as ratios between reagents are maintained, in order to suit local conditions. The appropriate batch size will also depend upon local conditions.

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

A.2 Method 1: Hot hydrogen peroxide

A.2.1 Apparatus

A.2.1.1 **Fume cupboard** or equivalent system.

A.2.1.2 **Hot plate,** sand bath or water bath.

A.2.1.3 **Beakers or boiling tubes** (one per sample).

A.2.1.4 **Means of measuring 20 ml volumes** of oxidizing agents.

A.2.1.5 **Clean Pasteur pipettes.**

A.2.1.6 **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.

A.2.1.7 **Centrifuge tube** (optional). These tubes shall be resistant to attack by the oxidizing agents or acids used to clean the diatoms.

A.2.2 Reagents

A.2.2.1 **Oxidizing agent,** e.g. hydrogen peroxide solution, H_2O_2, 30 % (volume fraction).

A.2.2.2 **Diluted hydrochloric acid,** e.g. c(HCl) = 1 mol/l.

A.2.2.3 **Antifoam agent,** (optional).

A.2.3 Procedure

Homogenize the sample by shaking and transfer 5 ml to 10 ml of the suspension to a beaker or boiling tube. Add approximately 20 ml of hydrogen peroxide (A.2.2.1) 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). Coarse 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. Antifoam agents may also be added.

Remove the beaker or boiling tube from the heat. Add a few drops of hydrochloric acid (A.2.2.2) to remove remaining hydrogen peroxide (A.2.2.1) plus any carbonates and wash down sides of beaker with distilled or demineralized water. Allow to cool in the fume cupboard.

The addition of hydrochloric acid (A.2.2.2) may be omitted if the sample comes from a region where carbonates are unlikely to be present.

Transfer contents of beaker or boiling tube to centrifuge tube, top up with distilled or demineralized water and centrifuge (see Note for details of centrifugation). Decant supernatant and resuspend pellet with distilled or demineralized water and repeat centrifugation.

The washing process should be repeated at least three times, or until all traces of hydrogen peroxide (A.2.2.1) have been removed. When all traces of hydrogen peroxide and acid have been removed, mix the diatom pellet in a small amount of distilled or demineralized water and transfer to a clean, small capacity vial. Add a few drops of 4 % formalin (5.1.1), hydrogen peroxide (A.2.2.1) or ethanol (5.1.3) to prevent fungal growth. The sample can then be stored indefinitely.

A.3 Method 2: Cold hydrogen peroxide

A.3.1 Apparatus and reagents

See A.2 (Method 1), but excluding hotplate, sand bath or water bath.

A.3.2 Procedure

Follow Method 1 (see A.2) but do not heat the beaker containing the sample. Instead leave the beaker (covered by a watch glass or similar) for at least four days. For acceleration of oxidation put the beakers in the sunlight or under a UV lamp.

Transfer the contents of the beaker to a centrifuge tube and continue with Method 1 (see A.2).

If this does not result in cleaned frustules, replace the hydrogen peroxide and leave for another period or use another method after washing.

A.4 Method 3: Hot hydrogen peroxide with potassium dichromate

A.4.1 Apparatus

See A.2 (Method 1).

A.4.2 Reagents

See A.2 (Method 1).

A.4.2.1 Crystalline potassium dichromate, $K_2Cr_2O_7$, or potassium permanganate $KMnO_4$.

A.4.3 Procedure

Homogenize the sample by shaking and transfer 2 ml to 5 ml of the thick suspension to a beaker. Add 50 ml of hydrogen peroxide (A.2.2.1) and heat under a fume cupboard on a hotplate at 90 °C until all organic material has been oxidized (0,5 h to 3 h). The samples may bubble vigorously. Caution is needed whilst pouring cold concentrated hydrogen peroxide (A.2.2.1) onto rich organic material and aquatic plants, and also during the heating process.

Remove the beaker from the heat. Add a spatula tip of potassium dichromate (A.4.2.1) grain by grain (note that this normally causes additional effervescence). This should result into a clear bluish-green solution after a few minutes.

If it is still turbid, add a few drops of hydrochloric acid (A.2.2.2) to remove remaining hydrogen peroxide (A.2.2.1) plus minor amounts of carbonates and wash down sides of beaker with distilled or demineralized water. If larger quantities of carbonates are present, add 20 ml of concentrated hydrochloric acid (A.2.2.2) and heat gently.

Transfer the contents of the beaker to a centrifuge tube and continue with Method 1 (see A.2).

When all traces of hydrogen peroxide (A.2.2.1) and acid have been removed, mix the diatom pellet in a small amount of distilled or demineralized water and transfer to a clean, small capacity vial. Add a few drops of 4 % formalin (5.1.1), hydrogen peroxide (A.2.2.1) or ethanol (5.1.3) to prevent fungal growth. The sample can then be stored indefinitely.

A.5 Method 4: Cold acid (permanganate) method of cleaning

A.5.1 Apparatus

A.5.1.1 Fume cupboard.

A.5.1.2 Means of measuring 5 ml to 10 ml volumes of acids and oxidizing agents.

Automatic pipettes, if used, should be periodically stripped down and cleaned to prevent corrosion.

A.5.1.3 Pasteur pipettes.

A.5.1.4 Centrifuge tubes.

NOTE The entire procedure can be performed in large (e.g. 30 ml) centrifuge tubes, as long as these tubes are made of a material that is resistant to the acid mixture used.

A.5.1.5 Centrifuge.

A.5.2 Reagents

A.5.2.1 Diluted hydrochloric acid, e.g. c(HCl) = 1 mol/l.

A.5.2.2 Concentrated sulfuric acid, H_2SO_4.

A.5.2.3 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).

A.5.2.4 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.

A.5.3 Procedure

Homogenize the sample by shaking and transfer 5 ml to 10 ml of the suspension to a centrifuge tube.

If calcareous material is present (or suspected) in the sample, this should be removed first. Add dilute hydrochloric acid (A.5.2.1) drop wise until effervescence, indicating carbon dioxide release has ceased.

Add distilled or demineralized water and centrifuge. Discard the supernatant.

This stage may be omitted if it is certain that the sample was not collected from a catchment where calcareous rocks are present.

Carefully add 5 ml of concentrated sulfuric acid (A.5.2.2).

Add approximately 0,1 g of solid potassium permanganate (or a few drops of saturated potassium permanganate solution) (A.5.2.3) 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.

Slowly add 10 ml of saturated oxalic acid (A.5.2.4) to the sample, which will result in strong effervescence. The end-result should be a suspension of bleached particles (mainly diatom valves).

Add distilled or demineralized water and centrifuge. Approximately 3 000 r/min for 5 min in a bench centrifuge is adequate to ensure that all the valves are precipitated. 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.

When the sample supernatant is neutral, mix the diatom pellet in a small amount of distilled or demineralized water and transfer to a clean, small capacity vial. Add a few drops of 4 % formalin (5.1.1), hydrogen peroxide (A.2.2.1) or ethanol (5.1.3) to prevent fungal growth. The sample can then be stored indefinitely.

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