Sampling protocol and audit benthic diatoms

1 Goal

The collection of benthic diatoms attached to stones, macrophytes, macroalgae or present in sediments of European river systems. Habitats are sampled and processed separately.

2 Principle

Benthic diatoms are sampled from different substrata in European streams and rivers in order to generate representative collections of natural diatom communities. The relative abundances of diatom taxa in these samples are used to assess water quality.

3 Applicability

European streams and rivers.

4 Definitions

Benthic diatoms: Bacillariophyta growing attached to a substratum. These substrata can be natural or artificial. The substrata considered in this protocol are stones (boulders, cobbles, pebbles), macroalgae and macrophytes (emergent and submersed) and sediments (mineral sand and silt).

The mineral substrata (Stones and sediments) considered in this protocol are classified following the STAR Site Protocol.

5 Recommendations on safety and environmental issues

Appropriate health and safety guidelines must be followed when using preservatives (e.g. formalin), oxidising agents (H_2O_2, strong acids) and mounting media. The name of the preservative should be marked on the outside of the sample bottles.

Used formalin must to be treated as chemical waste and should at no time get into contact with bare skin. H_2O_2 and H_2SO_4 have a strong oxidising effect and should also not get in tough with bare skin. Make use of a fume cupboard whenever possible, especially when preparing microscopic slides with mounting medium, as potentially carcinogenic fumes are released. It is recommend that prior to the application of a chosen method, a full risk assessment is made, as different preparation and preservation techniques present different hazards.

6 Equipment and materials

6.1 Field sampling equipment

- appropriate water safety equipment
- waders
- toothbrush to remove diatom film from stones and macrophytes
- plastic tray (approximately 30 × 20 cm or larger) (see comments below)
- pair of scissors or secateurs (or penknife) to cut off parts of macrophytes
- 1 large wide mouthed sample bottle (approx. 1000 ml) with lid to shake-off epiphytes from macrophytes

- wash bottle with distilled water
- preservative
- length of glass tube (large pipette, length approx. 50 cm, diameter approx. 5 mm)
- wide mouthed sample bottles (size is not critical, for example 50 or 100 ml)
- waterproof writing material

6.2 Laboratory processing

- petri dishes
- coverslips
- pipettes
- 50 ml beakers
- test tubes (optionally centrifuge tubes)
- microscopic slides
- heating equipment
- vials for storing of samples

6.3 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. See the draft CEN diatom sampling protocol for more detail (attached).

6.3.1 Preservatives

One of the following:

- Buffered 34% v/v (minimum) formaldehyde (HCHO) solution :
- Lugol's iodine:
- 70% Ethanol (C_2H_5OH)

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.

6.3.2 Reagents for cleaning diatoms

Hydrogen peroxide (H_2O_2, 35%), hydrochloric acid (HCl), sulphuric acid (H_2SO_4) or potassium pemanganate ($KMnO_4$) or a combination of some of reagents. See for methods Annex A from the draft CEN diatom sampling protocol (attached).

6.3.3 Reagents for preparing permanent slides

A diatom mountant with a refractive index > 1.6 is required. Propriety brands include Naphrax® and Hyrax®.

7 Methods

The sampling strategy differs, depending of the type of habitat. The objective is to sample a habitat as distinctly as possible in order to allow for habitat specific analyses. With information on the percent coverage of the substratum type at the site, it will be possible to assess the representativeness of the sample.

7.1 Timing of survey

The collection of benthic diatom samples should take place during periods of stable stream flow. These stable conditions will vary from region to region. It is therefore impossible to give an exact guideline on when samples should be taken. However, it is recommended that surveys are conducted at least four weeks after a period of extreme conditions like a major storm or drought. This guideline should also be considered when repeated surveys are to be conducted. Repeated surveys should take place approximately the same time each year, to minimise the influence of seasonal changes in the composition of diatom assemblages. Diatoms samples can be collected at any time of the year. However, spring is the preferred season as diatoms dominate the phytobenthos during that season (Moore 1977). Sampling in winter is not recommended as cell growth rates are lower and diatom communities will have less opportunity to reflect prevailing environmental conditions.

7.2 Establish the reach for sampling

See the AQEM STAR sampling protocol for the selection of the section of the river that is considered as the sampling site. The site should have suitable substrata for sampling benthic diatoms from stones, macrophytes or mineral sediments, depending on the type of river (see below). It is preferred that the sampled section combines riffles and pools to enable the sampling of a good variety of natural substrata.

It is recommended that samples are not taken too close near the riverbanks. On larger rivers try to stay away at least 1 meter from the river banks. On narrow rivers keep a margin of at least 10% of the river width.

7.3 Fill in field form and mark sample bottles

The field form for recording details of the sampling site is part of the STAR site protocol (v1.11, page 8). On this page mark the substratum type that has been sampled (Macrophyte/ -algae, Sediment (silt/sand) or Stone /man-made constructions). Also indicate the type of macrophyte/-algae that was sampled in the appropriate table. Space is allocated to add remarks.

Clearly mark sample bottles with the same site name, date and sample number that is mentioned on the field form.

7.4 Assess habitat conditions

Before sampling record the percent coverage area of all the substratum types in the reach that will be sampled. Complete the field data sheet (STAR site protocol) and mark the sampling bottles. Substratum types need to be recorded in steps of 5%.

7.5 Choose substratum

The following table indicates the type of substratum that needs to be sampled in each stream type.

Table 1 Substrata to be sampled in core- and additional stream types.

Stream type	Countries	Description	Substratum to be sampled for phytobenthos
Core stream type 1	UK, D, CZ, A	Small, shallow mountain streams	Stone (mesolithal=cobbles)
Core stream type 2	UK, D, DK, S	Medium-sized lowland stream	Sand (psammal / psammopelal) in DK, D or Pebble (microlithal) in UK, S
Additional stream types:			
	A	Small crystalline streams of the ridges of the Central Alps	Stone (mesolithal)
	CZ	Small streams in the central Czech highlands (Danube river basin)	Stone (mesolithal)
	D	Small Buntsandstein streams	Stone (mesolithal)
	F	small and shallow headwater streams in Western France	Stone (mesolithal)
	GR	Calcareous small sized mountain streams in Western, Central and Southern Greece	Stone (mesolithal)
	GR	Silicious medium sized streams on the Aegean Islands	Stone (mesolithal)
	GR	Silicious medium sized mountain streams in Northern Greece	Stone (microlithal)
	GR	Calcareous medium sized streams in Western Greece	Macrophyte or Sand (psammal / psammopelal)
	I	Small sized streams in the southern calcareous Alps	Macrophyte or Sand (psammal / psammopelal)
	I	Small sized calcareous streams in the Central Apennines	Stone (mesolithal)
	P	Medium sized streams in lower mountainous areas of Southern Portugal	Stone (mesolithal)
	S	Swedish additional stream type	?

Partners are required to sample one type of substratum as indicated in Table 1. However, it is recommended that partners take samples from more than one substratum type and keep these samples in case a later funding opportunity arises to process them. This should not take much extra time in the field and might prove to be very worthwhile. A reason for doing so is, that it might be unclear in some instances whether the proposed substratum type is truly representative of the stream type (do these substrata also occur on other sites of the same stream type?). It is not possible to compare diatom assemblages between sites or between moments of sampling when they have been collected from different substrata. This protocol explains the sampling of three types of substrata (stones, macrophytes and sediments). If samples are taken from these types of substrata, they need to be kept separate at all times.

When selecting suitable (extra) substrata, take these guidelines in mind:
- Sampling of stones (pebbles (microlithal) or larger) is preferred.
- If no microlithal stones are present or cannot be indicated as representative, macrophytes are the preferred substratum (if representative).
- Sand (psammal / psammopelal) can be sampled if none of the other substrata are truly representative.

Stones that are covered with filamentous algae need to be treated with caution (see paragraph 7.6). When the filamentous algae are completely obscuring the stone surfaces and have developed into

flee floating strains, these algae can be considered as separate substrata and can be sampled in concordance with the sampling of macrophytes.

7.6 Collect benthic diatoms

Sampling of stones
The objective is to collect epilithic diatoms from stones that are not moved by normal hydrological conditions. Cobbles (mesolithal) are generally preferred over boulders as they are easier to handle. Pebbles (microlithal) can also be used where cobbles are not available (diatom communities on pebbles are more readily disturbed by changing hydrological conditions). For a classification of stones see the STAR Site Protocol. A minimum of 5 cobbles should be selected at each site, so that the upper parts of the stone make up about 100 cm^2 of surface area that can be sampled (see below). Stones should be selected as randomly as possible from amongst those that are not smothered with filamentous algae and which have an obvious diatom film. When cobbles are not available and pebbles can only be sampled instead, at least 10 of such substrata should be sampled (again making up about 100 cm^2). Hold the stones briefly in flowing river water to remove loosely attached surface contamination (e.g. organic debris). Place the stones in a tray, add some (100-200 ml) distilled-or clear river water to the tray and rub the upper parts (exposed to stream flow) of the stones with a hard toothbrush. Collect the dislodged material from the stones and the toothbrush in the tray and finally decant the mixture into the sample bottle. In this way one combined sample will be collected, containing the diatom films of at least 5 stones.

If samples are not to be processed in the laboratory within 24 hrs, add a few drops of preservative to the sample. It is also possible to add preservative on return to the laboratory (within 24 hrs), which saves taking chemicals into the field. Store samples in a cool dark place. Clean sampling equipment thoroughly to prevent diatom contamination of subsequent sampling.

In some rivers, stones can be covered with filamentous algae. Sampling these stones would result in the collection of a mixture of epilithic and epiphytic diatoms. However, when all stones at the sampling site are covered with filamentous algae, collecting diatoms from the few stones that are not overgrown would clearly not result in a 'representative' sample. When stones are smothered with filamentous algae, remove the filamentous algae by hand and hold the stones briefly in flowing river water to remove loosely attached material. The stone surface can be is sampled as above. When the filamentous algae are completely obscuring the stone surfaces and have developed into flee floating strains, these algae can be considered as separate substrata and can be sampled in concordance with the sampling of macrophytes.

Sampling of macrophytes and filamentous algae
Submerged macrophytes are preferred over emergent ones. When emergent macrophytes are sampled, use only the submerged portions at sufficient depths to allow for fluctuating water levels With secateurs clip parts of live macrophyte stems and leaves, record the macrophyte's identity and estimated its percent abundance at the site. When several species of macrophytes are present, incorporate these species in a composite sample that is representative of the macrophytes present in the sampled reach. Filamentous algae (macro-algae)can be dislodged by hand. Place the collected macrophytes and/or macro-algae in a large wide-mouthed 1 litre sample container. Collect macrophytes until the container is about half full and add some (100-200 ml) distilled / clear river water. Close the lid and shake the container vigorously for about 60 seconds. Rub the substrata gently to remove remaining benthic algae. Finally, decant the suspension in a 250 ml sample bottle. In this way one combined sample will be collected, containing the diatom films of several macrophytes or macro-algae at the site.

If samples are not processed in the laboratory within 24 hrs, add a preservative to the sample. Store samples in a cool dark place. Processed macrophytes and/or macroalgae can be retained for further identification if necessary.

Sampling of mineral sediments
Draw a length of glass tube across the sediment allowing it to fill with a mixture of surface sediment and water. This can be done by initially keeping the top of the tube closed with ones thumb. Place the tip of the tube at the sediment surface. Release the thumb shortly while dragging the tube over the sediment surface. A mixture of sediment and water is collected in this way. Pour this mixture in the sample bottle. Repeat this action several times, while moving around the sampling site, so that material is collected from several spots at the sampling site. Keep on collecting material until approx. 200 ml of mixture has been collected in one sample bottle. Do NOT add a preservative as the sample will be treated further in the laboratory, preferably within 24 hrs. In this way one combined sample will be generated, containing sediment collected from several spots within the sampling site.

Fine sedimented particulate organic substrata (e.g. detritus) can be collected and processed like the mineral sediments. These samples should be marked clearly as containing organic sediments and treated and analysed separately.

7.7 Laboratory treatment

The collected samples need to be split-up on arrival in the laboratory, so that one part can be processed for diatom identification and counting and he remaining part can be used for identification and counting of non-diatom benthic algae (See "Sampling protocol non-diatom benthic algae"). This portion of the original sample should be preserved and retained in case problems are encountered during the preparation process and for later reference.

In the laboratory a visual assessment of subsamples at 400X magnification is required to estimate the proportion of living diatom cells. Samples with a large proportion of empty frustules (> 10%) should be discarded from further analyses. If sampling has occurred closely following this protocol, it will be unlikely that the samples contain large amounts of dead frustules. The quickcheck in the lab is therefore not compulsory. If samples are fixed with formalin of lugol, the check could also be done on preserved samples: comparing frustules containing chloroplasts with empty frustules. Preserving with alcohol will dissolve all chlorofyll, making a chloroplast check impossible. Treat the samples with dislodged material from each sampled substrata separately: care must be taken to ensure that the material from these samples are never mixed.

Epipelon processing
In the laboratory, sediment samples (organic and mineral) are placed in petri dishes. The sediment has to be allowed to settle overnight. The following morning the supernatant can be drawn off, with a fine Pasteur pipette, and 4 cover slips placed on top of the wet sediment. On the afternoon of the same day the cover slips should be removed carefully. In this way, only living cells that are attached to the cover slips are sampled. Four cover slips from each sample are placed in 50 ml beakers. The diatom material in the samples can be cleaned according to the methods described in Annex A.

Removal of a preservative from preserved samples
When samples have been preserved, the preservative should be washed from the samples before the samples are cleaned (oxidised) for microscopic examination.

- From each well shaken sample, pour 10 ml into test tubes and settle for 24 hrs, pour off supernatant.

If the samples contain a preservative, the samples should first be rinsed with distilled water:

- Add distilled water, shake and settle for 24 hrs. If the sample contains iron, add a few drops of HCl before settling. The sample will stain yellow. After settling pour off supernatant and add distilled water.

Cleaning (digestion) of diatoms for microscopic examination
The diatom material in the samples can be cleaned according to the methods described in Annex A.

Slide preparation
As for other stages in the preparation of diatom slides, there is no "right" way to prepare permanent slides, so long as the finished slides fulfil certain criteria. There is a definite "knack" to preparing slides, especially in judging the density of suspensions from which the slides are prepared. This comes with experience, and beginners may have to experiment to develop their technique.

The principle is that a drop of the cleaned suspension is evaporated gently onto a coverslip, which is then mounted onto a slide. It is important that the final suspension is of a low enough density to permit easy identification and counting, and that factors which contribute to non-random distributions (such as edge effects) are minimised.

1. Shake a vial of material and hold it up to the light. Fine particles should be just visible in the suspension. If the suspension appears milky or turbid, distilled water should be added to reduce their concentration. If no particles can be seen, then allow the suspension to settle and then carefully decant off some of the excess liquid.
2. Use a Pasteur pipette to remove some of the shaken suspension from the vial and put a drop onto a clean cover slip. Room temperature is recommended. Evaporation should take place in a warm, dust-free environment and takes upwards of one hour (depending upon ambient conditions). Once all liquid has been evaporated, the result should be a thin, whitish-grey film over about half of the cover slip. Common reasons for uneven strews of diatoms following evaporation include too rapid evaporation (caused by high temperatures) or because there is still some residual acid left in the suspension. Some clumping of diatoms is acceptable, so long as all valves can be identified reliably, and density criteria are not exceeded. If clumping of the suspension persists, try treating the coverslip with 0.1 M H_2SO_4, rinse with distilled water and wipe it dry before putting a drop of suspension on it.
3. The density of valves should now be checked by placing the coverslip face down on a slide and examining this under medium power objective (i.e. 400 ×). Assuming a maximum final density of about 15 valves per field of view at 1000 × magnification, this dry mount should have a maximum of roughly 30 valves per field of view. If the density is too high, repeat step 2 but with a more dilute suspension.
   Note that very gritty samples may necessitate dilute suspensions to ensure that the valves are not obscured by mineral particles.
4. Once a satisfactory density of valves has been achieved, put a small drop of naphrax onto a warm slide (e.g. using a warm hotplate) and place the coverslip face down on top of it. Heat the slide until the naphrax spreads and just begins to bubble. Apply gentle pressure (e.g. with the end of a pair of blunt forceps) to remove air bubbles. Repeat this 3 - 4 times then allow to cool. Ensure that the mountant has been properly cured by checking to see if the coverslip moves when pushed with a fingertip. If it cannot be moved, then check the valve density again under a microscope.

Three slides should be prepared from each sample: one for in house analyses, one for auditing and one as a reserve.

Slides, vials with cleaned suspension and preserved original samples, should be labelled with at least the following information:

- Institute: name of the organisation that takes the sample
- River name
- Location name
- Location co-ordinates: (xx°xx'xx'' E / xx°xx'xx'' N)
- Substrate: stone, macrophyte, sediment
- Date: day/month/year
- Collector: persons name
- Sample number

example (fits microscope slide):

STAR Phytobenthos
Inst.: Alterra.
River: Rhine
Loc.: Wageningen
co-ordinates: 5°40'01" E/
51°56'45" N
Subs: Stone. Date: 2-2-2002
Coll.: W. Alexander
Sample number: 1

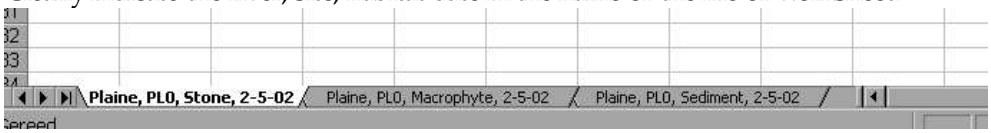
8 Counting and identifying

Complete the count sheet (an example is attached). 300 diatom valves (one half of a frustule) are identified and counted at a 1000x magnification. The slide is scanned so that successive fields are examined and that duplicate counting of the same field is avoided. Make sure that the examined fields are not all located on one small area of the slide.

Identify the taxa at least to species level. For nomenclature and encoding, the proposed standard taxonlist (see Audit procedure) must be used. Send the results of the counts in electronic format to the auditors. The preferred spreadsheet to be used in MS Excel 97. Use the following format of the table:

	A	B	C	D	E	F	G	H
1	**Taxon (authority)**	**Code**	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample …**
2	Achnanthes minutissima Kutzing var. minutissima Kutzing	AMIN	5		10	54	2	9
3	Navicula gregaria Donkin	NGRE						
4	…	…	…	…	…	…	…	…
5								
6	Total		306	332	321	311	332	305
7								

Clearly indicate the river, site, habitat date in the name of the file or worksheet:



To allow for a comprehensive comparison of important taxa during the course of the project for your own reference and also for the audit procedure, photographic records of abundant taxa should be generated and for those taxa that cannot be identified with a high degree of confidence. These photographs have to be sent electronically (by email or CD-ROM) to the auditors with the following requirements:

- minimum resolution 300 dpi
- Add a scale bar
- If more than one specimen is visible on the diagram, add an arrow to clearly indicate which specimen is referred to.
- State length and width in µm of the specimen. Optionally extra information can be added on the range of dimensions of the taxon.
- number of striae per 10 µm
- State the record number of the slide on which the specimen was recorded and the institute that collected the sample. Include the name of the river, sample site and type of habitat from which the sample was collected.
- Make sure that the aspect ratio of the electronic pictures is never altered.

Example:

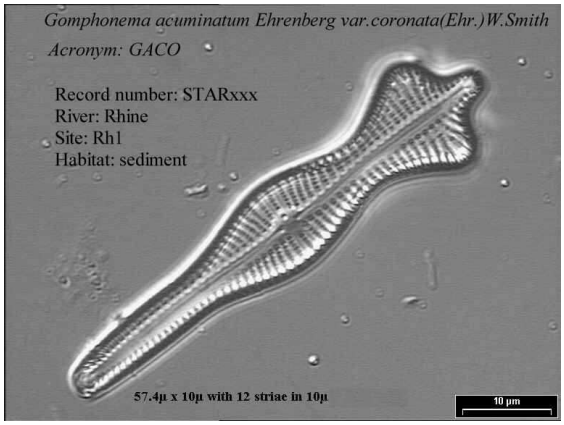


Figure 1 Example of micrograph.

9 Audit procedure

Participating research groups will send prepared permanent slides from of 10, or 5% of their sampled sites, whichever is the larger, to the auditors at Alterra. As described in the sampling protocol, samples taken from a maximum of three microhabitats at each site are processed and analysed separately. From the sites that are selected for the audit, all three samples should be sent to the auditors.

The nomenclature that will be used for diatom taxa must refer to the taxonlist that is developed in the EU-PAEQANN project. This taxonlist comprises 733 diatom taxa, each with a unique four letter code. Reference information on all taxa is part of the database and the literature it refers to in most cases (Krammer & Lange-Bertalot 1986-2000, part 1-5) is widely distributed.

A method to establish the similarity between samples, is the use of the Sørensen algorithm (Sørensen 1948). This methods not only compares the amount of similar taxa between two samples, it also takes the abundances of these taxa into account. The criteria to reject the hypotheses that two counts of the samples are different will be developed during two intercalibration exercises on samples collected during the workshop in La Bresse (April 2002). All participants will count and identify the samples taken from the same sites during this workshop and the results will be send to Alterra. This intercalibration exercise will enable us to establish the range of similarity that can be expected between the labs for two river systems in two different ecoregions.

A second audit method is based on the use of diatom indices (e.g. IPS or TDI) and the Hill's N_2 diversity index. The indices calculated on the basis of the original results and those based on the audit results, will be compared following Kelly (2001). This method uses the Bray-Curtis similarity index to compare diatom and diversity indices based on the original identifications and those based on the identifications and counts by the auditor.

10 References

References mentioned in the text

Kelly, M. G. (2001). "Use of similarity measures for quality control of benthic diatom samples." Water Research **35**(11): 2784-2788.

Moore, W. W. (1977). "Seasonal succession of algae in a eutrophic stream in ssouthern England." Hydrobiologia **53**: 181-192.

Sørensen, T. (1948). "A method of establishing groups of equal amplitude in plant sociology based on similarity of species content." Dt. Kong. Danske Vidensk. Selsk. Biol. Skr (Copenhagen) **5**(4): 1-34.

Wentworth, C. K. (1922). "A scale of grade and class terms for clastic sediments." Journal of Geology **30**: 377-392.

General recommendations on benthic diatom sampling and slide preparation

Kelly, M. G., A. Cazaubon, et al. (1998). "Recommendations for the routine sampling of diatoms for water quality assessments in Europe." Journal of Applied Phycology 10: 215-224.

Winter, J. G. & H. C. Duthie (2000). "Stream epilithic, epipelic and epiphytic diatoms: Habitat fidelity and use in biomonitoring." Aquatic Ecology 34(4): 345-353.

Draft European Standard: Guidance standard for the routine sampling an pre-treatment of benthic diatoms from rivers. CEN basic version 1.3. Contact person: M.G. Kelly, Bowburn Consultancy, 11 Monteigne Drive, Bowburn, Durham DH6 5QB, UK, +44 191 377 2077, email: Bowburn_Consultancy@ compuserve.com

Sampling of stones
Rolland, T., S. Fayolle, et al. (1997). "Methodical approach to distribution of epilithic and drifting algae communities in a French subalpine river: Inferences on water quality assessment." Aquatic Sciences 59(1): 57-73.

Sampling of macrophytes
Marker, A. F. H. & G. D. Collett (1997). "Spatial and temporal characteristics of algae in the River Great Ouse. II. The epiphytic algal flora." Regulated Rivers Research and Management 13(3): 235-244.
McCormick, P. V., R. B. E. Shuford, III, et al. (1998). "Spatial and seasonal patterns of periphyton biomass and productivity in the northern Everglades, Florida, USA." Hydrobiologia 362: 185-208.

Sampling of sediments
Round, F. E. (1981). The ecology of algae. Cambridge, Cambridge University Press.

Example: Benthic diatom laboratory bench sheet

Benthic diatoms project STAR		
Stream name:	*LAT :* *LONG:*	
Station # :	*sample # :*	
Date of sampling:	*slide # :*	
Taxonomist:	*archive # :*	
Habitat origin:	*remarks:*	
Microphyte/ Algae ☐ *Sediment* ☐		
Stone ☐		
taxon	*# of valves*	*total*
Total number of taxa:	*total*	

Date of analysis: …… *Signature:* ……

Annex A to Draft CEN Standard for sampling and pretreatment of benthic diatoms.

Cleaning diatoms for microscopic examination (informative)

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.

Method 1: Hot hydrogen peroxide

Apparatus

- Fume cupboard or equivalent system
- Hot plate, sand bath or water bath
- Beakers or boiling tubes (one per sample)
- Means of measuring 20 ml volumes of oxidising agents
- Clean Pasteur pipettes
- Centrifuge (optional)

Note: 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.

- Centrifuge tube (optional). These tubes must be resistant to attack by the oxidising agents or acids used to clean the diatoms

Reagents

- 30% (100 volume) hydrogen peroxide (H_2O_2) solution
- Dilute (e.g. 1 M) hydrochloric acid (HCl)

Method

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 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 can be removed after 30 minutes. Caution is needed while pouring cold concentrated hydrogen peroxide onto rich organic material and aquatic plants, and also during the heating process.
Remove the beaker or boiling tube from the heat. Add a few drops of hydrochloric acid to remove remaining hydrogen peroxide plus any carbonates and wash down sides of beaker with distilled or demineralised water. Allow to cool in the fume cupboard.
Note: The addition of hydrochloric acid can 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 demineralised water and centrifuge (see Note for details of centrifugation). Decant supernatant and resuspend pellet with distilled or demineralised water and repeat centrifugation.
The washing process should be repeated at least three times, or until all traces of hydrogen peroxide have been removed. When all traces of hydrogen peroxide and acid have been removed, mix the

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diatom pellet in a small amount of distilled or demineralised water and transfer to a clean, small capacity vial. Add a few drops of 4% formalin, hydrogen peroxide or ethanol to prevent fungal growth. The sample can then be stored indefinitely.

Method 2: Cold hydrogen peroxide

Apparatus and reagent
As Method 1, but excluding hotplate, sand bath or water bath.

Method
Follow Method 1 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.
Then transfer the contents of the beaker to a centrifuge tube and continue with Method 1.
If this does not result in cleaned frustules, replace the hydrogen peroxide and leave for another period or use another method after washing.

Method 3: Hot hydrogen peroxide with potassium dichromate

Apparatus
As Method 1

Reagents
As Method 1, with addition of crystalline potassium dichromate (or potassium permanganate)

Method
Homogenize the sample by shaking and transfer 2-5 ml of the thick suspension to a beaker. Add 50 ml of hydrogen peroxide and heat under a fume cupboard on a hotplate at 90 □C until all organic material has been oxidized (0.5-3 hours). The samples may bubble vigorously. Caution is needed while pouring cold concentrated hydrogen peroxide 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 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 (B.2.2) to remove remaining hydrogen peroxide plus minor amounts of carbonates and wash down sides of beaker with distilled or demineralised water. If larger quantities of carbonates are present, add 20 ml of concentrated hydrochloric acid and heat gently.
Then transfer the contents of the beaker to a centrifuge tube and continue with Method 1.
When all traces of hydrogen peroxide and acid have been removed, mix the diatom pellet in a small amount of distilled or demineralised water and transfer to a clean, small capacity vial. Add a few drops of 4% formalin, hydrogen peroxide or ethanol to prevent fungal growth. The sample can then be stored indefinitely.

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Method 4: Cold acid (permanganate) method of cleaning

Apparatus
— Fume cupboard
— Means of measuring 5 - 10 ml volumes of acids and oxidising agents

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

Centrifuge

Reagents
— Dilute (e.g. 1 M) hydrochloric acid (HCl)
— Concentrated sulfuric acid (H_2SO_4)
— Either Potassium permanganate ($KMnO_4$), as crystals (approximately 0.1 - 0.5 g per sample) or a saturated solution of potassium permanganate (1 - 2 ml per sample)
— Saturated oxalic acid ($C_2H_2O_4$) Dissolve approximately 10 g oxalic acid crystals in 100 ml distilled or demineralised 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.

Method
Homogenize the sample by shaking and transfer 5-10 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 drop wise until effervescence, indicating carbon dioxide release has ceased. Add distilled or demineralised water and centrifuge. Discard the supernatant.
Note: This stage can 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.
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.
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).
Add distilled or demineralised water and centrifuge. Approximately 3000 revolutions per minute for five minutes in a bench centrifuge is adequate to ensure that all the valves are precipitated. Decant and discard the supernatant.
Add distilled or demineralised 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 demineralised water and transfer to a clean, small capacity vial. Add a few drops of 4% formalin, hydrogen peroxide or ethanol to prevent fungal growth. The sample can then be stored indefinitely.

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