How to prepare diatom samples.

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This "primer" on cleaning and preparation of diatom materials is intended to assist two groups of diatomists in particular:
1) those who take up diatom studies as part of investigations rooted in some other discipline (plankton studies, wetlands research etc.)
2) investigators with limited laboratory facilities
Therefore, the overruling criterion has been simplicity. Methods have been selected that yield good results with the minimum of outlay and use the most widely available chemicals only. These "Diatom Handicrafts" notes will not deal with specialised methods as required for cytological work, for instance.

SAMPLE PRETREATMENT

Fossil "rocky" material (diatomites) may require specialised treatment and is treated separately further on. Assume that the sample consists of a gathering like:

- sediment from a pond or a coastal marine mudflat
- scrapings from stones or piles
- leaves of aquatic plants
- harvest from a plankton net

The sample will contain - apart from (hopefully) diatoms - mineral debris (sand, mud, silt) and organic matter (from plant debris to small animals). The aim is to remove both as well as possible while losing as few diatoms as possible. Mineral debris is removed by sedimentation, organic matter (including the diatom cell contents) is removed by oxidation. Sedimentation and oxidation will be treated further on.

Samples must be fixed with formalin immediately after collection. Add about a tenth of the sample volume of 40% formalin and swirl.

General note:
Throughout the text, the phrase "discard the supernatant" will occur. Do not use a pipette for this, or do not pour off the supernatant, as either action may disturb the sediment and you may lose material. The best way is siphoning with small diameter soft plastic tubing. The speed of draining can be sensitively controlled by pinching the tube.

Separate the coarse organic debris first. Objects like plant leaves or stems take ages (and gallons of oxidant) to decompose. On the other hand, they cannot be discarded because they may bear rich populations of diatoms (epiphytes!). The same is true for small stones or shells (epilithic species) and even for sand grains (epipsammic species).

Sample pre-treatment aims at detaching the diatoms from such substrata. Procedure:

1) *Remove excess water.*
If the sample contains a large volume of water, pour the entire gathering - leaves, stems, sand, algae, shells etc. - through a household sieve (plastic, mesh about 1 mm), collecting the "fluid" fraction (which also contains the sand/silt). What remains on the sieve (the solid fraction) goes into a generously sized glass beaker. Let the fluid fraction settle completely (check in direct sunlight, the supernatant should not be "milky"), discard supernatant, resuspend the sediment with just enough water and add to the solid fraction. These steps simply remove excess water and may not always be necessary: if the sample does not contain large plant fragments or floating stems, just let the material settle and discard the excess water.

2) *Detach diatoms.*
Pour some hydrochloric acid (household quality will do) onto the material. Calcareous matter (limestone, shells) will dissolve with production of foam. Stir and leave until foaming subsides, add some more hydrochloric acid until no foam develops anymore.
Add enough water to cover the sample by a layer of a couple of cms. Heat this gently and let simmer for about half an hour. Beware of fumes. This detaches the diatoms by dissolving mucus. The process can be assisted by scrubbing the leaves, stems or stones with a plastic toothbrush. Pour through a plastic sieve and collect the fluid, pour some more water over the residue in the sieve to wash out remaining diatoms. Collect this water too, discard contents of sieve.

3) *Remove acid.*
Let the fluid settle completely, discard supernatant. Add tap water, mix thoroughly with the sediment, let settle and discard supernatant. Repeat at least twice. This removes the acid and the calcium chloride into which the calcareous matter has been converted. Rinse twice with distilled water.

This pre-treatment procedure ensures that you will not try to oxidize more stuff than is necessary, whilst avoiding major loss of diatoms. You'll always lose a few! At this stage the volume of the sample has become much more manageable and the "raw" material can be stored for further processing if you add formalin. Incineration and mounting (see later)
allow quick examination under the microscope.

Not all materials require such pretreatment. The most unfavourable situation has been assumed (leaves, stems). Samples like scrapings from stones, plankton catches or rich harvests of

periphyton may not require anything but a rinse in hydrochloric acid just to be sure no calcareous matter is left. Then rinse thoroughly with distilled water and add some formalin.

DRY (HERBARIUM) MATERIALS

Type- and other materials from collections require special care. If the material has been oxidised already and is stored in distilled water, slides can be made without further processing. There are two special cases, however:

1) Tiny fragments of mica with specks of sample.
2) Samples in glass tubes that have dried out and form a hard cake that sticks to the tube.

Museum material is precious and there may be very little of it. Yet, it may be possible to collect specimens from tubes that are on record as being "empty" - and indeed look so! Some concessions may have to be made to cleanliness, the purpose is to at least
recover whatever diatoms may be left...

1) *Mica fragments.*
Place the fragment in a small test tube. Add a few drops of concentrated (30%) hydrogen peroxide. Let stand in sunlight for two days or so or gently heat to about 60° C. for an hour or so (water bath). Swirl gently every now and then. This will detach most of the diatoms from the mica. Take out the mica with pincers and transfer it to another test tube (#2) with some distilled water. Let the fluid in tube #1 settle completely and the supernatant can then be carefully (!) pipetted off, followed by two rinses of distilled water. Check critically (preferably in direct sunlight) that no material sticks to the wall of the tube! If it does, swirl briefly and let settle again.

If the sample is minute, it's easy to lose everything. In that case a slide can be made at this stage, without further rinsing. Some peroxide remains, but this evaporates when you make the slide. To remove any residual organic matter, use incineration (see further on). Note that if the material was of marine origin, rinsing may be unavoidable, to remove the salt.

ESSENTIAL: take out the mica fragment from tube #2, dry it and make a slide of it too! Place the mica on a small blob of mountant on a slide, the surface that carried the material uppermost. Put another small blob of mountant on a cover-slip, turn over the slip, drop it onto the mica and heat gently for an evenly spread and bubble-free layer of mountant. Apply some pressure (place a small weight on the cover before heating). The microscope image will not be perfect, but I've had a case where the only type specimen present in the entire sample had remained stuck to the mica! The water that remains in tube #2 is added to tube #1 - it still may contain the single diatom you're looking for ....

2) *Dried out samples.*

The natural inclination is to scrape a bit from the surface. NEVER (!) do this, probably the uppermost layer will merely contain the finest silt or the smallest diatoms in the sample and damage will also ensue.
Procedure: add a few drops of concentrated hydrogen peroxide. This softens the cake and after some time you can re-suspend the sample. Collect your subsample. For both subsample and the original sample replace the peroxide with distilled water (let settle and rinse, repeat 2x), add some formalin for storage.

CAUTION: if the dried-out sample is of "raw" (unoxidized) material, peroxide may lead to foaming, see "Beware of peroxide".

******** **BEWARE OF PEROXIDE!** *********

A lively reaction will occur when an oxidant is added to hydrogen peroxide (see further under "oxidation"). But even when used alone, peroxide has nasty surprises in store.

Adding hydrogen peroxide to unoxidized material may result in quite severe foaming and even "brewing up" of the lot. I have had to cope with truly explosive reactions after adding hydrogen peroxide to 40-year-old dried-out mud cakes! The phenomenon appears to be limited to muddy samples (including dirty periphyton) - do some muds contain katalytic minerals?

The most treacherous aspect of peroxide is that "brewing up" may take quite some time (several minutes) to start, when nothing much appears to happen. Then it may chain-react in almost no time to catastrophic intensity. Constantly keep an eye on a peroxide brew and if foaming seems to get out of hand, pour everything QUICKLY into a MUCH larger
wide beaker or better yet: a flat dish. NEVER (!) put a stopper on the tube or you may have to collect the remnants of the sample from the ceiling.

To my knowledge, no author has ever issued such a warning while describing the use of peroxide. SHAME indeed...

OXIDATION

General:

- For very small samples, see under "Dry (herbarium) materials".
- Just two methods of oxidation have been selected here. Both yield good results and require the minimum of widely available chemicals. Of course, many more have been described in the literature, but all seem to yield comparable results. Patience is more important than the chemical brew - do not hurry.
- Processing a small portion of the sample, say a layer of a few mm in a 50 ml beaker, is preferable to treating a larger quantity. Use beakers with a flat bottom and straight (not: conical) sides.
- If hydrogen peroxide is used, refer to "Beware of peroxide" first. Also, use wide beakers or dishes. Glassware must be heatproof (Pyrex etc.) as it becomes boiling hot very quickly.
- All oxidants are corrosive and fumes are toxic.
- Home-cooking of diatom samples knows no immutable laws but asks for flexibility, adapting your procedure to the material in question. Samples differ enormously in "difficulty" !

Peroxide method:

1) When the sample has settled completely, discard supernatant
2) Add a small quantity of concentrated (>30%) hydrogen peroxide
3) Let stand for several minutes. If alarming foaming already occurs, let this subside and only then add a little more peroxide. Repeat until foaming becomes less violent. If no serious foaming has occured several minutes after the first small amount of peroxide has been added, add peroxide until the volume is about 10x that of the original sample.
3) Heat gently (water-bath) for 30 minutes or so (depending on amount of organic dirt). Constantly watch, foaming can still get out of hand.
4) Take the beaker out of the water-bath and place it on the bench, preferably in a wide dish or on a plate. (If the reaction gets out of hand, you can then save the sample if it boils over).
5) Add a VERY SMALL pinch of finely powdered potassium bichromate, just a speck. A violent reaction will occur, swirl and let subside. Only then add a little bit more of the bichromate. Continue this until the reaction has stopped, the contents of the beaker must now be orange in colour.
6) Let settle completely, discard supernatant, resuspend with ample water and repeat this at least twice.

For plankton catches and other samples with very little organic dirt, steps 1-3 may be sufficient.

Sulphuric acid method:

This has the advantage of not causing violent foaming. Check that all calcareous compounds have been removed first, otherwise the sample will become totally useless because gypsum crystals will form.

1) When sample has settled completely, discard supernatant
2) Add concentrated sulphuric acid (battery acid from garages will do if you have to improvise) until the volume is twice that of the original sample.
3) Add potassium bichromate. In contrast to the H202 method, no special care is necessary as no violent reaction occurs. Just add enough bichromate to make for a saturated solution (a few orange crystals left on the bottom).
4) Let stand for 24 hours or more, or speed up the reaction in a water-bath (60 degrees or so). Even so, it may take several hours before the sample is clean. The sediment should look greyish and no plant fragments etc. should remain.
5) Let settle completely, discard supernatant and rinse several times as described above.

The sulphuric acid method seems to remove resistant "dirt" somewhat better than the H202 method, mainly because the oxidation reaction is not as abrupt as with peroxide. But again, the principal point is patience, not the chemistry involved.

SEDIMENTATION

General:
- After oxidation, sedimentation aims at removing as much of the mineral "dirt" (sand, silt, clay) as possible while losing as few diatoms as possible. Especially with very fine silt/clay, this may be difficult and some concessions may be necessary: the cleaner you want the sample to be, the greater the chance of losses. Attempts at getting "nice, clean" samples may be **incompatible with quantitative** investigations!
- Especially in sedimentation, you'll have to adapt your procedure to the nature of the sample. There are no standard time-schedules: some stuff settles in a few minutes, other samples may take hours. The only good method is individual checking, see further.
- It is assumed that the sample has been pre-treated (see "Pretreatment"), calcareous matter has been dissolved in hydrochloric acid and organic matter has been destroyed by oxidation, and that both the heaviest (very small gravel) and the floating (plant fragments) muck have been removed.
- When "suspending" or "resuspending" samples, do NOT violently shake the material as this may damage fragile diatoms. Instead, swirl the fluid around for as long as it takes to suspend the sediment.
- To discard the supernatant, use siphoning as described earlier.

- It may take many rinses to clean the sample sufficiently. To economise on distilled water consumption, the first rinses can be carried out with tap water. End with at least two rinses of distilled water, see further.
- Always check (preferably in direct sunlight) that no material sticks to the wall of the beaker glass. If it does, "twist" the beaker quickly by half a turn while it stands on the bench and let settle.

Centrifugation?

Sedimentation may be time-consuming. Although centrifugation speeds up the work considerably, it has a disadvantage: during sedimentation, the chemicals used for oxidation get a chance to slowly diffuse out. These chemicals have penetrated the diatom valves and are "trapped" in its minute cavities. Centrifugation may leave insufficient time for them to leach out so that oxidant residues may contaminate the sample. "Natural" sedimentation is slow enough for the chemicals to leach out.

Sedimentation procedure:

The literature contains recipes giving standard times for the sedimentation process, but the situation will differ for a small, clean epiphytic sample in a 2 ml tube and for a clay sample in a 250 ml beaker! The recipe given here is suitable for any volume, but it should be noted that processing of fairly small samples is always preferable. The aim is to remove the heaviest fraction (called "sand" here) and the lightest fraction (called "clay" here), the middle fraction (let's hope "diatoms") is retained. Before the "dirt" fractions are discarded, they are examined under the microscope to verify the ABSENCE of diatoms.
For this check, darkfield illumination and low power (100x) are ideal.

1) *Separate "sand":*
Suspend sample, let settle briefly (e.g. 20 seconds). Decant supernatant into another beaker ("#2") for further processing. Add water to "sand" in beaker #1, swirl, let settle briefly, decant supernatant into beaker #2. Keep "sand" in beaker #1.

Resuspend contents of beaker #2, let settle for 20 seconds, decant into beaker #3. Add water to "sand" residue in beaker #2, swirl, let settle briefly, decant into beaker #3. Pour contents of beaker #2 into beaker #1 - this is the "sand", beaker #3 will become the sample.

CHECK: add some water to the "sand" in beaker #1, swirl and check a drop of the suspended "sand" for diatoms. If none are present, discard the "sand". If diatoms are still present, repeat entire procedure for the "sand", shortening the settling-time.

When you read this, it may seem like juggling with too many balls (beakers), but it's self-explanatory when you do it...

2) *Swirling trick:*
Heavy diatoms like Trachyneis, some Centrics or Diploneis spp. may sink almost as quickly as the "sand". What may help is the "swirling trick".
Put a small quantity of the "sand" into a so-called watch glass. Add a few drops of distilled water. Place the watch glass on the bench and GENTLY swirl the fluid by shoving the watch glass with a circular motion over the bench . The sand will collect in the middle. Quickly collect the fluid with a pipette. Repeat and check for absence of diatoms in the "sand". Try faster or slower swirling speeds. If the diatoms cannot be separated in this manner, do not discard the "sand" fraction but make separate slides of it. Label these correspondingly, e.g. "heaviest fraction".

3) *Separate "clay*":
Suspend sample and let settle. When the supernatant about 1 cm above the sediment still contains diatoms (collect with pipette and check under the microscope), let settle for some more time. When the water about 1 cm above the sediment no longer contains diatoms, discard the supernatant. Resuspend the sediment and repeat until the discarded supernatant is no longer cloudy. "Clay" may be impossible to get rid of and you may have to settle for a "dirty" sample in the end.

NOTE:
A phenomenon I have seen with marine littoral mudflat samples is the following. When tap water is used for sedimentation, the supernatant will no longer be milky after a couple of rinses. Subsequent rinses with distilled water will nevertheless release large quantities of "clay". Sometimes 6 rinses were necessary and the volume of the sample was eventually reduced to less than a fifth! Apparently some physico-chemical mechanism is involved.

In extreme cases: sieving
After removal of "sand" and "clay", the middle fraction should contain the diatoms with a minimum of dirt. In some cases (notably marine littoral mudflats or saltmarshes) it may be impossible to separate the diatoms and tiny mineral grains in this manner. In that case, sieving through fine-mesh (20 microns) plankton gauze may be the last resort. This will, of course, also remove the smallest diatoms but it may be the only way to permit SEM studies. For these, you can finally place a drop of the sample on a Micropore filter.

Store:
To prevent fouling, add some 40% formalin, but to quote the curator of a renowned collection "we have finally succeeded in breeding a formalin-loving fungus". Formalin may cause some

erosion (only visible in SEM), addition of some hexamethylene-tetramine (buffer) prevents this. Alcohol (70%) is OK but it evaporates quickly unless sealing is really hermetic.
Label with full data (exact location, type of sample, date, collector etc.), use waterproof ink (if you use a printer check that the ink does not come off when wet!!) and varnish the label. Self-adhesive labels and transparent adhesive tape have been found to deteriorate quickly - sometimes in a decade - and if long-term documentation is desired it's better to stick to old-fashioned stuff like gummed paper. Secure the cap of the bottle by wrapping with tape. Dipping into molten paraffin is another good (old-fashioned...) method.

MAKING SLIDES.

Two key issues in making diatom preparations:

1) The slide and the cover-slip must be CLEAN. To test: a drop of water must spread evenly. Simplest: a household cleaner in powder form. Apply some powder to a small wad of moist toilet paper and rub both sides of slide and cover-slip. Rinse under the tap, dry with toilet paper.

NOTE: some luxury types of toilet paper apparently contain something like lanolin or whatever and are totally useless because the glass gets a nice coating of grease... Tissues may be equally useless. Buy cheap ...!

2) **NEVER**, repeat **NEVER**, make a preparation on the slide, always on the cover-slip. Preparation on the slide results in **severe** deterioration of image quality.

To make a slide:

- clean slide and cover, dry
- apply a drop (or two) of material to the COVER SLIP, spread evenly (breathing on the slip helps)
- dry WITHOUT disturbing the slip (diatoms will clot together otherwise). Gentle warming is OK. When the sample is dry, continue (!) drying for a few minutes to avoid droplets of condensation in the finished slide (moisture is "trapped" in the tiny cavities of the diatom valve).

- apply a SMALL blob of mountant to the SLIDE
- turn over the cover slip, place it on the blob of mountant
- gently heat until all bubbles have escaped and let cool.

Mountants:

For highest contrast use a mountant with high refractive index like Naphrax (RI= circa 1.7). Canada Balsam has an RI of circa 1.5 and is not suitable for the more delicate diatoms because it yields very low contrast. For very robust diatoms like heavily silicified Centrics or large Pinnularias, however, Canada Balsam may actually yield **better** results than high RI media because excessive contrast is avoided.

Varnishing of the edges of the cover prevents "cracking" of the mountant (after decades) but is not absolutely necessary. Canada Balsam slides keep for at least 150 years. Naphrax has been in use for several decades and no deterioration has been reported. Some exotic 19th-century mountants that have been used for slides in museum collections were unstable (crystallization).

NOTE: if a mountant with high RI is unobtainable, do NOT experiment with varnishes etc. but use Canada Balsam if you wish to assure survival of your slides for future reference. Personal examination has shown that well-made 19th-century Canada Balsam slides can still yield satisfactory images if contrast enhancement methods like DIC or phase-contrast are used - and at least, slides made with Canada Balsam do not deteriorate.

INCINERATION

A handy method to quickly examine samples. This is also the only procedure that leaves diatom frustules and aggregates intact, permitting verification of heterovalvarity (e.g. Achnanthes, Gyrosigma), growth in chains or sessile life-style. Procedure:

- fix fresh material in formalin
- let sample settle, decant supernatant
- pour on distilled water, let settle, decant
- repeat twice
- apply one (or two) drops to cleaned cover-slip, spread, dry
- heat cover-slip over (not: "in") a spirit or gas flame, the material will turn black, continue to heat until it becomes grey or white. Take care that the cover does not start to warp or melt!
- mount as usual

Organic matter (chloroplasts, blue-greens etc.) will be burnt nicely but mineral dirt will remain. You can partially remove this by first applying fractionated sedimentation to the fresh sample - see under Sedimentation, but beware of loss of material.

PROCESSING OF FOSSIL DIATOMITES.

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Problems working with this material tend to fall into these areas:
1. breaking up the block without breaking up the frustules
2. removing dissolved minerals from the frustules
3. removing contaminating organic materials
4. removing fine particles of broken frustules and other debris
5. further cleaning the frustules to remove particles that may be adherent to their surfaces

1. **Breaking up the block** is pretty easily done by repeated freezing and thawing of the diatomite in distilled water. It may take two or three freeze-thaw cycles or fifty; depends upon the degree of
compaction and the density of the diatomite. Some authors have suggested using a 30% boil in hydrogen peroxide to further the process but patience in the freezing and thawing helps. I have found that putting a block of diatomite into a labeled, water-tight, freeze-proof polyethylene container with a tight lid at home is the simplest; take it out when you arise and put into a bowl of hot water; place it back into the freezer when going out the door to work; repeat on coming home, and in a week one has 14 cycles completed (if need be).

2. **Removing dissolved minerals** is the next step, once the diatomite is a slurry. Transfer to a pyrex beaker and add, slowly, concentrated HCl, using the usual precautions. There may be a vigorous effervescent reaction if carbonates are present, or the liquid may go yellow. If there is no effervescence, boil the material for five minutes minimum in a fume hood and look for other changes of color.
If the supernatant goes very darkly colored, then you may need to allow the material to settle overnight or by centrifugation, then remove the colored supernatant, and add more distilled water down the side of the beaker to avoid splashing and more concentrated HCl. This may further break up any remaining chunks of diatomite. All of the mineral acid should then be removed by careful addition of distilled water.

3. **Removing contaminating organic materials**, such as plant roots, recent organic

contaminants. and insect parts or droppings, is best done by using a method described first, to my knowledge, by Meakin and Swatman, the hot sulfuric acid technique. When the material is rinsed clean of HCl, concentrated sulfuric acid is added at about five to ten times the estimated volume of the diatomite in the bottom of the beaker. This is heated, very carefully, in a fume hood until the water remaining is completely boiled off and the acid is fuming. The appearance of black specks in the diatomite is evidence of organic materials present. When the acid is hot and fuming, very slowly and carefully one drops small crystals of potassium chlorate into the fuming acid, swirling the material after it hisses into the acid. This is an extremely effective set of oxidizers, and when the fluid goes white or yellow from the dissolved chlorine dioxide gas generated, one has reached an endpoint. The material should be allowed to cool and then very carefully one washes down the side of the beaker using small aliquots of distilled water from a wash bottle. Swirl between additions and expect a considerable generation of heat as the reaction is highly exothermic. The goal is to fill the beaker with distilled water, then to allow the diatoms to settle. Subsequent additions of water will not have the same reaction, and one should fill and empty the beaker three to five times to wash the acid out of the solution.

4. **Removing fine particles** can only be done, to my knowledge, using a fine screen. The Global Gilson company has some very fine-mesh screening material in either stainless steel or nylon. Go to their website at www.globalgilson.com <http://www.globalgilson.com> and click under "Product Catalog" and then under "Sieving." The stainless mesh is of course resistant to acids but is much more expensive, and being a woven product, can trap diatoms that will contaminate the next batch. The nylon screening actually has holes punched in a sheet and has no such liability, although I fear that acids would yield holes in the fabric much larger than the nominal size! A 635-mesh screen has holes that are nominally 20 microns in diameter, and any sample (freshwater or fossil) will benefit from screening through this material, without a substantial loss of frustules, but with a remarkably cleaner appearance of the strew. Larger screens are also available, but a large number of frustules will pass through even a 400 mesh screen when one realizes that the nominal size of the pores is 38 microns.
If there is quartz or other sand or mica flakes present that has survived the acid ordeal, this may be removed by putting the sample in an evaporating dish, and directing a stream of water from the wash bottle to make a vortex. In a surprisingly short time the heavy solids will accumulate in the center of the evaporating dish, and the diatoms may be poured off. (I would suggest looking at what you pour off under the microscope to be sure you're not discarding some large forms.)

5. At the end of these processes you should have some clean diatoms, but microscopic examination may show either particulates adherent to the individual frustules, or fine flocculent material that is clearly not diatomaceous. You should have a 10% W:V solution of sodium hydroxide made up, and the way this should be used is like this: Heat up the diatoms in distilled water, keeping them moving constantly because at this stage of heating they will BUMP and blow fluid out of the beaker. While boiling, add an aliquot of the NaOH solution to the fluid in the beaker, where the size of the aliquot is 10% of the volume of the distilled water that holds the diatoms. This will reduce the NaOH concentration to about 1%, and the beaker should be boiled for about 30 seconds, then neutralized with HCl. Check a drop of the diatoms under the

microscope, and possibly repeat one or two more times, depending upon how adherent the diatoms are to one another.

I was taught this method of processing fossil diatomite by Klaus Kemp (www.distoms.co.uk <http://www.distoms.co.uk>) and it has worked very well with freshwater and saltwater frustules from Oregon, Nevada, California, Barbados, and New Zealand.

END.