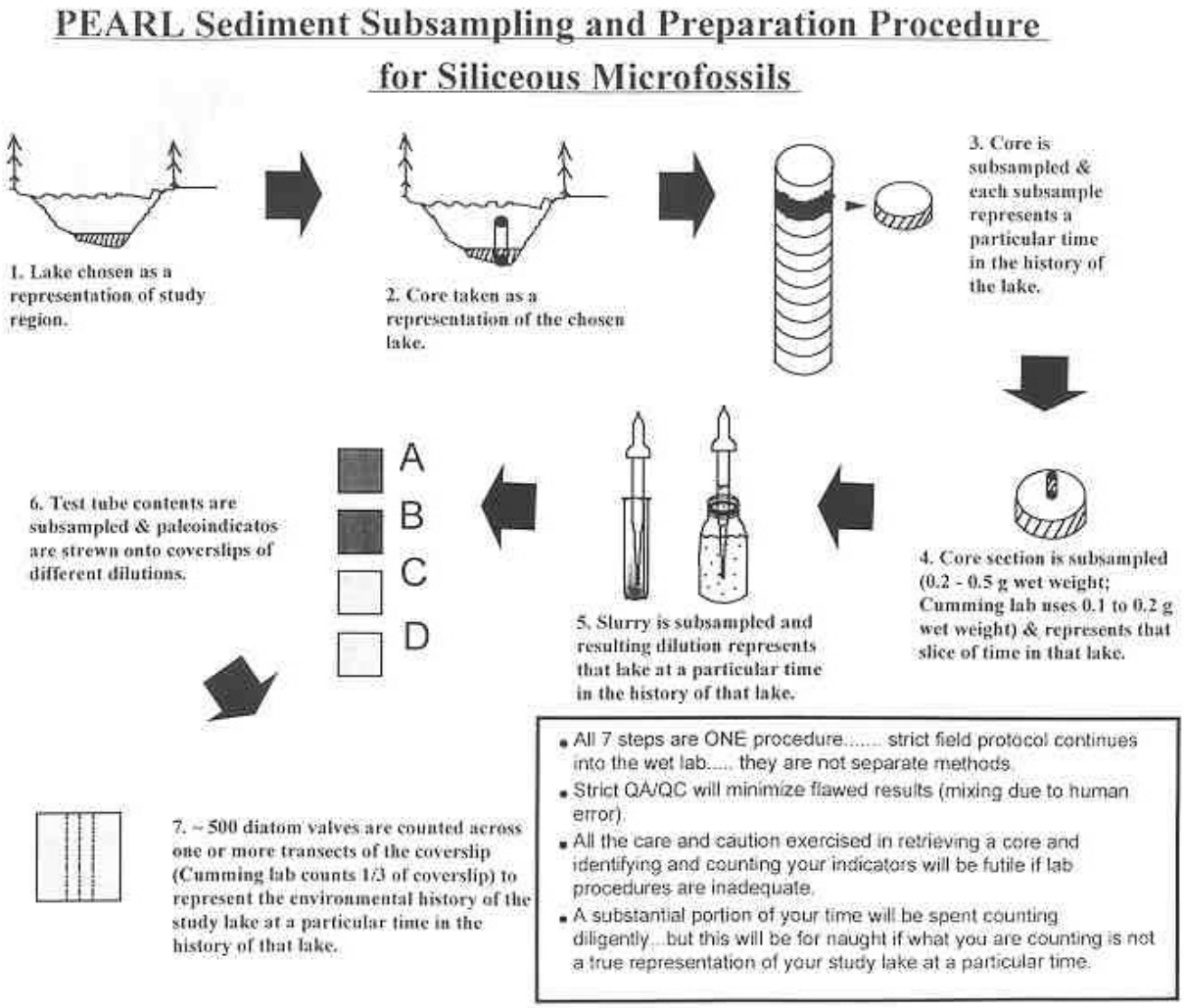
PEARL

Paleoecological Environmental Assessment and Research Laboratory
Department of Biology Queen's University

Standard Sediment Sample Preparation Methods for Siliceous Microfossils (Diatoms and Chrysophyte Scales and Cysts)

Prepared by Kathleen Ruhland, Tammy Karst, Andrew Paterson, Rene Gregory-Eaves, John P. Smol, and Brian F. Cumming

November, 1999



Step - by - Step Sediment Sample Preparation for

Siliceous Microfossils

****THESE STANDARD PROCEDURES SHOULD BE READ BY ALL NEW STUDENTS AND SHOULD BE FOLLOWED BY THOSE WHO ARE PASSING ON THIS INFORMATION TO NEW STUDENTS****

*** While working with concentrated acids, always wear the protection gear provided and work inside a fume hood. When adding acids together, always add in small quantities and slowly (see below).**

Protection gear: lab coat, rubber apron, acid gloves which can be replaced every term (*not* the rubber maid gloves), acid sleeves, face shield, safety glasses, shoes or rubber boots, long pants, and work inside a fume hood.

Fume hood: Avoid the temptation of getting close to your samples in the fume hood ....keep face outside of fume hood ...the fume hood is there to protect you, so by sticking your head in the fume hood to get a clearer look, defeats its purpose. Always ensure that the fume hood does not contain other chemicals etc. (e.g. the acid mix is highly reactive with the toluene contained in NAPHRAX...never work with the acids when NAPHRAX is in the hood!..see below). You must clean the fume hood before use (70% ethanol and a paper towel) AND when you have finished. Do not close the fume hood totally as it will not draw air (remember your WHIMIS). You can use a kimwipe as a "fly" at the bottom of the shield to let you know when you have the most draw (probably open about 10 cm would be optimal)....the Cumming lab has an optimal mark on their fume hood.

Logic: Whenever you are working with dangerous acids etc., make sure that there are other people around and inform them that you are working with acids in the fume hood.

Always allow yourself more time than you anticipate a procedure to take.....for certain steps (such as placing acid samples in hot water bath) make this task your priority for the day. If you don't think you will be able to chaperon your samples for the duration of the procedure, don't do start your digestion until you have the time. Consistency in preparations is of the utmost importance. In the past, unattended samples in a hot water bath have resulted in near disastrous consequences....the water evaporates, the samples continue to boil, the plastic digestion trays melt into an unrecognizable glob, the glass scintillation vials soon follow, and the resulting recipe can lead to a deadly explosion. Aside from the obvious safety hazards to you and your lab mates, you also lose your precious samples.

If this is your first time following any of these procedures: 1) READ the procedures carefully; 2) Arrange a time with an experienced lab mate to guide you through the steps..... it is good form to prepare yourself (read up on the procedure) beforehand and set aside enough time for this particular procedure.....be considerate of the person who has graciously volunteered their time to help you....let's continue to enjoy the "team spirit" present at PEARL.

Acids: Some acids react violently with sediment -- always add things very SLOWLY, in the fume hood. The mixing of H_2SO_4 and HNO_3 generates heat so it is a good idea to wait until the acids cool before adding them to the slurries (see below). Give reactions time to proceed slowly, before you rush them on. Always have test tubes and other reaction vessel openings pointed away from yourself, and anyone else that may be around.

***Remember to read the risks involved in all chemicals you use from the MSDS information**

(see the yellow binder where these sheets are contained.....if not there, check the MSDS web site or ask Jonathan Jones for the sheets you need).

1. ACID MIXING

Materials required:

- **-full protection gear (see first page)**
- **-large flask (of sufficient size for the amt. you plan to mix)**
- **-long glass rod**
- **-glass funnel**
- **-nitric acid (HNO_3)**
- **-sulphuric acid (H_2SO_4)**
- **-parafilm**
- **-glass graduated cylinder (there are a few that are used solely for this purpose that have plastic bags covering the openings to avoid lint buildup etc.).**

1. Work in a fume hood, wearing full protection gear and the glass shield pulled down to the optimal mark. Safety glasses are required under the face shield.

2. Be aware of your surroundings....look around and see what could possibly spill and how. Take action to avoid it and have contingency plans set in mind to deal with any problem that may possibly arise. Know in advance what you would do if an accident occurred. Take note of where the acid cleanup kits, the eye wash station, and the showers are located. Know how to run them.....it is not the best time to read directions on the use of safety gear during an emergency. Also, know where the fire extinguisher is located AND know how to use it.
*** The main acid spill kits are located on the shelves to the left as you enter the loading bay on the 2nd floor. PEARL has small spill kits next to the fume hood.**

3. Measure the nitric acid in a dry graduated cylinder (glass), using a funnel (glass).

4. Pour slowly into a large glass flask (or stainless steel container), using a glass funnel.

5. Measure the sulfuric acid into a glass graduated cylinder, using a glass funnel.

*** ALWAYS ADD THE MORE DENSE LIQUID (SULFURIC ACID) TO LESS DENSE LIQUID (NITRIC ACID*)**

	H_2SO_4 (conc.)	HNO_3 (conc.)
Molecular Weight	**98.075**	**63.01**
Specific Gravity	**1.84**	**1.42**

H_2SO_4 : HNO_3 ..... 50:50 solution by molecular weight

For 1000 ml solution: 530 ml HNO_3
470 ml H_2SO_4

For 500 ml solution: 265 ml HNO_3
235 ml H_2SO_4

If you add the less dense liquid to the more dense liquid, they can stratify, and build up heat at the interface between the two solutions, which can lead to splattering, etc. So, H_2SO_4 should be added to HNO_3 (i.e. this is why nitric acid is poured into flask FIRST, and sulfuric acid is ADDED slowly second). As well, the mixture should be slowly stirred while you are adding the sulfuric acid to the nitric acid (e.g. add a small quantity, stir, add a small quantity, stir etc.). As long as you don't let one sit on top of the other and accumulate heat. The same holds true for water - always add acid to water (A to W...alphabetical).

6. SLOWLY pour sulfuric acid into the large flask containing nitric acid, only adding a small quantity at a time and gently mixing after each addition.

7. Seal the flask opening with parafilm (make some puncture holes in the top) and leave in the fume hood, preferably in a corner out of harms way. The Cumming lab does not cover with parafilm but leaves the samples in the fume hood.
CLEARLY mark this flask with a label saying something to the effect of:
"*CAUTION* CONC. NITRIC/SULFURIC ACID MIXTURE: September 21/99, JPS".

This same logic should be used when leaving ANYTHING in the fume hood including distilled water.....a while ago an unmarked Ehrlmeyer flask was left in the fume hood for months.....no one knew what the flask contained and thus, it had to be disposed of with the assumption of it being a dangerous liquid. If this case should arise again, assume the worst (i.e. that this container is filled with acid). This is extremely costly; money that could be spent on other things like conferences.
8. Allow the acid mixture sufficient time to cool off ~ 1 - 2 hours (preferably over night), the flask should be cool to touch while wearing acid gloves.

9. Pour this acid mixture (using a glass funnel) into the glass acid dispenser (use this dispenser for sulfuric/nitric acid mixtures ONLY) using a spill tray. Allow to cool.

10. Clean all equipment when you are finished. Rinse funnels, flasks, graduated cylinders etc. and all paper towels used for acid wipe ups in the sink....let cold water run over the paper towels for 5 - 10 minutes before discarding into garbage can (stay in the room while rinsing to keep an eye on things). Note: Lab coats should be washed regularly (twice a year).

It is very important to rinse all equipment well, as the next person to use the graduated cylinder or funnel may be using it for a chemical that may react violently to these acids (such as H_2O_2 and toluene (present in NAPHRAX).

***PEARL is a relatively large group, so the chances of another person using the fume hood soon after you are done are high.....therefore, it is important that you CLEARLY LABEL what you have left in the fume hood (must include your name) AND that you clean up after you are finished.**

11. All acid waste must be put into GLASS bottles, NOT plastic bottles. Plastic bottles cannot withstand the exothermic reactions that can occur. Do not wait until acid waste is full....get rid of it early (i.e. when waste bottle is 2/3 - 3/4 full).
*** Acid waste bottles need to be packed in a cardboard box with vermiculite and labelled with a pink MSDS disposal sheet. Once the waste bottle is properly packaged, it can be taken down to the loading bay and placed with the toxic substances awaiting pick up for disposal (check to see on which day pick up occurs as they do not like the bottles sitting there for too long).**

*** Always wipe down the fume hood surfaces (glass shield, handles, front metal surface and the bottom of the hood), and wash off gloves after using acids.***

It is important to wash everything, even if you don't think that you have spilled a drop! If you are not convinced, have a look at some of the lab coats (esp. the blue acid-retardant lab coat) in the wet lab and you will notice the shocking number of holes in the sleeves.....SPLASHING DOES OCCUR..... PLEASE WASH UP COMPLETELY EVERY TIME!

2. Preparing the Scintillation Vials & Measuring Sediment:

Materials required:

- **sediment samples from coldroom**
- **glass scintillation vials and lids**
- **glass marking pen (or electric scriber)**
- **waterproof marker (permanent)**
- **weigh scale**
- **spatula**
- **deionized water**

-squeeze bottle (to be used ONLY for distilled/deionized water)
-kimwipes
-digestion tray

1. Label scintillation vials and lids with sample site code, sediment interval, core number, digestion date, and your initials. Etch this information on the glass scintillation vial with a diamond tip etcher (electric or pen). Mark this information on the plastic lid as well, using a water proof marker. Remember that these slurries will serve as an archive and should be easily identifiable a few years from now.

2. Weigh each sample (including the whirlpack) before any subsampling. This is extremely important if you intend to date these samples. It is a good idea to weigh these samples even though there is no intention of dating them when you subsample, as plans often change. Write down the weight of each sample AND the weight of an empty sample bag in a lab notebook... you may want to weigh 20 - 25 empty whirlpack bags to get an estimate of the mean empty bag weight.

3. Place clean scintillation vial on scale, without lid, zero the scale. Once you have gained experience and your work is solely for strewn mounts using relative % data, it is not necessary to weigh sediment. However, if you are ever unsure as to how much sediment you need to obtain 0.2 to 0.5 wet weight, WEIGH your sediment (it really does not take much time!). Using too much sediment can lead to incomplete digestion of your samples and many unnecessary headaches.

4. Sediment sample should be thoroughly mixed in the whirlpack/vial to obtain a homogeneous sample.

5. Subsample 0.2 - 0.5 (g) (Cumming lab prefers to use 0.1 to 0.2 (g)) wet sample, if available (if necessary, less sediment can be used....see JPS or BFC for details if you are in this situation) with a clean spatula (or a pipette for highly watery surface samples) and place in scintillation vial. This may seem like a very small amount but we can literally get away with the leftover scrapings of a sample bag to get ample material for diatom/chrysophyte preps. Try to avoid getting sediment around the neck of the vial as this makes it difficult to scrape off when digesting. Write down the weight of each subsample in your lab notebook.

6. Thoroughly CLEAN the spatula with distilled water and kimwipes (use a Nalgene squeeze bottle and a glass beaker) before proceeding to the next sample. Sloppiness at this stage will contaminate your samples with diatoms/chrysophytes from the previous sample.

7. If for some reason you are unable to add the acid right away, replace the lids on the vials until you are ready for the next step ....preferably the next day. In the mean time place these samples into the fridge.

8. Return unused sediment samples to the cold room.

3. Adding 10% Hydrochloric Acid (HCl)

Typically this step is done BEFORE the other acid steps

Materials required:
-10 % hydrochloric acid....remember acid to water (A to W) and SLOWLY
-4.5 % Calgon solution (optional, see section # 8)
-pipette
-squeeze bulb
-glass rods

***N.B. the 10% HCl is mixture is made in a glass flask, not in a squeeze bottle. Once a squeeze bottle is used for HCl, it cannot be used for distilled water etc. (label it carefully).**

***NOTE: The purpose for adding 10% HCl to your sediments is to eliminate excess $CaCO_3$ present in your samples. Sediment will effervesce (fizz) if $CaCO_3$ is present. Depending upon the geological setting of your sampling sites, this may be an important step (e.g. samples collected from regions with carbonate bedrock like limestone etc.).**

HOWEVER, the addition of HCl brings Ca into solution.....Ca can react with sulfuric acid to form gypsum, a hydrous calcium sulphate mineral, $CaSO_4.2H_2O$. At the 4^{th} or 5^{th} Annual meeting of the Arctic-Antarctic Diatom Workshop (AADW) this problem was brought forward by a few of the members whose sampling area contained was within highly carbonaceous bedrock . The gypsum tends to form a coating over the diatoms that makes it virtually impossible to identify them. A solution to this problem was to wash the sample with distilled water several times after the 10% HCl was aspirated and before adding the sulfuric/nitric acid mixture.

If you are working with surface samples from an area that is known to be on more acidic bedrock (such as granitic bedrock of the Canadian Shield etc.), add 10% HCl to be on the cautious side. One wash will probably suffice, especially if no effervescing occurs. Remember that calcareous glacial material is commonly encountered, especially in core samples.

Making a 10% HCl solution:

Work in the fume hood.
Wear safety equipment....eye glasses, lab coat, gloves, long pants
REMEMBER: add acid to water (A to W)
Mix acids in a glass container.

To make 1000 ml solution (Divide amounts in two for 500 ml):

-measure 772 ml of distilled water

-SLOWLY add 228 ml of concentrated HCL (Concentrated HCL is 36-38%)

-stir gently with a glass rod

This mixture can now be poured (using a funnel) into the HCl squeeze bottle that is used ONLY for this solution.....

DO NOT USE THIS SQUEEZE BOTTLE FOR DISTILLED/DEIONIZED WATER

Wipe down fume hood and wash all equipment when you are finished.

Procedure:

1. To be on the side of caution, do this in the fume hood. Slowly add 15 - 20 ml of 10 % hydrochloric acid (N.B. the reaction may be violent; add slowly), and 2-3 drops of 4.5 % Calgon solution (optional, see section #8) to each vial of sediment. Stir with glass rod, supplying each vial with its own clean, glass stirring rod.

HINT: To decrease the risk of contaminating neighbouring samples, be careful not to splash the samples you are working on, over the neighbouring samples (when adding acid and when stirring samples). Keep the sample you are working on away from the rest of the samples. To test how the samples will react with the HCl, start by adding only a few drops to each vial. Add the remaining acid SLOWLY by keeping the nozzle of the squeeze bottle close to the opening of the scintillation vial. Usually this step poses no problems, but there have been samples that have effervesced wildly and have flowed over the top of the scintillation vial. Exercise caution.

2. Place the vials in the digestion tray.

3. Cover with metal or plastic and leave undisturbed in the fume hood for 24 hours. * Leave a note on the outside of the fume hood indicating that samples are to left undisturbed.

4. ACID DIGESTION:

GENERAL INFORMATION

Sediment samples used for diatom and chrysophyte analyses are treated with strong acids (sulfuric/nitric mixture) to digest the organic sediment matrix. Basically, this means that the acid will chemically decompose or eat away (digest) any organic material contained in the sediment. This will result in a slurry of siliceous remains including the siliceous indicators that we are interested in. It will NOT eliminate clastic materials, as these have similar mineral compositions to the diatoms and chrysophytes (namely silica). If your sample contains excessive amounts of clastics that hinders diatom/chrysophyte

identification, re-digestion of your sample will do nothing for this problem..... refer to the section of this manual outlining procedures for SPT.

Aspirating:
a) Always check to see if the sediment in each sample has completely settled to the bottom of the vial BEFORE aspirating. It is very easy to overlook this step and you can suck up a lot of diatoms/chrysophytes this way. If there are still particles floating in the vial, give each vial a quick twist clockwise and then counter-clockwise. This will loosen particles from the sides and usually helps them settle.

b) Make sure that you can clearly see the vial contents when you are aspirating (don't just dip the pipette in and suck out the contents). Remove the vial from the digestion tray, move it away from the other samples, holding it securely in your hand and pointing the vial opening away from you. This will reduce the risk of dripping the contents of this sample into the neighbouring samples.

c) Aspirate with low suction (in the fume hood for the first 2 sulfuric/nitric acid washes and using the sink aspirator for the remaining washes, as well as for the 10% HCl washes). Aspirate the subsample until a little under ½ to no less than ¼ (be consistent in amt. you aspirate for all your samples) of the liquid in the vial remains (the more liquid you aspirate, the greater the chance of disturbing the sediment and sucking up diatoms/chrysophytes). Keep in mind that the 1st aspiration (nitric/sulfuric) will determine the number of aspirations that you will require later (i.e. if you suck up more acid at first, then your slurry is that much more dilute). Keep the pipette against the side of the vial and its tip below the liquid surface. This will minimize disturbance of the sediment. Loud sucking noises and bubbling liquid is a clear indication that there is too much suction and/or that the pipette tip is not below the surface of the liquid. If this is your first time aspirating, or if you are unsure of the strength of the suction, do a trial run with a vial containing pure water. Before proceeding to the next sample, dip the aspirator into a beaker of distilled water to clean the tip of the pipette (reduces risk of contamination).

d) Stir samples gently, with clean glass stirring rods, during the digestion steps (when you first add the acid and after you put samples in hot water bath). This is important as it will unclump the sediment (especially for core samples) allowing the acids to digest the sediment more thoroughly. This step will NOT cause diatoms to break.

*** Clean glass rods after use AND before starting to work.**

Washing: Keep the vial you are working on away from the remaining samples to avoid contamination. Add deionized water, applying force to the squeeze bottle to mix the sample; rinse the neck and inside of the vial to ensure that all remaining acid is washed down for the next aspiration.

Procedure:

Materials required:

-full protection gear
-acid mixture
-large glass rod
-glass rods
-acid dispenser
-water bath (deep pan onto top of hotplate OR full water bath if you have one)

*** Move the digestion rack, with subsamples, carefully so as not to disturb the subsamples.**

1. Aspirating the 10% HCl: Check samples to ensure that sediment has completely settled. Aspirate the samples as described in detail above.

2. If your sample contained $CaCO_3$ (samples effervesced after adding 10% HCl), it would be wise to wash your samples with deionized water several times before proceeding to the next step (see above).

3. Working in the fume hood, with full protection gear and the glass shield pulled down to the optimal mark, stir the acid mixture slowly to remix the acids.

4. Place the digestion tray with the vials of sediment on a solid prop to decrease the distance between the dispenser nozzle and the vial opening. Slowly add a small volume (2 - 5 ml) of acid to each vial to initiate the reaction. **Add acid dropwise (2-5 drops) if you did not treat with 10% HCl.

5. Stir each subsample with a glass rod (one for each sample) to mix the sediment and acids, scraping down any sediment clinging to the sides of the vial.

6. Slowly add more acid (small amounts) to each and stir again.

7. Keep repeating until the vials are approximately 2/3's full (15 ml) with acid.
8. Cover the digestion rack with plexiglass or, better still, stainless steel covers specifically made for this purpose. If no plexiglass covers are available, cover with a few layers of aluminum foil....rinse well and discard when finished.

9. CLEARLY mark your samples with the date, the sample name, your name, and the step you are on (i.e. pure acid or wash # 7 etc.). Failure to do this is not only poor form, but can be dangerous to fellow labmates with whom you are sharing the fume hood.....this is also a good way for you to keep track of which stage you are at in the digestion process.

10. Leave overnight in the fume hood.

11. Working in the fume hood, and wearing full acid gear, place digestion tray into the water bath (enamel pan filled with water set atop a hotplate...or full water bath in the Cumming lab). The reason this step is taken is that it speeds up the reaction. The water

level should be about up to 2/3 of the scintillation vials. Set the hot plate to about 6 (heat water to approximately 70- 80EC .....use a thermometer to check the water temperature).

12. Maintain this temperature for a minimum of 60 minutes. Carefully add more water (with a beaker) to the enamelled pan if necessary....DO NOT let the pan get dry.

If using the full water bath, be aware that the water bath does not get as hot as the hot plate and so the samples should be left in longer.....let samples sit for 6 - 7 hours).

13. Stir the samples gently with a glass stir rod as the water bath starts to heat up. Stirring at this stage greatly aids in declumping and digesting your sediments.

********NEVER LEAVE YOUR SAMPLES UNATTENDED DURING THIS STEP********

14. Turn off hotplate/waterbath.

15. If possible, leave the subsamples in the hot water to cool overnight. If someone needs the fume hood after you, remove the trays from the water bath and cover your samples with the plexiglass covers that are CLEARLY labelled. Wipe down the fume hood when finished.

*** If there is a high organic content in the samples, increase the digestion time accordingly. Continually check the water level and temperature, never let the water bath dry out.**

5. ASPIRATION:

Materials required:

- **-full protection gear**
- **-fume hood aspirator**
- **-deionized water**
- **-digested subsamples**

<u>First two aspirations</u>

1. Check to see that all the sediment has settled to the bottom of the vials. Aspirate, fume hood aspirator on low suction, the subsample to a little under half to no less than a quarter of the vial height...consistency in the amount aspirated across all of your samples is the key to getting consistent dilutions on your coverslips (see above for full details).

2. Taking care not to contaminate neighbouring samples, replenish the volume with deionized water, using a squeeze bottle. Apply force to the squeeze bottle to mix the sample; rinse the neck and inside of the vial to ensure that all remaining acid is washed down for the next aspiration.

3. Cover and leave the subsamples in the fume hood overnight.

4. Remove the stopper and with the aid of a funnel, slowly empty aspirator into acid waste (glass disposal bottles found in cupboard below fume hood...should be CLEARLY marked).

*** Always wipe down the fume hood surfaces (glass shield, handles, front metal surface and the bottom of the hood), and wash gloves after using acids.**
Paper towels used to wipe down the fume hood (or any other materials used with acids) should be washed thoroughly under running water (cold) for a few minutes before disposing of in the garbage can. Failure to do this can lead to the dry paper towels (containing acid) to ignite.

<u>Remaining aspirations</u> (can be flushed down sink with lots of water)
WEAR PROTECTIVE GEAR AT THIS STAGE AS WELL....acid splashed in your eyes at this stage of the procedure is dangerous.

1. Aspirate, using the sink aspirator on low suction, the subsample to approximately under ½ to no less than ¼ of the vial.

2. Replenish the volume with deionized water, using a squeeze bottle. Apply force to the squeeze bottle to mix the sample; rinse the neck and inside of the vial to ensure that all remaining acid is washed down for the next aspiration.
3. Cover and place in an area where it will not be disturbed overnight ....it does not need to be placed in the fume hood from the second wash onwards (at this point, having samples clearly marked becomes very important). There have been times when the fume hood was so congested with unlabelled digestion trays (upwards of 8 trays...some on their 9th wash, test tubes in racks etc.) that working in the fume hood became a hazard. Chances are that someone will need to mount their samples with NAPHRAX while you are digesting your samples....these are not good combinations so if you are finished rinsing your samples for acid (first two rinses), remove your trays from the fume hood. Be considerate of other people who need the fume hood after you.

4. Repeat until the acidity of the subsamples approaches that of distilled water.....when the litmus paper does not change colour (i.e. same pH as distilled water).....(8-12 washes and sometimes more, depending upon how much acid is left after aspiration.....how far down the vial you have aspirated for each wash...first aspiration of nitric/sulfuric acid is the key here...TRY TO BE CONSISTENT FOR ALL SAMPLES). The amount of washes necessary will vary from sample to sample so a litmus reading on one sample may not

necessarily mean the same reading on a neighbouring sample.

***N.B. Failure to achieve a neutral sample may result in problems with: 1) mounting (cover slips don't dry well), 2) counting (diatoms have a strange coating on them around the edges of cover slip), and 3) slide warmers (damage to the slide warmers when the samples are too acidic).**

5. Upon completion of aspirations, cap the vials and store the slurries for specimen mounting (see below).

6. If the vials are to be stored for long periods of time, it would be wise to add a few drops of formaldehyde to the slurries to prevent fungal contamination. Wearing protective glasses, rubber gloves, and a lab coat, add a few drops of formaldehyde, replace the cap, and then vortex the vial. CLEARLY mark that you have taken this step. If you need to use this sample in the future, aspirate the formaldehyde-containing slurries, and wash a couple of times.
***N.B. Splashing formaldehyde into your eyes can blind you.**

7. Slides should be mounted within 1 day to 1 week of preparing a clean slurry to avoid fungal growth.

6. SPECIMEN MOUNTING

GENERAL INFORMATION

1) The dilutions that you make for counting diatoms/chrysophytes are important to make your counting as smooth as possible. It is much better to spend an extra week or two in the wet lab perfecting your slides than it is to struggle with poor slides for months when counting. Don't forget that counting will consume the biggest portion of your time for your project, so it is worthwhile to spend time making quality slides.

2) As a general rule of thumb, you want to have approximately 4 - 5 diatom valves per field of view (2 - 3 for Cumming lab) for your counts so that one or two transects completely across the cover slip (for Cumming lab... to cover 1/3 of the entire coverslip) will yield your desired diatom/chrysophyte sum (- 350-400 valves for a core and at least 500 valves for a calibration study (Cumming lab typically count 400 valves for calibration)....To decide on

the best amount for your study .....SEE the section in this handbook on COUNTING. For some regions this is not possible. If you are in this situation, call your supervisor (JPS or BFC) over to discuss your slides and to determine if these slides are good enough to count. Perfect slides are not always possible for some regions (again, ask JPS or BFC for advice).

*** In the slide making procedure, a test slide should be completed to check for clastics, silt or other debris. If there is a significant amount of debris strewn throughout the mount, there are a few options, see section # 8.**

*** N.B. Four dilutions are usually made as the density of a full pipette of slurry will vary from sample to sample, especially for calibration samples. As well, in the future, these slides may be used for both chrysophytes and diatoms.**

Materials required:

-cover slips
-disposable pipettes
-rubber bulbs
-slide warmer
-deionized water
-small test tubes (15 ml) (disposable for Cumming lab)
-vortex
-10% ethanol (optional)
-surgeons's gloves (optional)

<u>Procedure</u>

1. Thoroughly clean the slide warmers with 10% ethanol and then wash with distilled water and kimwipes. Wash the test tubes (and test tube brushes) very well before (and of course after) starting the plating (use a test tube brush....this may be another source of contamination.....wash the test tube brush well before using). Rinse with distilled water several times. It is also a good idea to use the vortex mixer to better dislodge any attached contaminants. There is always the possibility of contamination of your samples with those of others that have used the test tubes (and slide warmer) earlier. A while ago, the Cumming lab experienced contamination as a result of the test tubes and wire test tube brushes not being thoroughly cleaned.....they found hyper-saline diatoms in ultra-oligotrophic samples. We could possibly shift to disposable test tubes (plastic) but we are supposed to be an environmental lab..... Wash the test tubes with a brush right after you are done, otherwise dried-up diatoms can leave a mess and are extremely difficult to clean (and hard to see of course). As well, thoroughly wash the test-tube brush to dislodge any diatoms. The Cumming lab will continue to use disposable test tubes to avoid contamination problems.

2. Place clean cover slips (cleaning with 10 % ethanol optional) on a slide warmer in columns of four. If you are inexperienced with this procedure, it would be best to widely space out your columns so as to avoid any spills. To avoid fingerprints on the coverslips when you are handling them, you can wear surgeon's gloves while placing them on the slide warmer...oil from fingerprints may affect the settling of the slurry.....but this should not be necessary after some practice.

3. Keep records of the location of each sample on the slide warmer.....either by putting a strip of masking tape along the edge of the slide warmer, marking the sample number beneath the column of cover slips and/or in a notebook.

4. Vortex the slurries to break up any clumps and diatom colonies.

5. Important! Make sure the slurry is very well mixed before taking a subsample with a pipette. Keep the slurry in constant motion with the pipette and then draw a sample from the bottom to the top of the vial to get an integrated sample of the whole vial. This takes some practice to master....ask an experienced person to demonstrate this procedure. Place this pipette-full of slurry into a clean test tube.

6. Add approximately 10 ml of deionized water (3/4 to 4/5 full).....use your judgement as some slurries are very dilute to begin with (especially surface samples).....this varies from region to region, lake to lake, and interval to interval. There is no set rule to determine how much to dilute your sample, if at all.

7. The sample should have a slightly cloudy appearance. If not, either add more slurry or remove some of the tube's contents and replace with deionized water. It is likely better to have one of your four samples too concentrated, than to have all four samples too sparse.
8. Again, mix the slurry in the test tube thoroughly and keep the slurry in constant motion with the pipette. Subsample the test tube contents by drawing up the slurry from the bottom to the top to get an integrated sample of the whole test tube. This is important as the heavier diatoms will start to sink immediately once the motion stops. YOU MUST HAVE A HOMOGENIZED SAMPLE TO DRY ON THE COVER SLIP.
*** N.B. This mixing should be done when you are taking slurry out of the test tube for the next dilution as well (as by then, diatoms have been differentially settling again).**

<u>Dilution A</u> (these steps are best explained with a demonstration)
9. Resuspend the test tube's contents thoroughly and remove ½ a pipette-full, starting from the base and drawing upwards.

10. Carefully dispense the pipette of slurry onto the cover slip, preferably keeping the meniscus intact. If meniscus breaks and the slurry draws underneath the cover slip, removing that cover slip when dried becomes a bit tricky. Be very careful when you are pipetting the slurries onto the cover slips....make absolutely sure that you do not splash neighbouring cover slips, otherwise the point of looking for changes in diatom abundances

is lost.
(Have this demonstrated to you by an experienced lab mate).

*** If your slurry spreads to neighbouring cover slips, you have no choice but to re-do these samples..... this can be done quite nicely by carefully drawing up the slurry from those coverslips with a pipette. Clean the slide warmer surface where the spill occurred, and replace with fresh cover slips.**

11. Resuspend the test tube contents and remove ½ of the test tube contents one pipette-full at a time, making sure that you maintain a homogenized sample each time.....dispose in a waste beaker.

12. Replenish the tube's contents with deionized water (again to about 3/4 full).

13. Repeat the above process for B, C, and D dilutions so that your four coverslips are all of different dilutions.
*** Remember to use a clean or new pipette for each slurry. Change or thoroughly rinse the rubber pipette bulbs between samples.**

14. Cover the slide warmer (clean/dust the cover...especially on the inside, as lint can rain down on you samples), so as to not produce condensation, and heat on a low level (1 - 1.25) until dry. Coverslips can be successfully dried at room temperature (i.e. with no heat from the slide warmers), however, if it is a humid day it is advisable to turn the slide warmer on low, so as to avoid condensation. Let the slurries dry slowly (don't crank up the heat on the slide warmer). Low heat helps, to some degree, to minimize the formation of an X pattern on the cover slips. This X occurs from the evaporation of the meniscus of the square cover slips and should not pose a big problem.

7. MOUNTING WITH NAPHRAX ®

General

NAPHRAX ®:
In the past, PEARL has used HYRAX ® as a mounting medium for diatom/chrysophyte preparations. We have replaced this with NAPHRAX ® (~1994) as the company that produced HYRAX is no longer in business (therefore, you may notice that some of the older papers/theses mention HYRAX ®).

NAPHRAX is a mounting medium with high refractive index (at least 1.74) that is ideal for mounting diatom and chrysophyte slides. It is a synthetic resin dissolved in toluene. This medium is ordered from England and usually takes a few weeks to arrive after it is ordered...therefore, make sure that we have enough NAPHRAX in stock (at least 4 - 5 bottles). If we start to run low, order some or ask the technician to order more....Cumming lab - please inform BFC. Because toluene-containing substances cannot be shipped by airmail, NAPHRAX arrives without the presence of toluene .....therefore, we must add the

solvent, toluene, to the resin before using a new bottle. Each new bottle comes with instructions as to how to prepare the medium and how to use the medium (refer to these instructions when making up a new batch).

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***N.B. TOLUENE IS CARCINOGENIC, TERATOGENIC, AND MAY CAUSE REPRODUCTIVE EFFECTS (see MSDS information in yellow binder).....work in the fume hood AND wear protective gloves (surgeon gloves) AND a lab coat when handling this material (as outlined in Material Safety Data Sheet). Chronic exposure through contact with skin and inhalation can lead to dermatitis and may cause cardiac sensitization and severe heart abnormalities. Allow your freshly mounted slides to dry in the fume hood for at least 24 hours before removing.**
In other words, wear the safety equipment and work in the fume hood with the shield at the optimum mark..

******TOLUENE (solvent used in NAPHRAX) IS AN INCOMPATIBLE MATERIAL WITH BOTH NITRIC AND SULFURIC ACID (used in our digestion process)******
In other words, don't keep NAPHRAX and freshly mounted slides in the same fume hood when there is acid digestions in progress. This can be very dangerous!!

Materials required:

- **labelled slides (site, core #, date collected, sediment interval, date mounted, your initials)**
- **NAPHRAX mounting medium**
- **hotplate**
- **forceps**
- **labcoat**
- **rubber surgeon's gloves**
- **10% ethanol (optional)**

1. Label your slides with site #, sediment interval, date of preparation, dilutions (i.e. A, B), etc. Scribe this information on the back of the frosted side of the slide and as well on the opposite end of the cover slip (cover slips break most easily along the frosted end thus information scribed at the opposite end will ensure that you do not lose this important information if this occurs). Avoid getting oily fingerprints on the slide (especially where you will be mounting the cover slips as that fingerprint will be there permanently). Clean slides using 10% ethanol (optional). Use a waterproof marker, and write this information

on the front of the frosted side of the slide.
*** Hint: to avoid getting fingerprints on the slides when you are marking/etching them, wear surgeon's gloves.**

2. Work in a fume hood.

3. Place a small drop of NAPHRAX centrally onto a slide (either with a glass rod or with a disposable pipette). The amount needed is surprisingly small.....it may take a bit of practice to get it right. Ideally you want to mount your cover slips in the minimal amount of NAPHRAX to minimize the thickness of the mountant. As well, this will shave hours off your time in cleaning the slides once they are thoroughly dried.

4. Carefully pick up the cover slip using TWEEZERS (place other hand beneath the sample in case coverslip should fall). DO NOT use your fingers, as a smudge on the cover slip can eliminate a high percentage of the precious diatoms that you have so carefully plated. As well, you are more likely to contaminate the next coverslip you are going to mount. This can be easily mastered with a bit of practice.

5. Briefly heat the cover slip, on the hotplate (hotplate set at medium to low heat), with the sample side up. This is a good idea as it removes any unwanted, residual moisture that is left on the cover slip AND this adheres the diatoms onto the cover slip (see instructions given with the NAPHRAX supplies).

6. Place the cover slip, sample side down, onto the NAPHRAX.

HINTS: 1) For best results, aim to place the cover slip so that the drop of NAPHRAX is squarely in the middle of your cover slip....this will aid in the NAPHRAX spreading evenly over the entire cover slip. You can give the top of the coverslip a gentle tap with the tip of the glass rod/forceps to help spread the NAPHRAX. 2) Allow the NAPHRAX to spread (without your aid) for about a minute.....you can prepare the next sample in the meantime.

7. Repeat for the second cover slip for that slide.

8. Heat the slide on the hotplate until the NAPHRAX starts to bubble. Remove from the hotplate and allow to cool.... the bubbles will disappear. DO NOT let the NAPHRAX bubble vigorously. There is a fine line between heating the NAPHRAX for too long and for too short a time.....by heating it in steps ensures that you will not burn the resin, and that you will slowly drive off the solvent. Heating of the NAPHRAX will drive off the solvent (toluene) and is therefore, an important step in drying/setting the mounting medium....if you do not succeed in driving off enough of the toluene, your coverslips will take a long time to dry, as toluene (solvent) is what keeps the resin supple.

9. Return the slide to the hotplate and watch until the bubbles appear again....remove and let cool. Repeat (usually two more times) until bubbling appears slower, with larger

bubbles forming. Remove and lie on a perfectly flat surface. The timing of this steps depends upon the temperature of the hot plate and the amount of NAPHRAX used....this step is best demonstrated by an experienced lab mate.
*** Coverslips need to be heated long enough to drive off the solvent (what keeps the medium soft) but not too long or the resin will burn (turns darkish brown and very hard).**

10. Watch closely and allow the NAPHRAX to spread by itself (usually the bubbles should disappear on their own). Before the medium cools, press down gently with a glass rod so that the minimum thickness of mountant remains under the cover slip. Again, it takes a bit of practice to master this skill....especially in terms of timing.

If, by chance, you need a bit more mountant for that particular slide (some samples are more grainy than others), this is best added after step #8 (after the first time you place the slide on the hotplate).... add NAPHRAX carefully along an edge of the cover slip and allow the medium to draw under the cover slip when you return the slide to the hotplate for the second time.

11. Place aside to cool, on a tray, in the fume hood, with the NAPHRAX pipette, overnight.

12. Dispose of NAPHRAX pipette in glass waste.

13. When the NAPHRAX has dried completely (about one day) and wearing surgeon's gloves, carefully remove any spots of NAPHRAX on the top of the cover slip with an exacto blade flat on the surface of the slide (this step should be minimal if you have mastered the skill of mounting). You can then clean the slide with a bit of ethanol and a kimwipe, making sure not to touch the information you have written in waterproof ink on the frosted side of the slide, as the ethanol will remove this writing.

*** Once the slides are made and satisfy your requirements, and any other remaining work is completed (eg. S.E.M. stubs), there are several storing options for the remaining slurry. 1) Cap tightly and store in a cool, dark room, 2) Add 2-3 drops of formaldehyde to the slurry and vortex. 3) Alternatively the slurries can be evaporated and stored. The latter two options are useful if there is a risk of fungal infection.**

8. Troubleshooting Solutions for Slides

*** Treatments should only be used as a last resort and be tested on a sub sample of the slurry for its effectiveness before treating all slurries.**

8-1. Dilution Alterations.

If the test slides are laden with debris, try making a weaker set of dilutions for mounting. Making sure that the weaker dilutions still have an adequate number of diatoms to count. Alternatively, if the slides are "thin" (not enough diatoms), try increasing the amount of slurry used for making the first dilution.

8-2. Treatment of Slurries with 4.5 % Calgon

This treatment is normally done on those samples which have a high clay content. Try to only use this as a final option. Two to three drops of 4.5 % Calgon should be added to the slurries, preferably when the 10 % Hydrochloric acid is added. If the solution has to be added following the digestion procedure, mix the slurries and let settle. Aspirate the slurries 8 times before mounting in Naphrax.

8-3. TREATMENT OF SLURRIES WITH SODIUM POLYTUNGSTATE (SPT)

For more information on this procedure, check the www at the following address: (http://www.indiana.edu/~diatom/siltsort.dis).

Sodium Polytungstate is a non-toxic heavy liquid used for density gradient centrifugation to concentrate diatoms from silt size particles in samples.

Materials required:

-sodium polytungstate (very expensive material)
-small beakers (20-250 ml)
-centrifuge
-parafilm
-plastic centrifuge tubes
-deionized water
-disposable pipettes
-latex gloves (use is optional but may be preferred)

1. Aspirate, sink aspirator on low suction, 3/4 to 4/5 of the water in the vials.
2. Prepare the SPT solution checking for the desired specific gravity (SG).
3. In a 150 ml beaker, weigh the amount of deionized water.
4. Measure out the amount of SPT using a small glass beaker.

*** Do not use metal as this can cause a reaction.**

5. Tightly seal the Zip-lock bag when finished.

*** It is important to keep out any moisture so check and double check the bag's seal before putting it away into the tupperware container.**

6. Always add SPT to water, and add the SPT slowly and always stirring the water.
7. The solution should have a clear appearance before use.
8. Cover with parafilm when the solution is not in use.
9. Add 2-3 ml of the SPT solution to the clean centrifuge tubes.
10. Vortex the remaining slurry and add, using a pipette, 0.5 - 1 ml of slurry on top of the

SPT solution in the centrifuge tubes.

11. Balance the centrifuge tubes (metal holders and tubes) by carefully adding deionized water to the tubes until weights are equal.

12. Centrifuge for 5 minutes at a low speed (500 RPM).

*** A faster RPM may force the diatoms through the SPT.**

13. Pipette out the slurry lying above the SPT interface, trying to pipette minimal amounts of SPT.

14. Dispense into a separate set of centrifuge tubes.

15. Remove the SPT, leaving the bottom pellet, and dispense into a beaker.

16. Discard the pellet, or following the below washing techniques, prepare a slide to determine diatom loss (should be minimal).

17. Resuspend the material in the centrifuge tubes with deionized water. Mix thoroughly.

18. Balance and centrifuge for 3 minutes at 1500 RPM.

19. Remove the water, replenish with deionized water, mix and centrifuge as in # 18.

20. Repeat this 4 times to remove the SPT.

21. Add the wash water to the beaker containing the waste SPT beaker for recovery.

22. After washing, place the samples into clean plastic scintillation vials.

23. Make slides using the standard technique (see section # 7).

SPT Recovery

Set up a filtering kit, see instructions, with 0.45 micron filter paper. Take the waste SPT beaker and remove the upper layer of liquid. Filter this liquid and repeat with clean filter paper. Take the clean liquid and place in an oven to evaporate the all the water. Once it is in powder form, place the recovered SPT powder into a zip-lock bag marked Used SPT, labelled with the number of times it has been used. If you preparing numerous samples, re-use the SPT of your previous work. The SPT can be reused approximately 4 times.

8.

Calculating Specific Density for Sodium Polytungstate (SPT)

Density (g/ml)	Grams of SPT	Grams of Distilled Water
2.25	700	300
2.3	723	277
2.35	740	260
2.4	752	248
2.45	763	237
2.5	772	228
2.55	782	218
2.6	790	210
2.65	797	203
2.7	805	195
2.75	815	185
2.8	820	180
2.85	827	173
2.9	835	165
2.95	840	160
3	850	150

*** The above measurements supply SPT solution for approximately 100 samples when using 2.5 - 3.0 ml per sample processed.**

NOTE: ONLY USE DISTILLED WATER
USE ONLY GLASSWARE OR PLASTIC
ONLY ADD SPT TO WATER, DO NOT ADD WATER TO SPT
DO NOT USE ANY KIND OF METAL

MATERIAL SAFETY DATA SHEETS (MSDS)

Please read these data sheets carefully BEFORE starting a procedure. The following pages include important information on:

1) Sulfuric Acid

2) Nitric Acid

3) Toluene

4) Hydrochloric Acid

*** N.B. Carefully read the sections on:**

a) <u>Incompatible materials</u> .....this will help avoid serious accidents!

b) Personal protective equipment

c) Potential Health Effects