

## LIENSs Stable Isotope Facility

### Appendix 2: Sample preparation protocols

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This appendix details all the technical protocols used at LIENSs Stable Isotope Facility (LIENSs SIF) for stable isotope analysis of various sample types. Sampling strategies and methodologies depend on research objectives, sampling sites and sampled organisms or materials. As such they are under the responsibility of the researcher and are thus not described here.

Besides sample preparation protocols, this appendix concerns also sample encapsulation and sample submission to our laboratory.

This appendix is focused on C & N analyses. However, a large part of it can be applied to other isotopes or other matrices which specificities are (or will be) given in additional appendices.

#### 1. Sample preparation

Sample preparation depends on their type first : Particulate Organic Mater or phytoplankton on filters are not treated a same way than sediments or organisms. Extraction of dissolved nutrients, as carbonate analysis, require specific techniques that will be described apart.

##### 1.1. Plant and animal samples

Most samples analyzed by LIENSs SIF are in this category : higher plants, marine phanerogames, microalgae, macrofauna, meiofauna, tissues from birds or mammals, etc... Treatment line from sampling is not as much constraining as for other types of analysis. Most samples can be analyzed just after drying and grinding, others can need additional treatments to remove some unwanted components such as carbonates (much more enriched in  $^{13}\text{C}$  than organic matter, they must be removed in food web studies) or lipids (depleted in  $^{13}\text{C}$ , it is often necessary to remove reserve lipids that can rise to bias in the estimation of food origin).

##### 1.1.1. Collection, preliminary treatment and storage of the samples

It was formerly advised to use only glass containers (and even acid-washed) to collect samples in the field. However, plastic containers are much more convenient in the field and can be used without any problem if they are cleaned (even new ones), and if a high care is taken not to scratch them with metallic tools, that could introduce plastic particles.

Samples must be taken to the laboratory in a cool box, and animals should be kept alive as to be put in filtered seawater to empty their digestive tracts, which content could interfere

with the animal isotopic signature. This is particularly important for animals ingesting sediment.

The organisms must be cleaned from any sediment or detritus before freezing. Macrophytes should eventually be dipped during a few minutes in HCl 0,1 N tout remove any carbonate associated with some epibionts, then rinsed with pure water.

Samples can then be frozen at -20°C, or dried and stored in a freezer or even in a desiccator under vacuum at room temperature.

**Special cases** : *in some sampling sites where no equipment is available to keep frozen or dry samples, these ones can be stored in (pure) 70% ethanol, that should be evaporate once back to the laboratory.*

*Ethanol is the least bad preservative (never use formaline !), but it's necessary to make sure that it has no effect (or quantify its effect) on the type of analyzed sample (For your information, tests have not shown any effect on different terrestrial plants, and a slight effect on seeds. Ethanol 70% is often used to preserve blood samples taken on birds).*

**Sample storage is easy in view of isotopic analyses, as long as no bacterial or fungi development occurs : dried samples can stay at room temperature, better in a dessicator, or kept in a freezer (in this latter case, they may get back some humidity and are better dried again).**

### 1.1.2. Sample drying

Drying can be obtained through various ways : in an oven or by freeze-drying (or even by sun exposition in some areas where no electricity or equipment is available, the main point is to avoid bacterial development).

Oven-drying should not be done at a temperature >50°C (some volatils lipids can be lost). Contrarily to freeze-drying, oven-drying generally lead to very hard dried material, that is difficult to grind using a mortar and pestle.

*Very fat samples (such as marine mammal samples) generally cannot be neither oven-dried nor freeze-dried thoroughly. Except if they are further ground frozen in liquid nitrogen, it is better to perform a preliminary delipidation by cutting samples in tiny pieces before drying (better by freeze-drying).*

In case of grinding using of ball mill (see below), samples must be extremely dry. It is better to keep them overnight in a desiccator under vacuum and over P<sub>2</sub>O<sub>5</sub> before grinding.

### 1.1.3. Sample grinding

For isotopic analyses, grinding should be done as to obtain a very homogenous and fine powder (as flour), as to ensure i) a better combustion and ii) a good homogeneity.

However, if the sample can be considered as homogenous (blood, invertebrate muscle), a thorough grinding is less necessary. Very small organisms can also be analyzed *in toto*, if their weight does not exceed the upper limit.

Numerous types of grinding equipments exist. For isotopic analyses, ball mills are especially efficient in obtaining very fine powders. However, samples must be very dry or the grinder must be used under liquid nitrogen.

In our laboratory, we use a mixer ball mill Retsch MM400 (Haan, Germany).

Various grinding jars are available : inox 10 ml (1 or 2 balls of 9 or 10 mm in diameter) , inox 25 ml (1 or 2 balls of 12 mm), zirconium 35 ml (1 ball of 12 mm), and supports for 20 x 2 ml microtubes (1 ball of 3 mm + 1 ball of 4 mm).

*Zirconium jars are kept for samples grinding in view of lipids and metals analysis.*

*Grinding within microtubes is useful for small samples (of volume no more than 1/3 of the microtube volume) : even if grinding time is longer (3 minutes against 1 in inox jars), 20 samples can be ground at a time. Another interest is that samples can be stored with the microtube, that saves the cleaning time of jars (take care that hard samples, for examples pieces of mollusc shell, cannot be ground in plastic microtubes).*

The use of the grinder is very simple but some points must be considered :

- never use the grinder with only one jar : even only one sample have to be ground, the second jar of the same size (with the equivalent balls) must be installed.
- never use balls of a different material than the jar.
- the two jars must have approximatively the same load.
- the sample must represent <25% of the jar volume
- never overtighten the tightening screws : the clamping device is sufficient to maintain jars in the right position.

The full grinder user's guide and a synthetic note (see the end of this document) are available close to the equipment.

***Reading of this note is absolutely required before using the grinder for the 1st time.***

#### **1.1.4. Lipid removal**

Lipid removal from animal samples is necessary in food web studies when the lipid content is high or/and when it shows large variations (e.g. lipid reserve accumulation for reproduction). Lipids are depleted in  $^{13}\text{C}$  relative to other biochemical constituents, and thus affect the global isotopic signature, while accumulation of lipids is not always linked to a change of food resources.

In any case, only reserve lipids, the ones that can exhibit large variations, should be removed. The delipidation methodology used at LIENSs SIF, using cyclohexane at room temperature, is convenient in that aim. It presents much less drawbacks than other methods generally used, like chloroform-methanol mixtures that have secondary effects on the  $\delta^{15}\text{N}$ . Indeed these methods extract also other molecules than lipids, in peculiar containing amines.

**Warning : even if cyclohexane shows less effect than chloroform-methanol, it may have a more pronounced negative effect on some tissues like digestive glands or liver.**

**It is always better to check this effect, and in case of large effect, to perform 2 analyses, one with and one without delipidation.**

In case it is needed to carry out both a delipidation and a removal of carbonates, it is generally better to delipidate first, especially if the lipid content is high.

### Delipidation protocol

- 1 - About 80 to 100 mg of sample powder are placed in 10 ml glass tubes with screw caps (2 x 20 samples can be prepared at a time)
- 2 - Add 4 ml cyclohexane (analytical grade) using an automatic dispenser, tightly close the tubes, shake using a vortex and eventually sonicate in an ultrasonic bath during 1 minute (take care to the warm-up)
- 3 - Agitate the tubes during 1 h using a rotator
- 4 - Centrifuge at 2500 rpm (1200 g) during 10 min at 10°C
- 5 - Have a look to the color of the supernatant and dispose it with care within the bottle "Cyclohexan trash"
- 6 - If the supernatant has some color\*, restart at step 2
- 7 - Rinse the pellet by adding 2 ml cyclohexan, close the tube, shake using a vortex or sonication
- 8 - Centrifuge again (1200 g, 10 min, 10°C), dispose the supernatant in the cyclohexan trash
- 9 - Dry the pellets by placing the tubes in a dry bath at max 45°C. Drying is quickly done (~ 2 h)
- 10 - Transfer the samples in 2 ml screw cap microtubes.
- 11 - Wash and dry the screw glass tubes

\* Warning : supernatants may be not coloured, even in case of high lipid content (e.g. some fish). Incomplete delipidation is shown by a C:N (by weight) ratio >4.

#### 1.1.5. Carbonates removal

This treatment is necessary for isotopic analysis of samples that may contain some carbonates. Their <sup>13</sup>C content is always much higher relative to organic matter and can distort the results, even if the inorganic carbon content is low.

Muscle of some fishes that have very small fishbones, some crustaceans and some invertebrates have to be decarbonated. Some macrophytes can also support some calcareous attached epibiontes.

##### On fresh organisms :

Removal of carbonates can be carried out on fresh organisms if the acid can diffuse all the way to carbonates (e.g. macrophytes, see § 1.1.1) : dip samples into HCl 0.1 N and watch to bubble production. When no more bubbles are formed, rinse thoroughly with pure water.

On dried organisms :**Protocol for carbonate removal (dried and ground organisms)**

- Place max 100 mg of sample powder in a glass vial (8ml, flat bottom) or a 10 ml Pyrex tube
- Add 1 ml HCl 0.1 N (or 0.5 N for samples with a high carbonate content)
- Sonicate in an ultrasonic bath during 1 min
- Notice bubbles production : when it stops, add 100 µl Hcl more to check for complete removal of carbonates
- Place the vials in the **Techne dry bath at >60°C** to evaporate the acid
- Adjust the system of evaporation by filtered air flushing : lower the plunger tubes by 1 cm deep in the vials and turn on compressed air. After ½ h, check that the flux is still high enough
- Left till dry (many hours, can be left overnight)
- Homogenize in 1 ml pure water (1 min sonication)
- Freeze the samples (not at -80° for the vials with flat bottom as they break easily) and freeze-dry them
- Using a spatula, scratch the vial wall to recover all the sample and homogenize it
- Transfer to a 2 ml screw cap microtube
- Clean and dry vials

**Warning : Carbonate removal may have an unwanted effect on nitrogen isotopic signature. This effect is normally insignificant for animals or plants with low carbonate content, but may increase if the sample is rinsed (loss of organic material). It may also be more important for some type of biological samples and sediments highly carbonated, for which more concentrated acid must be used.**

**Check for this effect. In some cases, it may be better to perform 2 analyses, with and without removal of carbonates.**

## **1.2. Sediments**

### **1.2.1. Sampling, preliminary treatment and sample storage**

Sediment samples are more often collected by coring. If different depths have to be analyzed, layers of the sediment core must be cut directly in the field and slices taken in plastic bags or Petri dishes, depending on their volume.

Samples should be wrapped in an aluminium foil, especially if pigments have also to be measured on, and kept cool.

At the laboratory they are then freeze-dried, roughly ground using mortar and pestle and sieved on a 250 µm net to remove large particles.

*Note : Oven drying is possible but may lead for some types of sediment (mud) to a material very hard to grind and sieve.*

Samples are then finely ground (see § 1.1.3) and stored dry at room temperature in a desiccator or in a freezer.

### 1.2.2. Carbonate removal

Sediment contain very variable carbonate content, the acid strength should thus be adjusted by preliminary tests to completely remove carbonates without adding a large volume of acid that would be long to evaporate.

Follow the standard protocol using this acid strength (see § 1.1.5).

**Warning :** Most coastal sediments contain high carbonate contents, and thus large acid quantities are needed to remove their carbonates. However, the reaction leads to the formation of  $\text{CaCl}_2$ , that has 3 consequences that should be taken into account :

- the  $\text{CaCl}_2$  contained in sediment samples may give chlorine compounds, that damage the reactivities in the combustion reactor and even can damage the quartz reactor itself, that becomes porous and can even break.
- The weight of the  $\text{CaCl}_2$  produced is higher to the weight of carbonate decomposed : that weight change should be taken into account if a precise measurement of the carbon content (%C) is required.
- $\text{CaCl}_2$  is very hygroscopic : acidified samples must be stored in a desiccator under vacuum and their weighing should be done quickly.

***Note :*** *Acidified sediments, even after evaporation of the acid, still stay very acid and quickly damage tin capsules. To avoid it, analyses should be performed very quickly after weighing (maximum 8 to 10 j), or samples should be first packed in silver capsules, themselves packed in a second tin capsule to ensure a better combustion.*

*In any case, samples should be kept in a desiccator under vacuum over  $\text{P}_2\text{O}_5$ .*

***Note :*** *Carbonate removal may affect the nitrogen isotopic signature. This effect should be measured relative to the quantity of acid used and the type of samples. It may be better to perform 2 analyses, one with carbonate removal (for organic C) and one without acidification (for N).*

## 1.3. Phytoplankton and SPOM (Suspended Particulate Organic Matter) on filters

### 1.3.1. Sampling, preliminary treatment and sample storage

Phytoplankton and SPOM are collected on glass fiber filters (GF/C or GF/F type) or on quartz fiber filters, which materials do not contain any carbon, nitrogen or sulfur.

One drawback of glass fiber filters over quartz fiber filters is that they melt during the combustion of the sample, that sometimes ends in a block inside the insert which is difficult to remove without breaking the insert. A more frequent removal of ash is therefore needed.

However quartz fiber filters are more fragile and less easy to manipulate during the filtration process.

In any case, filters must be previously cleaned from any organic matter by burning at 450°C maximum during 3 h (some studies in very oligotrophic waters may require a preceding rinse using HCl 0,1 N and a thorough rinse with ultrapure water before drying and burning, to be sure that no carbonates are present).

The volume to be filtered highly depends on the size and the abundance of suspended particles and of the surface of the filter as well. The filtered volume must be recorded if the absolute amount of C and/or N has to be determined. In that case, some points about sample encapsulation should be considered (§ 2.5 et 2.6).

After filtration, filters must be individually placed in cleaned containers (Cleaned Petri dishes, glass vials) and kept cool, then stored in the lab at -20° before further treatment.

### 1.3.2. Carbonates removal

That step is necessary to remove carbonates from the seawater absorbed on the filter or on organisms or particles that have been retained on the filter.

Filters are first freeze-dried or oven-dried.

Carbonates removal is performed using HCl fumes under a hood : vials or Petri dishes containing the samples are placed (open) in a glass desiccator along with a small beaker filled with 5 to 10 ml fuming HCl (37%), under a slight vacuum during 4 h.

***Warning : Never manipulate strong acids without wearing a coat and gloves, and outside a hood.***

Remove the beaker containing the acid, and left the desiccator open under the hood overnight. At this step, take care to any contamination due to any nitrogenous volatile compounds (ammonia, quaternary amines ...) in the ambient air.

Filters can be stored then either in a freezer or in a desiccator under vacuum at room temperature.

## 2. Sample encapsulation

### 2.1. Sample weight

The sample weight is not strictly necessary to get the stable isotope ratio values. However, these values are obtained with a high precision and confidence within a certain weight range, and if the measurements of the %C and %N have to be determined, the sample weight has to be precisely known ( $\pm 1 \mu\text{g}$ ).

The mass spectrometers available at LIENSs SIF are fitted with the Smart-EA option, that allows an individual adjustment of the sample gas to the level of the corresponding reference gas peak. Even if that configuration allows the analysis of samples within a wide weight range with the same precision, it is better to try to have sample weights within the « optimal » range. Below the minimal values of this range, measurements are less precise, and above maximal values, saturation of the ion collectors can occur.

The following table gives the minimal, maximal and optimal weights for various types of samples (as mg DW) for C and N isotope ratio measurements. For sediments, tests on a few samples must be done to determine the optimal weight, if no data on their C and N content is available. For filters, the weight is not useful since it includes an unknown quantity of glass fibers relative to the organic matter : experience is needed to know how much organic

matter has to be scratched from the filter, and for %C and %N calculations, they are done relative to the filtered volume, if all the filter can be analyzed (see § 1.3.1)

Sample type	Minimum	Optimal	Maximum
Animal tissue	0.1 mg	0.4 mg	1.5 mg
Micro or macroalgae	0.2 mg	0.6 mg	1.8 mg
Marine phanerogams	0.3 mg	0.7 mg	2.0 mg
Terrestrial plants	0.3 mg	0.7 mg	2.0 mg
Sediment (high content in organic matter)	0.2 mg	0.6 mg	1.8 mg
Sédiment (low content in organic matter)	1.0 mg	-	> 30.0 mg

## 2.2. Capsule selection

For animal or plant samples, the analyzed quantity is very small, 8 x 5 mm capsules are convenient (although smaller capsules can be used, they may rise to problems with our automatic sampler if they are too tightly pressed and too small). For sediments and filters the same capsules can be used but it can be necessary to use larger ones.

Tin capsules are the ones commonly used, but they can be rapidly damaged by acidified samples of sediment (especially if they are not stored under vacuum). If the analysis cannot be done within about 10 days after encapsulation, it is better to use silver capsules.

## 2.3. Weighing : warnings

Encapsulation and sample weighing are the steps that require the highest caution : any contamination, even by the smallest particle or fiber may rise to high risks to get a wrong result.

- Wearing cotton coat is highly recommended, to avoid in a large part contamination by fibers from clothes (in peculiar from pull-overs) and static electricity (static eliminators, either included in the balance or as an independant equipment are generally very efficient).
- Never use ordinary paper towels, but use ones that are specially intended to avoid fibers, and if you use gloves, use only no-powder gloves.
- All of the tools (spatula, scissors, pliers ..) used must be first cleaned with ethanol, as the aluminium plate where capsules are placed during encapsulation.
- **never touch capsules or parts of tools in contact with capsules with your fingers.** Use gloves or work using pliers.

## 2.4. Weighing

The microbalance user's guide is available in a display box fixed on the wall right above the balance.

***Before weighing samples, take a sample list form and fill in first all the fields on the top of the sheet (please read instructions in § 3).***

The routine use of the balance is quite simple :

- open the balance door : press one of the 2 large buttons on the left or right sides. The sliding door will open on the right side (for left-handed people, this can be changed, see the user's guide),
- Put the small plastic stand on the weighing plate, and using the plier with fine and bent tips, place an empty capsule in the stand,
- Close the door by pressing one of the 2 big buttons,
- Wait for balance stabilization, indicated by the display of "mg" after the number. then press "Tare",
- Open the door, take out the capsule using the bent plier, place it at the low right corner of the aluminium plate (this 6 x 6 cm part should never be touched with your hands) : **never load capsules with the sample inside the balance.**
- Using a small spatula, take some sample powder and put it in the capsule, taking care it falls down right to the bottom and not on the walls of the capsules,
- Put back the capsule on the stand in the balance, look at the weight (without closing the balance, the precision is only at 2 decimals, but it is sufficient to evaluate if it is enough or not). If the weight is not within the right range, get out the capsules and add in or remove samples from the capsule to end in the wanted weight,
- Close the balance door and wait for the stabilization,
- Notice the weight et write it down in the sample list form (see § 3).

**Note 1** : *it is much more difficult to remove part of the sample from a capsule than to add some.*

**Note 2** : *if samples are electrostatic, turn on the static eliminator and place the sample tube in front of it during about 10 s.*

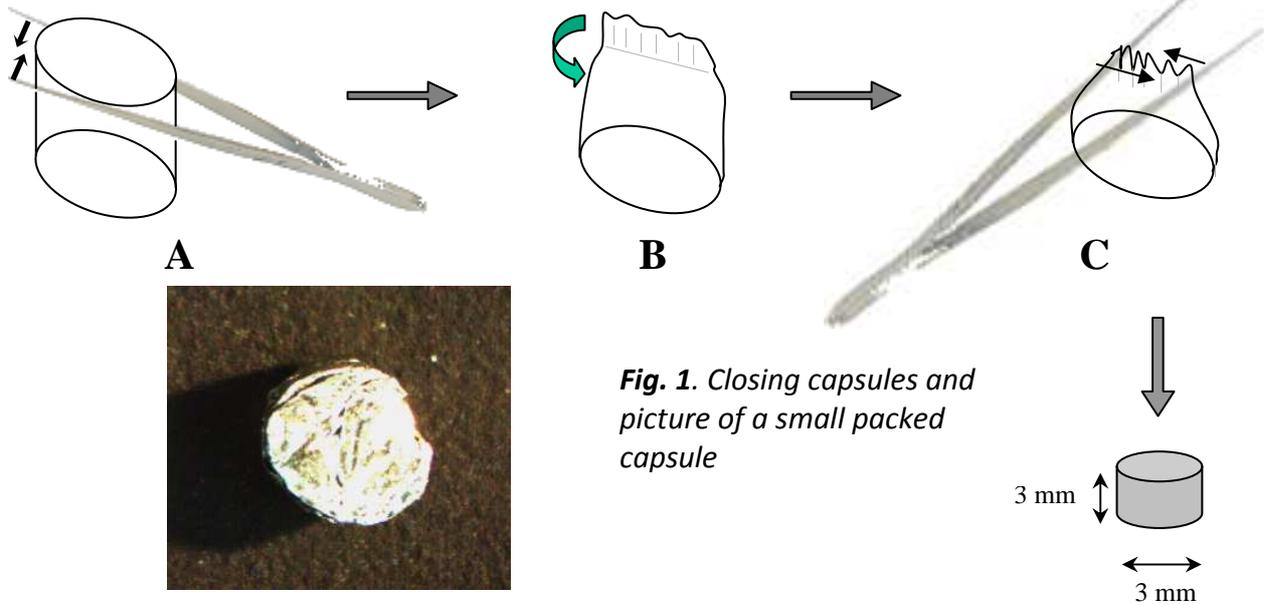
## 2.5. Closing the capsules

Place the capsule vertically in the working zone on the aluminium plate. Close it by pressing firmly its top 1 or 1.5 mm using a flat ended plier (Fig. 1 A) then fold this pressed part down (Fig. 1 B). Fold again in the perpendicular direction (Fig. 1 C), then press the capsule using 2 flat ended pliers to end in a packed capsule in a shape of a section of cylinder (max diameter 3 mm, height of about 3 mm, for animal or plant tissues).

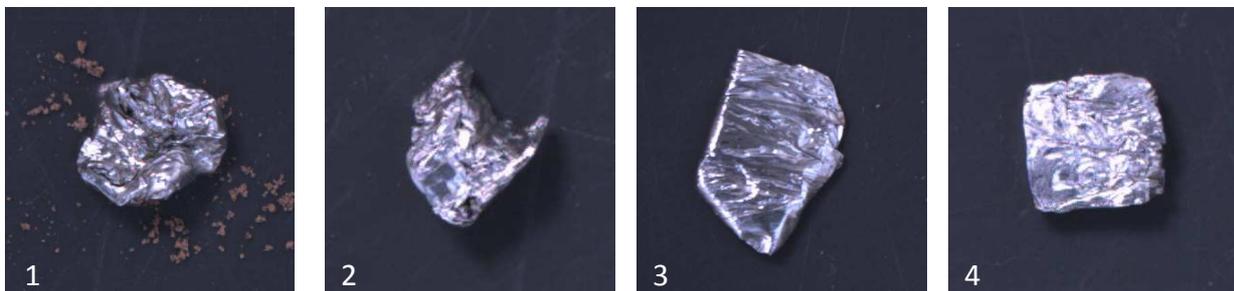
For sediments and filters, packing down to that size is difficult, sample volume being generally more important. In that case packed capsules are more spherical, they however should never be larger than 7 mm in diameter.

**Note 1** : *It is preferable to make small packed capsules, that can be used with the 100-positions tray on the automatic sampler, and thus to analyze about 30% more samples per day.*

**Note 2** : *Warning : Packed capsules must not be too small (not <1.5 mm in diameter) since they can stay caught in the automatic sampler.*



**Note:** It is very important to respect the recommendations concerning the shape and size of the packed capsules. Badly packed capsules (cf . fig 2a) can either leak if they are not well sealed (sample lost and contamination of other samples), or get caught in the well of the automatic sampler tray (and being not analyzed), or even jam the automatic sampler (with in worst cases, lost of all of the samples in the tray).



**Fig. 2a.** Examples of badly packed capsules : 1) Badly sealed : lost of part of the sample; 2) Not rounded enough : risk to get caught in the tray ; 3) Too flat : risk of jamming and blocking the automatic sampler ; 4) Square : risk to get caught in the tray



**Fig. 2b.** Examples of well packed capsules : 5-6) Small capsules (animals and plants); 7) Big capsule (sediments and filters)

## 2.6. Analysis of filters

The sizes of the wells of the automatic sampler trays available at LIENSs SIF (3 mm and 7 mm) do not allow to analyze capsules containing a full 25 mm filter (and even more so 47 mm filters). Several other ways may be used :

- scratching the filter surface to recover the organic matter : in any case, the recovery cannot be guaranteed as quantitative. Minimizing the quantity of glass fibers is important since it hampers the combustion and increases the ash volume. This way of organic matter recovering rises also the problem of the heterogeneity of the sample and the non-representativity of the recovered part.

- cutting out a filter fraction : parts of filters can be cut out using a punch, that allows through a surface ratio to estimate the total quantity of material on the filter, in case it is needed. However, distribution of the organic matter on the filter is generally heterogenous and for both the quantitative estimation of the %C & %N, and isotope ratios, this protocol may give a higher variability.

- use of mini-filters : filtration can be performed using filters of 12 mm in diameter (to cut out from larger filters) thanks to 13 mm filter holders (e.g. Sweenex). After cutting out of the white external part (very easy simply by strong tightening of the holder just after filtration when the filter is still wet) and after sample treatment (see 1.3), peel as much as possible the back side of the filter, that does not hold any organic matter.

The rest can easily be contained in a small capsule. This protocol allows to obtain a value for all the filtered sample, it works well, but needs to have a minimal load of suspended organic matter.

## 2.7. Storage, preservation and sample sending

### 2.7.1. Storage

Packed capsules are stored in 96-well trays (Fig. 3), identified by rows (A to H) and columns (1 to 12). Sample list forms (Excel files) are organized to correspond to this storage (see § 3).

**📌 Group samples of similar material or similar sizes** (small capsules : animals/plants together and large capsules : sediment/filters together) : putting a large capsule among small ones forces to analyze the whole serie with the large well tray of the automatic sampler, that does not allow to analyze more than 30 samples at a time instead of 80.

**📌 Never left empty wells between series !!**

96-well tray cover must be hold in place using **reusable laboratory tape**. It is indeed necessary to open the trays many times to put in or remove capsules, this should be easily done.

**📌 Never use ordinary adhesive tape to maintain the covers ! : high risk of sudden opening and of mixing or knocking over of the capsules (and glue traces are also a problem)**

Each 96-well tray must be clearly identified by noting some indications on a piece of **reusable laboratory tape put on the cover**. The indications must allow the right association

of the sample tray and of the Excel file of the sample list form. It is therefore necessary to indicate on the cover at least the name of this file and the name of the sender (Fig 3).

**The best is to give the name of the file as reference for the tray.**

**📌 Please do not write directly on the cover but on a piece of reusable laboratory tape !  
Trays are re-used after cleaning.**



**Fig.3.** 96-well tray used for storage and posting of the samples : notice the 2 pieces of reusable tape to hold the cover and the one to note the identification informations

### 2.7.2. Preservation

Except for acidified sediment tin capsules, that should be quickly analyzed, other capsules can be stored during long periods, better under vacuum in a desiccator (at room temperature) than in a freezer where samples can get back some water.

### 2.7.3. Sending samples to LIENSs SIF

96-well trays can be sent by postage services, enclosed in a bubble envelope.

To avoid that capsules, and in peculiar the smallest ones, get out the wells and mix, **place an aluminium foil (in 2 or 3 layers) over the tray before securing the cover.**

**Never use Parafilm or adhesive tape or any type of paper (that may release fibers) to cover the wells of the tray.**

Hold the cover by 2 pieces of reusable laboratory tape (see Fig. 3), that are normally largely sufficient. However, to better secure the cover, wrap the tray within an aluminium foil.

## 3. Filling in the sample list form

Each tray must be associated to a separate sample list form (submitted as an Excel file). The form includes an information sheet, that includes various fields for the administration, and a sample list sheet.

Samples must be identified following the instructions below :

- The tray reference must be indicated in the upper right field (Fig. 4) and should be the same as indicated on the tray itself and the same as file name of the sample list form. This reference should be very specific (avoid generic terms as Box 1, Tray 3 ..).
- The name of the sender must be indicated in the upper left field.

Sender	<u>P Richard</u>	Project :	<u>FoodWeBio</u>	Tray ref. :	<u>FWB 4</u>
Prepared by	<u>MZ</u>	Date :	<u>14/01/09</u>		

Well #	Sample reference	Weight (mg)	Type	Comment
A 1	FWB-LR-M4	0.346	muscle	
A 2	FWB-LR-M5	0.309	muscle	
A 3	FWB-LA-M1	0.441	muscle	
A 4	FWB-LA-M2	0.288	muscle	
A 5				

**Fig. 4.** Sample list sheet

- Each reference of a sample must be unique, even for replicates that should be separately identified. It must have more than one character. Avoid generic references as 'Sample 1', 'Sample 2' or 'A1', 'A2' ...
- Do not use any special characters like : . , / ; ( ) ?+ @ # \$ % ^&\*. However spaces and hyphens (-) can be used. Avoid accented letters.
- Save the Excel file under the same filename that the tray reference.

#### 4. Enriched samples

Stables isotopes can be used in experimentations based on enrichments from products that can have > 95% of the heavy isotope.

- ❗ **The highest caution must be taken at any step of the preparation of enriched samples : any contamination may have disastrous effect on measurements at the level of natural abundance.**

Users of enriched products must consider the following points before submitting enriched samples to LIENSs SIF :

- the abundance of the heavy isotope ( $^{13}\text{C}$ ,  $^{15}\text{N}$  ...) must be below **3%**. In case of higher enrichments, as it is the case of labelled preys in grazing experiments, samples must be diluted with the same sample but non-labelled whose natural abundance is measured apart and taken into account in the calculations. Any other compound can also be used but the corrections should then include the relative proportions of the element (C or N).

***Warning : Between 1 and 3% of enrichment, a "memory effect" may be found on samples following a sample with a high enrichment. Samples must be arranged from low to high enrichments, and separating samples with high enrichment from non-enriched compounds should be considered.***

***Warning : It is of the responsibility of customers to be sure that the level of enrichment is <3%. Higher enrichments may lead to an irremediable contamination of the reactors (and even of the CPG column) whose replacement may be charged to the customer.***

- For **enriched** samples, arrange samples from low to high enrichments to avoid possible memory effect. Place non-enriched samples ahead of enriched ones. Use separate trays for enriched samples and samples at natural abundance.
- When working with enriched products, it must be taken a great care of all possible contaminations and all the equipments and tools used with enriched samples must be thoroughly cleaned.
- A special attention must be taken when weighing enriched samples using the microbalance : since this latter one is used to weight the standards at natural abundance, its contamination (and of the associated tools as well) may end in wrong results for all samples including the ones of other users.

 **Enriched samples in a tray must be indicated on the tray and in the sample list forms !**

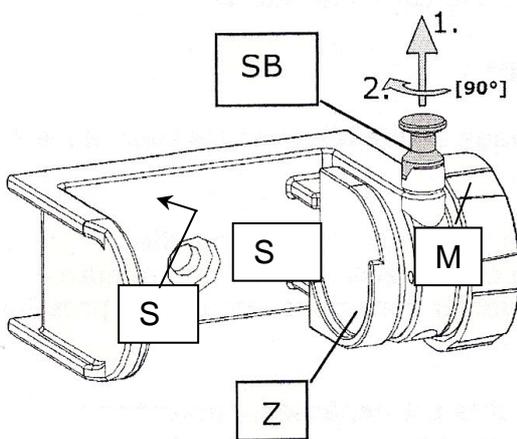
## Short instructions for the use of the ball mill Retsch MM400

(see user's guide in the display box fixed on the wall)

- Select the bowls to use according to the size of the samples : 2 same bowls must always be used (small samples can be ground in 2 ml microtubes : place one microtube rack on each side, with the same number of tubes)
- Place the samples and the corresponding balls within the bowls :

Bowl type / volume	Balls per bowl or microtube	Use
Inox 10 ml	1 or 2 inox balls 9 or 10 mm	General
Inox 25 ml	1 or 2 inox balls 12 mm	General
Zirconium 35 ml	1 zirconium ball 12 mm	Lipids or metals
Microtubes PE 2 ml (x10)	2 inox balls (3 mm + 4 mm)	Small samples

- Close the bowls et place them on their holder :



- unscrew the handwheel knob M, remove the locking pin SB upwards and turn it through 90°, then place the bowl between the two socks SM and SF.

- screw the handwheel M **without tightening**
- turn the locking pin SB through 90° downwards,
- screw again the handwheel M through 6-8 easily audible "clicks".
- **NEVER FORCE THE TIGHTENING**

- shut the hood
- Adjust grinding parameters (see user's

guide) : the only useful adjustment is the duration of the grinding (to modify it press "SET", then adjust to the wanted time, and save by pressing again "SET").

- Start grinding (pour very dry samples, 1 to 3 minutes are generally sufficient),
- Recover the sample powder
- Wash the bowls (warning : washers cannot be easily removed, they must be lift a little to be cleaned with a small brush), rince them using pure water then ethanol
- put them in the specific drying oven
- fill in the notebook with your name, time of use, number and types of samples

### Points that must absolutely be respected :

- never use the grinder with only one bowl installed, and always load bowls with about the same weight
- do not use balls in a different material than the bowls.
- do not strongly tighten the holding system : the clamping system is largely sufficient to hold the bowls.