3. Preparation of Samples

3.5 Homogenisation

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Environmental samples are often very heterogeneous. Especially small weigh-ins would be affected by high standard deviation for repetitions of P determinations without sample homogenisation. This is especially true for **suspensions, solid plant** or **mineral samples** and **animal tissues**. In a lot of cases a multi-step homogenisation is necessary.

3.5.1 Homogenisation of suspensions

Because suspensions are not real solutions, solid particles sediment in dependency on time, which means a certain separation between solid and liquid phase. Examples for suspensions are liquid manure, digestates, slurry and sewage sludge. If digestion and P determination is planned from wet matter (cannot be recommended), the suspension has to be mixed by vigorous stirring. This should be done before every subsample. Parallelly, beside samples for P determination, subsamples have to be taken for determination of dry matter percentage (DM-%). Extremely heterogenous samples (e.g. undecomposed, bulky plant parts) cannot be digested as wet samples. Such samples have to be dried and further pre-treated. There are different possibilities for preparation/homogenisation. The selection of possibilities depend on scientific questioning and the character of the suspension (e.g. DM-%). After the drying of suspension, the sample can be treated such as a solid sample (see 3.5.2 homogenisation of solid samples). For samples with high concentrations of organic matter (OM) a homogenisation by ashing is possible (chapter 3.3). Take care that masses of the complete suspension and of the wet and dry solid and liquid phases are determined.

(a) Separation between **solid** and **liquid phase** of suspensions
Especially for suspensions such as liquid manure and digestates, which are used for fertilisation, questions arise from distribution of nutrients between solid and liquid phase. Under these circumstances a separation between solid and liquid phase is urgently necessary before drying.
Separation is possible by **(a.1) gravity-related sedimentation** of the solid phase. However, a long waiting time is necessary and the separation

is incomplete. Additionally, microbial conversions are possible due to long waiting time. After separation solid and liquid phase have to be treated separately. This method cannot be recommended due to the incomplete separation and long waiting time.

The second possibility of separation is for example **(a.2) screw-press** (Mönicke et al. 1978). This type of separation is especially used in the professional agricultural sector for large sample volumes. The solid and liquid phase can subsequently be used separately in farming. After separation by a screw press the solid phase has a DM concentration between 18 and 25 % and the liquid phase of 3 to 7% (Burgstaler et al. 2017). In digestates P is more accumulated in the solid phase compared to the liquid phase (Bachmann et al. 2015). The consistency of the solid phase is comparable to that of bought potting soil or peat. This solid phase only needed to be dried and eventually ground and ashed. The liquid phase is a suspension, in which particles can sediment. This suspension has to be stirred vigorously if digested in a wet state (parallel determination of dry matter) or has to be dried before further analysis (points b and c).

Separation of solid and liquid phase can also be done by **(a.3) centrifugation**. For representative sampling larger sample amounts should be centrifuged. This is much faster than gravity-related sedimentation and separation is more completely than with a screw-press. In the labs of the working group soil science (in AUF) up to 750 ml per vessel can be centrifuged by the centrifuge (Heraeus Multifuge 3 SR+ Centrifuge, Thermo Scientific). In one pass 4 vessels of 750 ml can be processed in this centrifuge. Alternatively, adapters can be used (4 per rotor) for filling with 12 x 15 ml centrifuge tubes (48 per rotor), 5 x 50 ml centrifuge tubes (20 per rotor) or 2 x 100 ml centrifuge tubes (8 per rotor). Other adapters, for example for microtiter plates, are not available. Since the centrifuge has a swing-out rotor the maximum centrifugal acceleration is 4500 x g. Additionally, a cooling (≤ -9 °C) is possible in this centrifuge. In case of uneven loading the centrifuge stops automatically due to their imbalance detection. Acceleration and deceleration can be set separately. After centrifugation the supernatant has to be decanted carefully or has to be removed by a syringe. When solid and liquid phase are separated after centrifugation the solid phase can be dried completely (see points b and c). Decision has to be made if liquid phase has to be dried or can be digested in the wet state.
A separation of phases can also be done by **(a.4) filtration**. The most complete separation between solid and liquid phase is possible by appropriate selection of filters. Direct filtration of suspension can only be recommended for suspensions with very high percentages of the liquid

phase. Otherwise, the filters will be clogged by the developing filter cake. A previous centrifugation is therefore recommended. After filtration the filter cake has to be separated from the filter. Therefore, selection of the filter is also affected by this possibility. Before filtration the mass of the filter has to be determined. After filtration the filter can be dried and weigh with the filter cake. The dried filter cake can be brushed off carefully from the filter.

(b) Drying of sample **in drying oven**

If the suspension is not separated in solid and liquid phase, it is recommended to dry the suspension completely or a large subsample of it (vigorously stirring as well as possible). If the solid phase was separated previously, this solid phase has to be dried completely. It has to be decided individually if the liquid phase has to be dried as well or can be digested in the wet state. Take care that for all samples the weigh-in and out before and after drying is noted (note empty mass of the vessels), to determine DM-%. Flat bowls should be preferred for drying in the drying oven (if possible, with circulating air). Drying with circulating air is much faster than without. The selected temperature depends on scientific questioning and subsequent analyses. If only total element concentrations have to be determined, a temperature of 60 °C can be recommended. To prevent samples from "sticking/caking", the samples have to be stirred from time to time during drying or should be crumbled by hand. If liquid manure, digestates or something similar is dried, an odour nuisance is possible. For such samples lyophilization (c) is recommended or the drying oven has to be in a separate room. After drying the sample has to be grounded (see chapter 3.5.2 homogenisation of solid samples). In the working groups soil science, soil physics and agronomy several drying ovens are available. One of the drying ovens (with circulating air) is separated in the basement.

(c) Lyophilisation (freeze drying)

If a freeze dryer is available, it should be selected instead of oven-drying for odorous samples or for subsequent temperature-sensitive analyses (e.g. microbial decomposition of samples). Depending on sample amount and type of freeze-dryer the samples can be filled in centrifuge tubes (e.g. 50, 100 or 500 ml). For all samples weigh-in (or samples volume) and weight out after drying have to be noted (note also empty mass of tubes), to determine DM-%. Tubes are closed with a lint-free paper (laboratory cloth) which is fixed by a rubber band. Tubes are set in a holder and frozen (at -20 °C) in a freezer. Note that frozen water has a larger volume than liquid water. Therefore, sufficient volume in the tube is necessary. If samples are completely frozen, the paper can be removed and the tubes can be set in the freeze dryer. It is not allowed to use the freeze dryer alone. Before usage a detailed briefing by a professional laboratory assistant

is mandatory or the equipment is operated by the laboratory assistant. Depending on the sample volume, complete drying can take a week or longer. After drying the sample is mostly a powdery substance. The solid can be digested directly or has to be homogenised such as peat (chapter 3.5.2). However, sieving to < 2 mm is not necessary. In the working group soil science (AUF, University of Rostock) is a freeze dryer (Alpha 2-4 by Christ Gefriertrocknungsanlagen; Martin Christ Gefriertrocknungsanlagen GmbH; https://www.martinchrist.de/en/). The ice condenser has a maximum capacity of 4 kg and a temperature of -85 °C. That means that it is possible to dry solvent-based or other low-eutectic samples as well.

3.5.2 Homogenisation of solid samples

Solid samples such as plants, peat, < 2 mm sieved mineral soils, sediments and bio chars can be very heterogeneous as well. In plant and peat heterogeneity is caused by bulkiness and/or different tissues. In soils and sediment heterogeneity is mainly caused aggregates and/or different sized individual particles (mainly sands).

Plant material and **peat**
After drying, plant material has to be milled (if necessary stepwise: first coarse, then fine). **Herbaceous plants** (surface biomass, roots), **seaweed** and similar can be crumbled by hand and subsequently milled fine (e.g. Ultra-Centrifugal mill ZM1000 by Retsch, Pulverisette 23 and 6 by Fritsch) or ashed directly (see chapter 3.3). In the working group soil science for fine milling the "Ultra-centrifugal mill ZM1000" by Retsch can be used. The mill ZM1000 has a speed of 15000 rotations per minute and a sieve < 0.25 mm. Material for this mill has to be < 5/10 mm. The real size depends on the structure and strengths of the material. In the working group Agronomy (AUF, University of Rostock) the ball mill Pulverisette 23 (by Fritsch) can be used for volumes of up to 5 ml (grain size < 6 mm) to mill to final grain size of 5 µm. Generally, this mill can be used for cryo-milling, but this has not been tested before. For larger sample volumes the planetary ball mill Pulverisette 6 (by Fritsch) can be used. This mill holds a sample volume between 10 and 225 ml; the maximum grain size is 10 mm. The grinding fineness is < 1 µm. Fine grinding is not necessary if larger sample amounts will be ashed (see chapter 3.3).

Cereal whole plants and suchlike are separated before drying in straw and corn by threshing. The straw is cut by scissors in short pieces before grinding. Alternatively, the straw can be grinded by a coarse shredder. In the working group Soils Science (AUF, University of Rostock) the mill "Schneidmühle SM200" by Retsch can be used. The final fineness is between

0.25 and 0.20 mm, depending on hole wide of the sieves. It was possible to grind corncobs with this mile. The coarse crushed straw and suchlike can be further milled with fine mills (see above). For a representative straw sample several subsamples should be milled and pooled together again. Cereal corns can be grinded with different mills such as knife mills for animal tissue (see below) or grinder for nuts and alike or can be ashed without further grinding. Grinding of oil-containing seeds can be problematic (e.g. rape), if samples warm up during grinding. This has to considered for time of grinding and other settings for the mills. In the working group Agronomy (AUF, University of Rostock) the ball mill Pulverisette 23 is used for grinding of rape seed. For these seeds 35 rotations min^{-1} and a time of 20 sec is used. The milling chamber is filled to a maximum of ¾, so that mill balls are well visible. Additionally, in the experimental station Satower Strasse a cutting mill combination Pulverisette 25/19 by Fritsch is available. In this mill corn cobs can be grinded as well. It is a combination mile for pre-crushing and fine grinding in one step. Pieces of a maximum of 120 mm x 85 mm can be processed. After completely automatic two-step crushing the final fineness is 0.2 to 0.6 mm. The sample is cooled during grinding. The crushing is as follows: in the mill Pulverisette 25 the whole sample is pre-crushed, subsequently the sample fell via a hopper on a sample divider, where the sample normally is subdivided in the relation 1:13. The split ratio is variable. This amount-reduced subsample is automatically milled in the Pulverisette 19 to a final fineness of 0.2 mm and automatically aspirated by a Fritsch Zyklon into the sample vial. It has to be decided specifically if fineness is sufficient or if further grinding with a fine mill (see above) is necessary. Request for theses mills is possible at the working group Agronomy or the leader of the experimental station (chapter 10).

Woody plant material with leaves (e.g. branches/twigs from willows) has to be separated by hand before drying in woody parts and leaves. Subsequently, it has to be milled separately. The wood is grinded in a coarse mill and subsequently in a fine mill (see above). According to the producer the mill SM200 by Retsch is suited. The combination mill Pulverisette 25/19 might be suited as well. This has to be requested in the working group Agronomy. For grinding the wood in the fine mill, the material must be of adequate fineness (< 5/10 mm). Only small samples amounts should be milled, because these fine mills warm up very fast. Leaves of the twigs are processed such as herbaceous plant material. That means that normally no coarse pre-grinding is necessary; fine grinding or direct ashing is possible.

Tubers such as from potatoes are often peeled and separated in peel and crushed potato pulp (depending on scientific question). Peel and potato pulp are crushed and subsequently dried separately. Grinding of potato material in common mills for plant material, such as the ultra-centrifugal mill, is not

possible. In the working group Agronomy, the cutting mill combination Pulverisette 25/19 by the firm Fritsch is used (see above). It can be tried to crush potatoes in a knife mill (see chapter 3.5.3) as well. If this is not possible the material has to be ashed (see chapter 3.3).

Peat material has to be processed differentially after drying due to fibrousness. Ideally, peat material is sieved to < 2 mm after drying such as mineral soil. Subsequently, it is milled in a mortal mill (see below). If this is not possible due to long/coarse fibres it can be tried to process peat in knife mills. If this is not possible, the peat samples have to be ashed (chapter 3.3).

Mineral soil, sediments, biochar (also bone char)
Minerals soil has to be sieved < 2 mm after drying. If very large and solid aggregates are in soil samples (e.g. in clay soils) it may be necessary to crush the aggregates with mortal and pestle by hand. Crushing of large aggregates may never be done on the 2 mm sieve because the sieve would be destroyed! Soils and sediments cannot be milled in mills for plant material. After sieving < 2 mm subsamples of the soil are milled in a mortar mills (e.g. in the working group Soil Science 2 mortar mills: RM100 by Retsch and Pulverisette 2 by Fritsch), to destroy small aggregates and homogenise the sample. The final fineness of the Pulverisette 2 is 10 to 20 µm. Sample volumes of maximum 190 ml with a particle size of maximum 6 to 8 mm can be milled. For RM100 no more details were found, because only successor model RM200 is on the market. According to own experience the sample volume, particle size and final fineness is similar to the Pulverisette 2. In the working group Soil Science in both mills soil volumes of around 1 to 2 tablespoons are milled.
Mineral **sediments** can be processed like mineral soil. Sediments with too high OM percentages and from which subsamples cannot be milled have to be ashed. **Bone chars** and probably other biochar cannot be milled in mortar mills for soils according to own tests. Such samples have to be crushed by mortal and pestle by hand dust-finely, if possible. This crushing is not necessary if different sieve fractions of chars are processed and the digestion of single particles is planned.

3.5.3 Homogenisation of animal tissue

Animal tissue can be differentiated in water-rich soft tissue (e.g. muscle meat), (fish) skin and solid tissues such as bones, cartilage, horns and mussel shells. Only a few experiences are available for these materials. It has to be kept in mind that for all pieces the wet mass, the dry mass and the ash mass have to be determined.

If not separated previously, birds have to be plucked and other animals have to be skinned. Muscle meat has to be separated from bones and offal. First, the animal is opened, and the offal are removed separately. Offal (especially stomach and intestines) have to be washed separately to remove for example digestive residues. Subsequently, the muscle meat has to be removed from bones by a sharp blade. Bones have to be processed separately. Fishes are processed as whole fish or separated in pieces according to the scientific question. If the fish has to be separated, the head has to be removed first, followed by the offal. Afterwards, the fish is fileted to separate fish meat (filet) and bones (carcass). The fish skin either remains with the filet or has to be removed before fileting.

Soft tissue such as fish meat and **fish skin**

Water-rich soft tissue has to be crushed separately as well as possible (muscle meat, skin, offal) before drying. A first pre-crushing can be done by hand with scissors or a knife. Generally, fish muscle meat can be digested in a fresh state in a microwave with HNO_3. This should only be done if the material is relatively homogeneous. Parallelly, the DM-% has to be determined. In the working group Aquaculture & Sea-Ranching (AUF, University of Rostock) fish were frozen as whole fish but also crushed. Whole fish were frozen at -20 °C, subsequently sawn coarsely in small pieces and finally minced 3 times. For fish pieces the frozen filet (due to the leathery skin) as well as the carcass were minced separately. According to the experience of the working group Aquaculture, fish meat has to be crushed in a frozen state, because in a fresh state too much water would be lost! Crushing in knife mills was not possible, because fish pieces rotated in the mill but were not crushed. After crushing, the material was frozen again at -20 °C and subsequently lyophilised for around 3 days. The lyophilised material was milled first by an Agate ball mill (at 200 rotations per minute for 20 minutes) and subsequently milled finely in a ceramic mill (IKEA 365+ IHÄRDIG)

Barrento et al. (2009) crushed fresh fish meat with a knife mill (Grindomix GM200 by Retsch; 5000 rotations per minute) until complete visible destruction of material. Polypropylene cups and stainless steel knifes were used. Subsequently, the material was frozen at -20 °C. A subsample was lyophilised for 48 hours at -50 °C and low pressure (10^{-1} atm). Finally, samples were ashed and digested by HNO_3. Ersoy and Çelik (2009) also crushed fresh fish samples in a mill by stainless steel knifes but digested the homogenised material directly without drying.

Generally, fishes can be dried in a drying oven or lyophilised (see chapter 3.5.1 points b and c). In both cases the weigh-in and out must be noted to determine DM-%. Drying in a drying oven causes a lot of odour. Therefore, drying by lyophilisation is recommended (chapter 3.5.1 point c).

According to Schoo (2010), larvae of lobster can be completely frozen directly after harvest, lyophilised subsequently pulverized and digested. No further information is presented about pulverisation by Schoo (2010).
Generally, fish meat can also be ashed in a muffle furnace in a wet state at 450 to 550 °C for homogenisation (e.g. Engmann and Jorhem 1998, Jorhem et al. 1996). According to experiments at the Biological station Zingst this causes a lot of odour and a strong sooting of the oven (see chapter 3.3).

Hard tissue, e.g. bones, cartilage and horns
Bones, **cartilage** and **horns** cannot be milled in common mills or crushed by hand with mortar and pestle for homogenisation. Special bone mills are necessary. According to the experience of the working group Aquaculture, carcasses of fishes can be crushed by a mincer. Possibly, the jaw crusher (https://www.retsch.com/products/milling/jaw-crusher/) of the firm Retsch is suited to crush bones, since with this crusher ores, ceramic and suchlike is crushed. The mill BB50 by Retsch processes material < 40 mm and mill to particles of < 0.5 mm, whereas the BB200 can mill particle sizes of < 90 mm to < 2 mm. Bone material < 10 mm can partly be crushed in ball mills. If bones, cartilage and horn are chips < 5 mm they can be digested directly with HNO_3 + H_2O_2 in a microwave. If this material is visible heterogeneous it has to be crushed like coarse material or should be ashed in a muffle furnace for homogenisation. Ashing of bones has not been tested before in the labs. Since during pyrolysis (free of O_2) at 500 °C bone chips form a relatively crumbly material, it is supposed that during ashing in a muffle furnace a material is formed, which can be crushed by mortar and pestle by hand.

Carbonate-containing biogenic material, e.g. mussel shells, chitinous carapace of arthropods
Mussel shells mainly consist of calcium carbonate (in the form of aragonite), which is stabilised by the organic substance conchiolin (Wikipedia conchiolin). The first reference for conchiolin is Frémy (1855). It has to be considered to clean mussel shells before crushing from adhesive dirt and other organic substances, according to the scientific question. Generally, mussel shells can be crushed by hand with mortar and pestle (Pilkey & Goodell 1963, Ragland et al. 1979). Alternatively, they could be ashed in a muffle furnace at 550 °C, since organic substances would be ashed leaving only a fragile calcium carbonate, which could be crushed easily (400 °C, in Elsaesser 2014). Analyses of mussel shells have not been done before in the labs. According to Kost (1853), a strong formation of ammonia could be possible during ashing.

Chitin-containing exoskeleton (e.g. crustacean) can be coarsely crushed by hand, and, according to Cárdenas et al (2004), dried in porcelain cups at 105 °C (to weight constancy) and subsequently ashed at 900 °C for homogenisation. Boßelmann et al. (2007) milled exoskeletons of lobsters in a ball mill with 500 rotations per minute for 3 h at room temperature and with liquid nitrogen in a mortar. They found no chemical differences due to different forms of homogenisation.
A further possibility to mill samples being difficult to mill and heat-sensitive is milling in **cryogenic mills**. During milling, samples are cooled by immersion of the milling cup in liquid nitrogen. Additionally, the sample is embrittled by the nitrogen cooling and volatile substance remain in the sample. For these mills special safety regulations have to be considered during processing and the staff has to be trained in handling liquid nitrogen. Such mills are for example the cryogenic vibrating tube mill CryoMill by Retsch or the cryogenic mill C3 (Prozess- und Analysentechnik GmbH). The cryogenic mill from Retsch has a closed LN2-system (autofill), which avoids direct contact of the user with liquid nitrogen and can therefore increase safety. Until now, there is no experience in the labs with such cryogenic mills.

Comparison of P concentrations between differentially pre-treated soil samples: < 2 mm and not mortared vs. additionally mortared (homogenised) soil samples

In this experiment 4 soils (BS1 to BS4) each < 2 mm sieved, either not mortared or additionally mortared for homogenisation (n = 5). Soil samples of 0.50 g were digested with aqua regia in a microwave. P concentrations were measured at ICP-OES at a wave length of 214.914 nm.

For all 4 soils no significant differences in P concentrations between soil < 2 mm and additionally mortared soil were verified (Tab. 3.5-1). However, relative standard deviation was lower in additionally mortared samples compared to the of only sieved samples. This is also visible in the smaller range between minimum and maximum for mortared samples. The smallest range between minimum and maximum for < 2 mm sieved samples was 32 mg P kg^{-1} (BS1) and the largest was 1214 mg P kg^{-1} (BS2), whereas for mortared samples the smallest range was 16 mg P kg^{-1} (BS2) and the largest 43 mg P kg^{-1} (BS3). Additionally, no outliers were found for mortared samples. Mortaring of soils samples increased homogeneity of samples, since differentially big soil aggregates were destroyed. This effect was also evident for other elements such as Al, Ca, Fe, K, Mg, Mn, Zn (not shown).

Table 3.5-1 Minimum, maximum, mean and relative standard deviation (S %) of P concentrations in mg kg^{-1} for the soils BS1 to BS 4 each < 2 mm sieved and additionally mortared

soil	pre-treatment	minimum	maximum	mean	S %
BS1	<2 mm	598	630	615	2.1
	mortared	602	620	612	1.2
BS2	<2 mm	792	2006	1051	51
	mortared	797	813	803	0.8
BS3	<2 mm	938	1004	975	2.7
	mortared	936	979	962	2.2
BS4	<2 mm	655	719	681	4.3
	mortared	672	691	678	1.1

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