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A. Castelli[1], C. Lardicci[1], D. Tagliapietra[2]

[1] Dipartimento di Scienze dell'Uomo e dell'Ambiente, Università di Pisa, Via A. Volta, 6 - 56126 Pisa, Italy.
[2] Istituto di Scienze del Mare, C.N.R., San Polo 1364 - 30125 Venezia, Italy.

CHAPTER 4

SOFT BOTTOM MACROBENTHOS

Contents

4.1. Introduction

The sea-bed is mainly covered by sediments; only a relatively small proportion is formed of primary or secondary hard bottoms (Gray, 1981). The soft bottom features a number of systems that are of interest on account of their widespread distribution and the importance and diversity of benthic infaunal communities (Gray, 1997; Snelgrove, 1997). This chapter will consider soft bottom macrobenthic communities, defined here as organisms broader than 0.5 mm, or restricted to this size class by sieve

dimensions (Holme and McIntyre, 1971, 1984; Gray, 1981). This minimum limit is not universally accepted, as some Authors believe that 1 mm is more appropriate (Gray, 1981; Cognetti and Sarà, 1972; Cognetti *et al.*, 2000, and Chapter 3, this volume). The communities in question consist mainly of polychaetes, followed by bivalve molluscs, amphipod and decapod crustaceans, and echinoderms.

Soft bottom macrobenthic communities are mainly composed of infaunal organisms (Fig. 1). One of the most important aspects considered in this chapter will therefore be sediment sampling methodologies and subsequent organism extraction. *In situ* studies based on acoustic techniques (see Chapter 11) or sediment profile imagery are sometimes useful for rapid monitoring (Gray, 2002), but they are still at the initial stages of development (Rumohr and Karakassis, 1999; Legendre *et al.*, 2002; Ellingsen *et al.*, 2002).

The study of soft bottom communities requires special instruments to collect the sediment that contains the organisms and to treat it so that any of the organisms present are separated. It is vital that the most suitable tools are chosen to protect the environment and the communities. Soft bottoms are colonized by different kinds

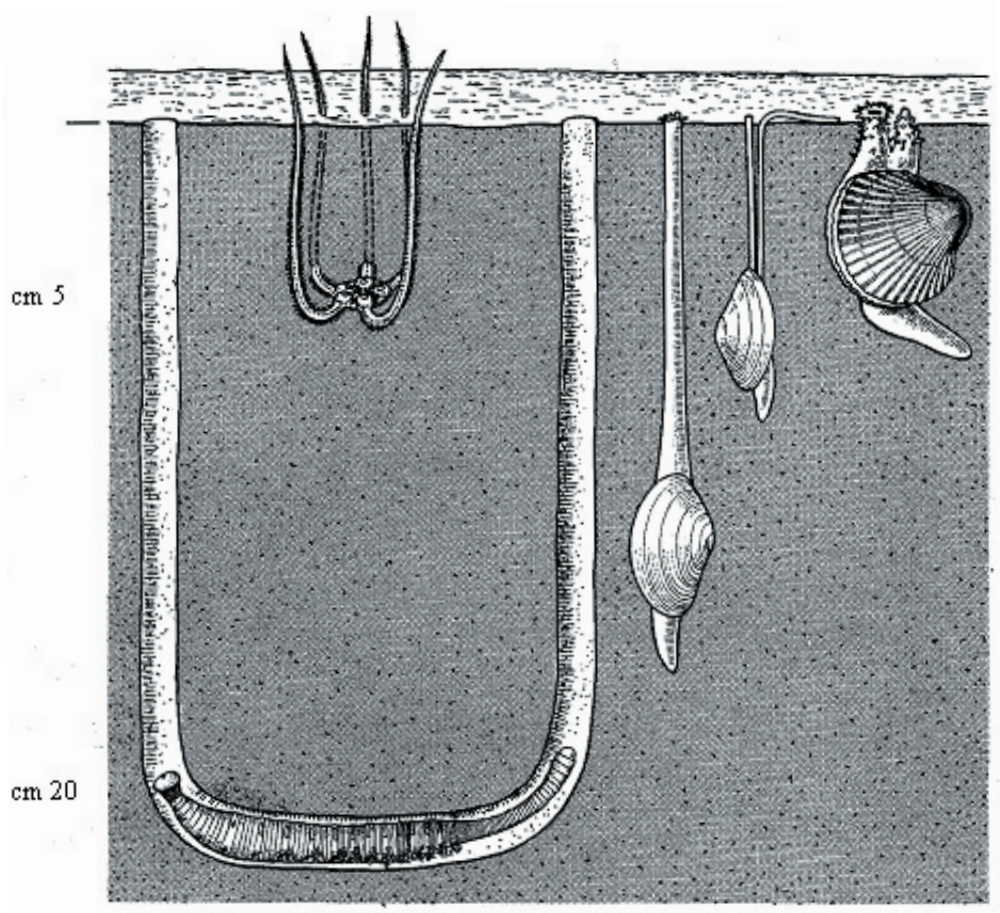

Fig. 1 - Soft bottom endofauna (modified from Cognetti and Sarà, 1972).

of organisms that can penetrate at varying depths into the substrate, favoring its oxygenation by such activity as the construction of tunnels. The depth colonized varies considerably in marine and brackish environments, in relation to grain size distribution, organic matter, and so forth. The choice of tool for sample collection also depends on the characteristics of the substrate, as well as the purposes of the study. The most important methodologies for the collection, treatment and laboratory study of the soft bottom macrobenthos in marine and brackish environments will therefore be described. Methodologies more suitable for the study of other soft bottom components (e.g., meiobenthos, see also Chapter 3) or those useful for specimens that will be treated for chemical analyses (in the latter case, sample contamination must be avoided) will be described in other sections of this Manual (see Chapters 15, 16); the same holds for aspects concerning research organization with adequate sampling strategies and data analyses (see Chapters 13, 14). Soft bottom macrobenthic communities are particularly important because of their ability to adapt according to natural or anthropogenic environmental changes.

Macrobenthic community structure strongly depends on a range of biotic and abiotic factors characterized by elevated spatial and temporal variability, such as hydrodynamics, substrate granulometry, organic matter concentration, pollution and, not least, the biological and ecological requirements of the species forming part of the community. These factors must be taken into account during research planning, both in sampling design formulation (see Chapters 13, 14) and in the specification of sampling strategies and instruments. The study of macrobenthic communities is therefore highly suitable for the evaluation of environmental quality, as modification of such communities may be directly traceable to natural or anthropogenic environmental variations (Pearson and Rosenberg 1978; Gray, 1981; Gray *et al.*, 1990; Warwick and Clarke, 1991).

Since this is a manual for operators who analyze the marine environment starting from different background knowledge, this chapter will mainly focus on the soft bottom of coastal zones, where the methodologies described may be more frequently applied. While it should be kept in mind that soft bottoms are widely distributed from the surface to the abyssal depths, the coastal zone as a whole (marine and brackish environments) is the system most crucially involved in environmental variations: it is therefore the most commonly studied and monitored to evaluate environmental quality.

4.2. Importance of soft bottom macrobenthos in environmental monitoring

The soft bottom macrobenthos represents an important tool for marine environment monitoring. Soft bottom benthic communities are widely used as "indicators" of environmental characteristics and make it possible to study changes to the marine environment resulting from anthropic activity (Crema *et al.*, 1983; Bilyard, 1987; Thomson *et al.*, 2003). In order to analyze and predict changes to the structure of communities along gradients of various kinds of environmental disturbance, a number of models have been developed, one of the most important of which is the 'Species-Abundance-Biomass model' (Pearson and Rosenberg, 1978). Such models have allowed the development of a series of methodologies suitable for describing and quantifying the effects of different levels of environmental disturbance on the structural parameters of the communities (Gray, 1981; Lambshead *et al.*, 1983; Heip *et al.*, 1988; Clarke, 1993; Elliott, 1994 and Chapter 17 of this volume). The most

recent methods for the study of marine pollution have been developed precisely through analysis of the structure of soft-bottom macrobenthic communities (Clarke and Warwick, 1994).

Given the importance of soft-bottom macrobenthic communities, they have been taken as the basis of numerous studies designed during recent decades, focusing on the Italian coastal area both in marine and brackish environments. These communities have for example been used to assess how the marine environment has been affected by urban sewage, industrial and thermal waste, the dumping of dredged materials and of drilling muds, and so forth. Thus in the early 1980s, interesting research was conducted by Crema and Bonvicini Pagliai (1980) and Fresi *et al.* (1983) along the Tyrrhenian coast in the Gulf of Follonica and at the mouths of the Rivers Ombrone and Tiber, yielding results that contributed to assessment of the effect of punctiform sources of pollution - such as industrial plants or river mouths - on communities and therefore on the marine environment. These studies then formed the basis for subsequent in-depth research and/or for research based on analyses of temporal series of data (see, for example, Lardicci *et al.*, 1992, 1999, for problems relating to heat pollution in the Gulf of Follonica). Also worth mentioning are the studies carried out by Crema *et al.* (1991, 2001) investigating the effect of punctiform sources of environmental change in the North Adriatic (off-shore platforms). These studies included an analysis of the modifications that have occurred over the last century in this basin (linked among other things to the increase in agricultural and industrial activities in the Po Valley), and evaluated the development of benthic populations in comparison with the early picture described by Vatova (1949).

Brackish environments likewise represent very important coastal systems (Bianchi, 1988; Carrada and Fresi, 1988; Cognetti, 1988). In such environments, soft-bottom macrobenthic communities have been found especially useful in highlighting the development of environmental features and have thus been the object of investigation by numerous research groups and organizations entrusted with supervision of the area. The study of macrobenthic communities has also contributed to highlighting the phenomena affecting numerous brackish basins along the Italian coast in recent decades. In the Orbetello Lagoon and in the Comacchio Valleys, for example, it has been possible to track developments that arose between the 1970s (Cognetti *et al.*, 1975; Cognetti *et al.*, 1978; Lardicci *et al.*, 1993) and the 1990's (Lardicci *et al.*, 1997, 2000; Lardicci and Rossi, 1998; Crema *et al.*, 2000, Mistri *et al.*, 2000), just as it has also been possible to track changing conditions in the Venice Lagoon (Giordani-Soika, 1973; Pranovi *et al.*, 2000; Sfriso *et al.*, 2001; Tagliapietra *et al.*, 1998, 2000) and the Po Delta (Mistri *et al.*, 2001).

The above studies are based on extensive knowledge of the taxonomy of soft-bottom macrobenthic communities both in marine and brackish environments. Such knowledge is absolutely vital for the management of correct programs of environmental monitoring and (see paragraph 4.4.2.2) should be adequately developed by research groups working in this sector.

In some cases, however, the aim of the research and/or of the monitoring program may make it necessary to restrict the time and costs of research both as regards sampling and laboratory work, although care should always be taken to adopt adequate methodologies as far as possible (Ferraro *et al.,* 1989; Warwick, 1993; Thomson *et al.*, 2003). From this perspective, the possibility of using 1 mm instead of 0.5 mm sieves to separate macrofauna has been explored (see paragraph 4.3.2.1). Moreover, some Authors have contested the "need" for identification at species level, arguing

that in some cases it might be sufficient to analyze communities at higher taxonomic levels and suggesting that this could be done without losing a significant part of the information, while gaining considerably in terms of time spent in identification, due to the resulting lower risk of identification errors (Warwick and Clarke, 1998). In this regard, the concept of "taxonomic sufficiency" has been introduced, involving the identification of organisms to a taxonomic level deemed adequate for the purpose of the study (Ellis, 1985). The aggregation of macrobenthic organisms at higher levels than the species has in some cases permitted the detection of differences between communities along environmental gradients, although the possibility of using this approach is, however, a function of the extent of the differences existing along the environmental gradients examined and of the data treatment methods. In particular, 'taxonomic sufficiency' has been shown to be valid for analysis of highly variable environments, of strong environmental gradients or of areas subjected to a marked level of pollution, while it has not proven adequate for studies on the biodiversity existing in a certain system (Warwick, 1988a, 1988b; Ferraro and Cole, 1992; James *et al.*, 1995; Olsgard *et al.*, 1997, 1998; Lardicci and Rossi, 1998; Mistri and Rossi, 2001).

These abbreviated methods, although useful for monitoring, should be carried out in parallel with a development and expansion of knowledge on the taxonomy of the most important groups present in the macrobenthos. In effect, identification at species level is particularly important not only because it allows detection of the possible presence of species that act as indicators of special environmental conditions (Cognetti, 1978; 1982; Castelli *et al.,* 1988), but also because it provides a picture of the species present in a given area, which may be useful for biogeographic studies (see for example the problem of the introduction and spread of exotic species), and for considerations on the medium and/or long term development of the macrobenthic communities in the area.

Finally, information on benthic communities can be used in conjunction with other information, such as data on toxicity and on the chemistry of sediments in what is known as the "Sediment Quality Triad" (SQT) (Long and Chapman, 1985; Chapman, 1990, and Chapter 17 for more detail) recently also applied in Italy (Volpi Ghirardini *et al.*, 1999).

4.3. Methodologies and sampling devices

Once the sampling design has been defined, based on the aim of the study (see Chapters 13, 14), it is necessary to define the most suitable sampling instruments, including size and replicate number, sampling techniques, treatment of samples, and sediment characteristics.

Collection and treating of samples must be divided into several principal phases: collection, sieving, fixation, preservation, sorting, and identification. The subsequent phases, consisting of data organization, data analysis and result discussion, are described in Chapters 13, 14, and 17, as previously stated.

4.3.1. Sample collection

Several devices may be used for soft bottom macrobenthos sampling. Grabs, dredges, and box-corers, while various kinds of core samplers with handles may also be used by SCUBA divers (up to depths of 40 m). Grabs, box-corers and other core samplers are particularly useful for quantitative studies, necessary for adequate

characterizations of benthic communities by the methodologies described in other chapters of this Manual (see Chapters 13, 14, 17). Use of these devices leads to collection of a well-defined amount of sediment and wide sample reproducibility. In addition, they yield more precise information concerning the distribution of individuals, and by collecting more complete specimens they allow more accurate biomass evaluation.

The most important models of grabs, box-corer and various kinds of core samplers have been described by several Authors; for a general review, see Holme and McIntyre, 1971, 1984; Eleftheriou and Holme, 1984. Eleftheriou and Holme (1984) described 14 different grabs, choice among them depending on various factors including working conditions (i.e. type of boat), substrate and type of habitat. They concluded that if moderate weight and adequate depth penetration are prime factors of consideration, then Van Veen, Smith-McIntyre and Day grabs are very suitable.

Fig. 2 - Ekman grab, modified by Pessa, used in Venice Lagoon (Photograph by G. Pessa).

The Day grab has the advantage of simpler, safer construction and is now widely used in soft bottom macrobenthos collection (Gray *et al.*, 1992; Davies *et al.*, 2001). Mc Donald *et al.* (1997) described the principal characteristics of different kinds of grabs (ideal habitat, advantages and limitations) and Van Veen and Smith-McIntyre also commented on other grabs such as the Petersen and Eckman (Fig. 2). Because of its ease of handling the Ekman-Birge grab is particularly suitable when used with small boats for sampling on bottoms with a fine granulometry (muddy bottoms); modified versions of this grab are suitable for manual sampling in the littoral zone or at shallow depths (1-1.5 m) (Fig. 3). The Ekman-Birge grab is very small and therefore not properly suitable for macrobenthos sampling; however, together with an appropriate sampling design, in certain environments, a relatively small device may also be used, increasing the number of replicates, if necessary. The Hamon grab

Fig. 3 - Modified version of Ekman-Birge grab suitable for manual sampling in the littoral zone or at shallow depths (1-1.5 m).

is very suitable for collecting coarse substrata; note that this is the only sampler able to collect reproducible samples on coarse bottoms (Pohle and Thomas, 2001).

Choice of a type of grab of a given size must lead to the collection of relatively undisturbed samples down to a depth that gives an adequate sediment profile. According to Gray *et al.* (1992), a grab that covers a surface area of 0.1 m^2 should penetrate at least 10 cm (preferably 20 cm) and collect at least 4 l of sediment; other Authors prefer slightly different values; inadequate samples should be discarded (Davies *et al.*, 2001). The grab size usually varies between 0.03 and 0.55 m^2, although the most common sizes are between 0.1 and 0.2 m^2 (Eleftheriou and Holme, 1984; Riddle, 1989).

Grab dimensions should be defined on the basis of boat characteristics and sampling design, keeping in mind that a high number of relatively small sized samples yields greater information than a small number of large samples, when analyzing communities with a wide spatial variability such as macrobenthic communities (see Chap. 13). The collection of a sample with a sediment depth layer of at least 20 cm seems more useful for obtaining data concerning the principal components of the community because of the occurrence of digger organisms in soft bottom deep layers.

Research groups often adapt the devices described above to meet local requirements. Device suitability should be evaluated and inter-calibration should be performed when studies carried out with different techniques are compared.

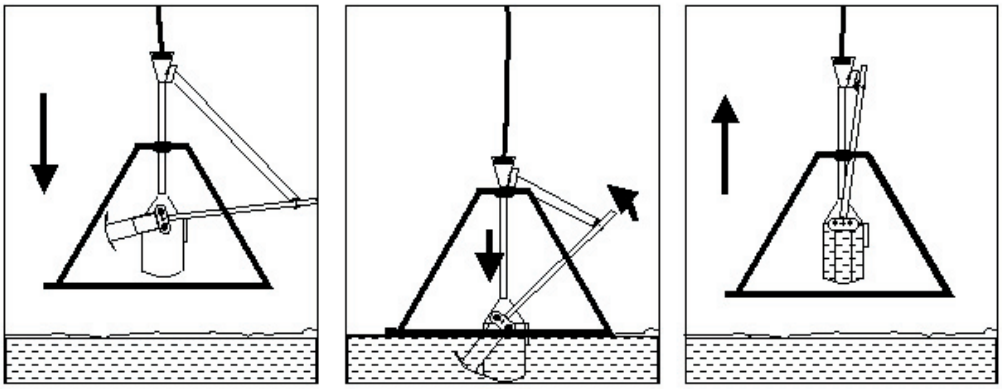

Fig. 4 - Scheme of *box-corer* functioning (modified from Della Croce *et al.*, 1997).

Core samplers and box-corers (Fig. 4) are particularly suitable for macrobenthos sampling, because they collect relatively undisturbed sediment and water, and only slight quantities of other material are washed away. Moreover, these devices maintain the substrate stratification and are therefore suitable for research on the vertical distribution of organisms down to the bottom profile.

Small box-corers that cover a surface area of about 0.02 m^2 (Fig. 5) are suitable for manual sampling in the littoral or shallow sublittoral zone, on fine substrates and in particular those occurring in brackish environments, as previously reported for Ekman-Birge grabs (Lardicci *et al.*, 1997; Crema *et al*., 2000).

Small Plexiglas corer samplers may be easily used by SCUBA divers and lead to adequate sampling of the coastal zone soft bottom macrobenthos (Fig. 6). Lardicci *et al.* (1999) proposed, for instance, devices that are easily handled (8 cm diam. 25 cm length), capable of penetrating the substrate down to 15 cm, and particularly suitable

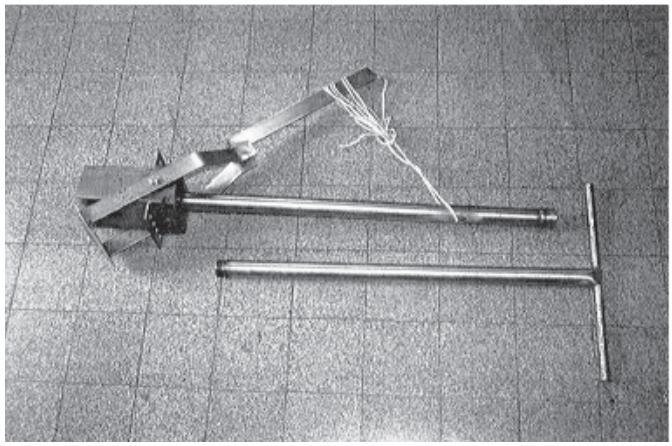

Fig. 5 - Manual *box-corer* (Photograph by A. Floris).

for collection on shallow sandy sediments. Sampling by dredges obtains greater and more general information about the macrobenthos occurring in a marine zone, but dredges are not suitable for quantitative analyses, as they collect an undefined amount of sediment. Their dragging on the bottom leads to a generalized fragmentation of specimens, which in turn raises difficulty with regard to such aspects as taxonomic determination and biomass definition (Gambi *et al*., 1987). In some cases, "suction samplers" may be used for collecting soft bottom macrobenthos.

These devices, handled by SCUBA divers, suck and filter sediment; their functioning is described in Chapter 5 and 6 because of their extensive use in the study of other benthic biotopes. At the end of this and subsequent paragraphs, instruments necessary

Fig. 6 - Sampling by corer samplers used by SCUBA divers:
a) on soft bottoms near a *Posidonia oceanica* meadow (Photograph by A. Floris).
b) in a brackish environment (Photograph by S.E.L.C.).

for an accurate development of field and laboratory activities and their appropriate care will be emphasized where necessary. During sample collection, for instance, it is necessary to avoid (wherever possible) any sample contamination. In the S&Q (Safety and Quality) sections, the procedures required for operation safety and quality will be outlined.

S&Q

- Samplers must be carefully washed and rinsed before subsequent sampling.
- When the sampler comes to the surface, rapid treatment is necessary to prevent any stress that might modify its content.
- The exterior surface of the sampler must be carefully rinsed to prevent sample contamination with external sediment.
- The sample must be carefully examined to check that its general condition is good, its surface is not washed away, and the minimal depth is reached.
- Care must be taken to prevent loss of the first 1-2 cm of surface water and consequent modification of the sample surface.
- Operators involved in sampling must be carefully trained in recognizing any potential contamination. They must know safety procedures, sample treatment procedures (general and personal activities) and, finally, the purpose of the study.

4.3.2. Sieving and temporary storage

Samples must be sieved in order to eliminate water, fine sediments and any other extraneous materials not necessary for the study.

After it is recovered on board, the sampler must be suitably treated in order to collect the entire sample and sieve it without leaving any material. After a preliminary description of its surface, the sample must be completely removed from the gear and sieved as soon as possible. When small box-corers or approximately 10-l grabs are used, it is best to place the sample in a plastic bowl and treat it by passing it through mobile sieves.

The procedure must be carried out according to the following steps. First, the sample description must be recorded on the appropriate card, specifying not only general data concerning the sample but also all information observed on the "fresh" sample: surface characteristics, individual density, occurrence of organic detritus, etc. A sampling card, suitable mainly for brackish-water sampling, is outlined in Table 1.

Then the sample must be completely removed from the gear, and placed in an appropriate container (Fig. 7). Any water occurring on the sample surface must be put in the bowl with the sample. Used sampling equipment must be carefully washed and the washing water must also be collected in the bowl, after carefully sieving it with a mesh smaller than 0.5 mm to avoid accidental introduction of other organisms.

A washing desk equipped with a wide smooth surface, on which samples may be washed and slightly dissolved before being placed in the container, can be used for dissolving the sample sediment material.

A valid alternative, most suitable for clay sediments, is to slightly dissolve the sediment inside the bowl itself, after adding filtered seawater (3-4 times the volume of the sample). Water may be directly sprinkled onto the sample with a low-pressure nozzle in order to avert damage to animals. When the required level is obtained, the sample should be shaken slightly until almost complete dissolution. Small clay nodules, which usually remain aggregate, will be dissolved later during sieving.

Tab. 1 - Sampling card.

Date Ship

Starting time: Ending time:

Station

Label: Name

Progressive number:

Coordinates

X = Y =

Other criteria suitable for station localization

Depth:

Bottom morphology:

Water column

Transparency (Secchi):

Temp. [°C]	Salinity [PSU]	Cond. [uS]	pH	DO [mg/l]	DO [% sat]	ORP [mV]

Tool used:

Operator

Meteo

Air Temperature [°C]: Barometric Pressure [mb]:

Wind: Direction: Speed:

Sky Conditions:

				Sea conditions:			
sunny or slightly cloudy	slightly cloudy or partly cloudy	irregularly cloudy or cloudy	very cloudy or overcast	0	1	2	3
				Calm	Quite Calm	Smooth	Slight
0 - 1 - 2	3 - 4	5 - 6	7 - 8	-	0 - 0.1	0.1 - 0.5	0.5 - 1.25
Number of covering (from 0 to 8)				Waves height [m]			

TIDE:	Ebb	Flood	Still water

follows

(*follows*) Tab. 1 - Sampling card.
MACROBENTHOS

N° Replicates: **Tool Used:**

Operator:

	Sediment										
	Sediment consistence			Burrows			Microalgae		Macrophytes		
	Plastic	Dense	Fluid	Absent	Few	Many	Yes	No	Absent	Few	Many
Repl. A											
Repl. B											
Repl. C											
Repl. D											
Repl. E											

	H_2S Smell				RPD	**Sieving**
	Absent	Low	Medium	High	Layer	N° Containers and notes
Repl. A						
Repl. B						
Repl. C						
Repl. D						
Repl. E						

Card Compiler:

Notes Drawing on the back Yes No

If boat characteristics or lack of sufficient time do not permit immediate sample treatment, samples can be kept in watertight plastic bags after being adequately described. However, it is important to treat them in the above-described manner as soon as possible in order to prevent significant modifications.

4.3.2.1. Sieving

This process consists in sieving the sediment sample dissolved in sea water. In order to separate macrofauna, the sieve must have a 1 mm or 0.5 mm mesh (Eleftheriou and

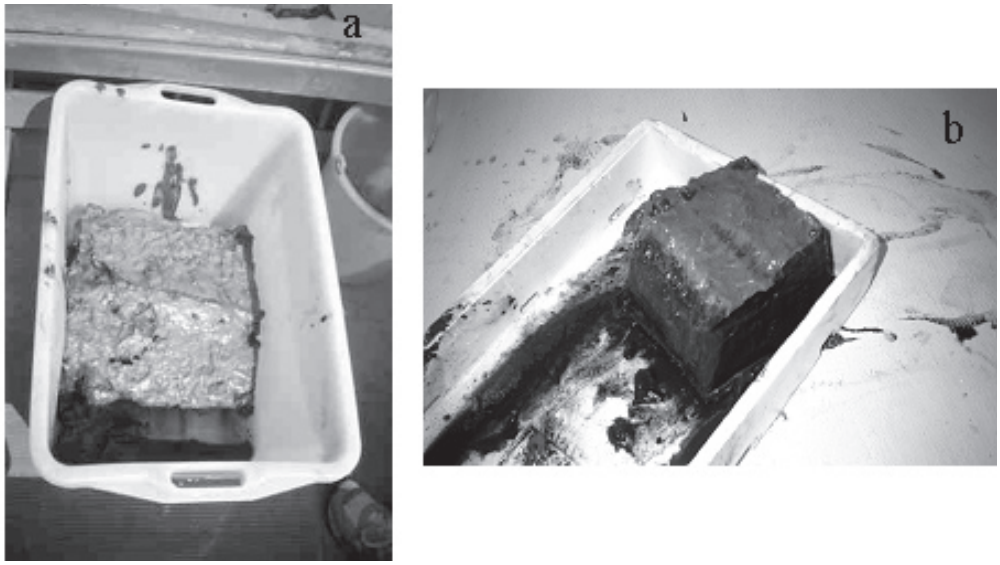

Fig. 7 - a) Container with a soft bottom macrobenthos sample collected with a Van Veen grab;
b) Container with a soft bottom macrobenthos sample collected with an Ekman-Birge grab. (Photograph by D. Tagliapietra).

Holme, 1984; Kingston and Riddle, 1989). For large scale environmental monitoring, a 1 mm mesh is usually suitable, as the best compromise between reliability of result and research costs (Buchanan *et al.*, 1974; Hartley, 1982; Ferraro *et al.*, 1989), given that macrobenthic fraction loss does not cause significant distortions of the main community parameters (Kingston and Riddle, 1989; Cognetti and Cognetti, 1992).

In certain cases, when the macrobenthic component measuring less than 1 mm might be important (i.e. when a large amount of organic matter favors the abundance of "opportunistic" forms - usually small in size), or when analysis of juvenile recovery is important, then use of 0.5 mm mesh sieves in addition to the others is appropriate (Cognetti and Cognetti, 1992). It is recommended that the fraction held back by the 1 mm mesh sieve be kept separate from the material obtained in other sieves. Naturally, use of different sieves leads to separate counts resulting in different numbers and different kinds of macrobenthic organisms.

Ideally, steel mesh must be used, with wire thickness comparable to that of the US Standard sieve.

Tab. 2 - Sieve characteristics according to main standards.

Mesh dimension [mm]	ISO 3310	ASTM E-11-95	UNI 2331/2332	Wire diameter [mm] (ASTM)	Mesh/cm^2 (ASTM)	Mesh/cm^2 (UNI)
2.000	2.00 mm	# 10	# 7	0.900	13	11
1.000	1.00 mm	# 18	# 13	0.580	45	35
0.500	500 µm	# 35	# 20	0.340	158	140
0.250	250 µm	# 60	# 26	0.180	575	590
0.125	125 µm	# 120	# 32	0.091	2,200	2,400
0.063	63 µm	# 230	# 38	0.044	8,800	9,450

If the sieve is 1 mm mesh, wire thickness must be 0.58 mm (AAVV, 2002) (Tab. 2).

The sieve surface must hold the sample without deforming it. If the sample is likely to be too heavy, a reinforcing steel net (with ca 10 cm mesh) may be used.

The sieve must have an adequate surface to avoid clogging. Choice of the appropriate surface size depends on sieve mesh, sieved volume and type of sediment being worked on. For volumes less than 10 l of silt or sandy sediment, using 1 mm mesh, a sieve with a diameter of 30-40 cm may be sufficient. For sediments rich in organic detritus, a considerably larger surface is required (50-60 cm diameter). Sieves are usually about 1000-2500 cm^2 in surface area; their dimensions vary from 30 × 30 cm (or about 35 cm in diameter) to 50 × 50 cm (or about 55 cm in diameter) (Fig. 8). Small circular sieves are suitable for manual sampling treatment, whereas square ones are suitable for being fitted into sieving desks. Moreover, in circular

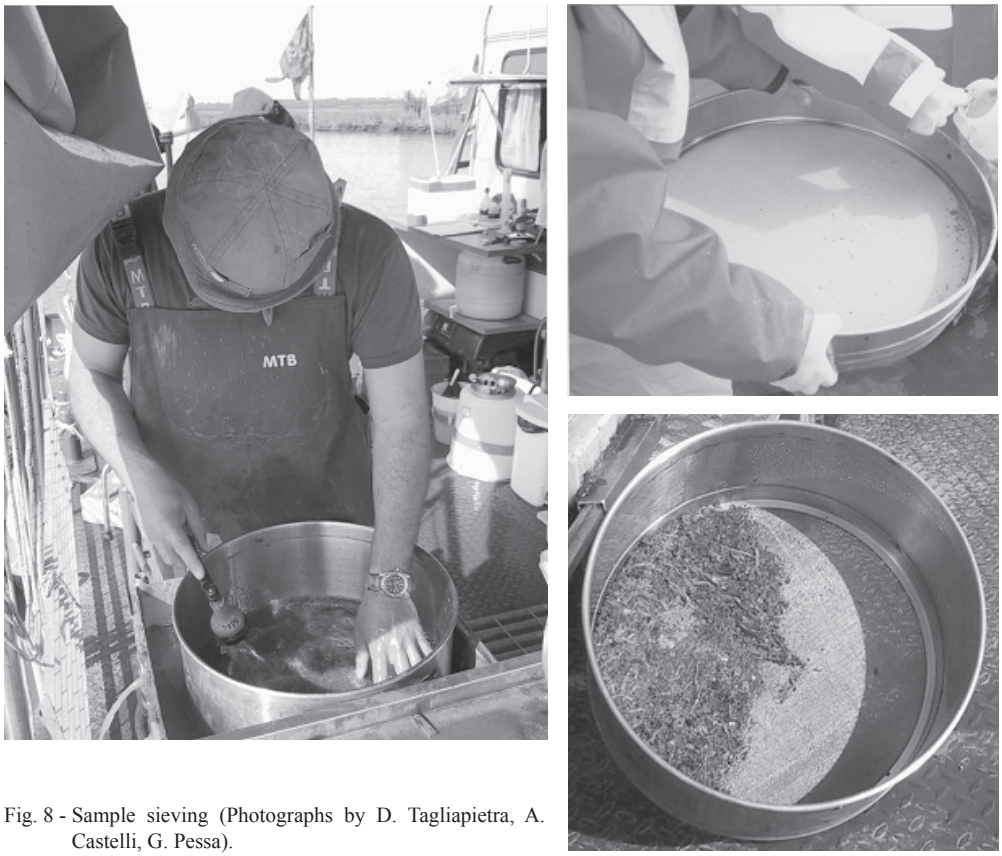

Fig. 8 - Sample sieving (Photographs by D. Tagliapietra, A. Castelli, G. Pessa).

sieves water facilitates the dissolving of sediment, especially when a large number of plant fragments occur and obstruct the sieve. The depth of the sieve must enable the sample to settle and prevent any loss of material that might occur with too vigorous washing. The spray nozzle allows animals to be separated from the sediment without being damaged. Pressurized seawater is recommended, using a pump with a filter to avoid sample contamination.

Sediment must not be rubbed against the sieve surface, in order to avoid organisms being damaged. If small-sized animals pass through the sieve mesh, it is advisable to collect the washing water in a suitable basin and sieve it again in order to recover these organisms.

S&Q

- The pressure of rinsing water must not be so high as to risk damaging organisms.
- Sieves must be washed and thoroughly re-cleaned after sieving each sample.
- Rapid execution is vital to prevent organisms becoming fastened to the sieve mesh or being washed away with rinsing water.

4.3.2.2. Temporary storage

After the sieving operation is completed, the remaining material (organisms, shell fragments, vegetal debris, coarse sediment and any other matter) must be transferred to the appropriate containers. The whole sieved material must then be made to flow towards the edge (or a corner) of the sieve using a low-pressure jet; it should then be removed extremely carefully, after which the sieve must be carefully inspected in order to find any organisms caught in the mesh. The sieve mesh may be washed with a low-pressure jet, or with a pressure nozzle similar to the kind used for gardening; to this end, the sieve may also be soaked for approximately ten minutes in a solution to loosen any remaining organisms (see also paragraph 4.3.3.1) and then washed again with sea water. Once the material has been carefully and completely removed, the sieve must be thoroughly washed and cleaned with a bristle scrubbing brush to prepare it for the next sample.

The material removed from the sieve must be conducted towards a wide-mouthed funnel using the water jet (a polypropylene "dust funnel" with a 20 cm (approx.) diameter mouth and a 5 cm (approx.) diameter stem is also suitable). The outlet of the funnel must be inserted in the storage container, ensuring that the latter is of a size that allows easy handling; large quantities of sample may be stored in several containers.

Excess water in the container must be removed; to avoid accidental loss of material it is advisable to filter the water through a small 250-μm sieve over a suitably sized plastic bowl.

For rapid retrieval of small organisms, usually occurring mostly in the surface centimeters of sediment, surface rinsing of the sample has been found advantageous. Once the sample is placed in the bowl, the top stratum may be carefully removed with a jet of water and filtered through a small sieve. The technique is rapid and enables most of the specimens present, especially small ones, to be very carefully collected. If the sample is full of vegetal debris or shell fragments in the lower strata, the organisms removed with this method should be stored separately from the rest of the sample.

The presence of vegetal debris often produces a felt of bits and fibers on the surface of the sieve, which may reduce the size of the mesh so that smaller organisms are also retained. The thickness of the felt may thus make it impossible to compare samples in which the felt is present with those where it is absent.

When this occurs it is advisable to assess the need for a further sieving of the sorted material.

When there are large quantities of organic debris, procedures subsequent to sieving (see paragraph 4.3.3) may be accelerated by using the so-called "stocking method". The technique consists in transferring the sieved material into a 200-μm (approx.) mesh tulle bag or nylon stocking, the material thus being kept together during subsequent phases. Collecting the sample in a permeable bag prevents it from spilling into a sieve when solutions are replaced (e.g. replacement of fixing solution with preservative) (see paragraph 4.3.3), and allows safer, quicker washing procedures. Note that the time spent during sorting in the cleaning of the wire mesh does not make this phase longer. When this method is used, the material is removed from the sieve by directing it with a jet of water towards a very wide-mouthed funnel (approx. 20-25 cm mouth and approx. 5-7 cm outlet, usually described in catalogues as a "dust funnel"), to which the tulle bag is tightly attached. Before closing the bag, the organisms and material left trapped between the mesh of the fabric must also be placed in the funnel. The bag must be closed by tightly knotting or by using a self-tightening plastic clip. The material contained in the sample must not be closely packed, that is to say, it must have enough room inside the bag so that it rapidly comes into contact with the solutions. The bag must then be placed in a suitable container and labeled. It is strongly recommended that the entire sample should be placed in a single bag and a single container; if the sample has to be split up, the divided samples must be appropriately labeled with "split numbers" (see paragraph 4.3.2.3).

Containers for storage must have an airtight closure and be large enough to hold the sample and treatment solutions (narcotics and fixatives, see above). For macrofauna study, High Density Polyethylene (HDPE) containers have been found to be a good compromise between cost and strength. Square containers are the most practical shape for storage. Stackable square HDPE containers with a screw top and fitted with a base plug are also available commercially. If storage and transport are not a problem, cylindrical HDPE containers fitted with stopper and base plug can be used; these have a proportionally wider mouth and may be more easily cleaned because they have no corners.

If some organisms need special treatment they may be stored in small Polyethylene or Prolyproylene sample test tubes with stopper. The container holding the sub sample should be appropriately labeled and placed inside the container that contains the rest of the sample.

4.3.2.3. Labeling

Containers must be labeled both internally and externally for greater security. The external label may be written with a permanent marking pen or attached to the container using a strip of transparent parcel tape; the internal label must be made of transparency paper (written in lead pencil or Indian ink) or of acetate (photocopied beforehand). The labels must record: the station code, the replication code, sampling date and split number. The split number refers to a number in a series, for example, "one of four", "three of five" etc., and is written as a fraction 1/4, 3/5 (1/1 if there is just one container).

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- Labeling must be performed by two people.
- Data on labels must be recorded in special field files.
- Labeling procedure must be checked by the area operations manager.

4.3.3. Fixation and preservation

4.3.3.1. Narcosis

Before fixation, the sample should be treated with an anaesthetic and/or relaxant solution, for example a solution of 7% Magnesium Chloride or 10-15% Ethanol (see paragraph 4.3.4.1 for a list of the main solutions used and their formulation). The sample should be treated for about thirty minutes, although the time can vary according to taxonomic group and organism size as well as sample organic matter and clay content.

The use of such a solution has the double purpose of relaxing the organisms and anaesthetizing them to avert the risk of contraction, since excessive contraction could lead to breakage and make them difficult to identify.

In addition, the anaesthetic prevents the organisms from suffering when they are immersed in the fixative solution.

Once the sieved material is placed in the storage container, it must be covered with plenty of relaxing solution, and then stirred well so that the organisms come into contact with the solution; after a suitable interval, sample fixation can be commenced (see paragraph 4.3.4.2).

When fixative has been added, the relaxing solution obviously loses its effectiveness. The quantity of relaxing solution used may be substantial in the case of research that requires the collection of large numbers of samples. If the "stocking method" is used, the relaxing solution can be used several times. The sample is then placed in a wide container that may hold as much as ten liters or so of relaxing agent, and which is fitted with a screw lid. At the set time the bag is taken out, left to drip over a sieve (which may be a broad mesh sieve) that is placed over the container itself.

The bag is then placed in the storage container, where the fixative solution is then added.

4.3.3.2. Fixation

Fixation of a biological sample prevents *post-mortem* degeneration of tissue induced by autolysis; it also prevents microbial attack and preserves the structure as unchanged as possible. Chemical fixatives denature, precipitate or bind proteins to one another, resulting in the hardening of tissues. Best results in fixation are obtained when the animal is still alive but anaesthetized. For each taxonomic group there are special anaesthetizing/relaxation and fixation methods depending in particular on the different chemical composition of the various organisms (Tab. 5). For practical reasons, however, block fixation of the entire sieved sample is usually resorted to.

To fix the sample, the container must not be filled more than half full with sieved material; the container must be filled 3/4 full of the fixative solution. A ratio of 3:1 in volume between the fixing solution and the volume of the sample is however recommended. As already described for the relaxing solution, the sample should likewise be well mixed so that all the organisms can come into contact with the fixing solution. The presence of considerable quantities of organic matter obviously requires a larger quantity of fixative solution. A good fixation of macrobenthic organisms may be obtained within a couple of days, but even after just a few hours, specimens are protected from the worst damage.

The most common fixative is a 10% formalin solution (or 4% formaldehyde) in seawater. Formaldehyde is a gas produced by the oxidation of methanol while formalin is the saturated solution of this gas in water; formalin is therefore a commercial

aqueous solution of 40% formaldehyde. This solution should always be expressed as a percentage of formaldehyde and not of formalin (a 10% formalin solution or 4% formaldehyde solution is the same thing).

The formaldehyde content in formalin readily oxidizes to formic acid, producing a solution with pH variable from 2.5 to 5. Such a degree of acidity would cause damage to the calcified parts and other tissues of the organisms; the formaldehyde must therefore be buffered or neutralized.

The addition of seawater, with pH of 8.2, already has a degree of buffering effect on the formalin, but the buffering effect of the carbonates contained in seawater does not last long. A practical way to buffer formalin is to saturate it with Calcium Carbonate ($CaCO_3$) or Borax (Sodium Tetraborate, $Na_2B_4O_7$). The neutralizing effect of these common buffers may be reduced over time, and Borax may lead to lysis of tissues and a strong discoloration of pigments. For this reason, and also because of the toxicity of formaldehyde, we would advise against the use of formalin as a preservative solution. If it is particularly crucial to preserve the sample in formalin for a longer period, the use of a phosphate buffer is recommended (Sörensen Buffer) composed of monohydrate Sodium Phosphate and Dibasic Phosphate of Anhydrous Sodium. In any case, calcareous matter or shells can be kept in the formalin container as a precautionary measure.

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- Caution: it must be remembered that formaldehyde is toxic and carcinogenic and should therefore be used with extreme caution, preferably in the open air or, failing this, in a well-ventilated area and under an extractor fan; in the laboratory, a fume mask should be worn.

It is to be hoped that materials far less toxic than formaldehyde will be used in the future (Tab. 3). In the U.S.A., "Formalternate" is sometimes used, but this product is on sale only there. EPA is actually testing the "NoTox" Biological Preservative, apparently achieving very positive preliminary results. Paraformaldehyde, used in histology, is likewise toxic, albeit slightly less so than formaldehyde; our advice therefore is to avoid this substance as well. Solutions containing formaldehyde may,

Tab. 3 - Most common substitute solutions for formaldehyde, preservatives and detoxifiers in the USA.

Product	**Manufacturer/Supplier**
Fixative Solutions	
Formalternate	Flinn Scientific Inc., P.O. Box 219, Batavia, L 60510
HistoFix	Trend Scientific Inc., P.O. Box 120266, St. Paul, MN 55112
Prefer	Anatech Ltd., 1020 Harts Lake Road Battle Creek, MI 49015
S.T.F.	Streck Laboratories Inc., 14306 Industrial Road, Omaha, NE 68144
Products for the detoxification of Formaldehyde	
Formaldetox	Anatech Ltd., 1020 Harts Lake Road, Battle Creek, MI 49015
DeTox	Earth Safe Industries Inc., Belle Meade, NJ
Vytac	Trend Scientific Inc., P.O. Box 120266, St. Paul, MN 55112
Formaldehyde-free or low formaldehyde Preservatives	
NoTox Biological Preservative	Earth Safe Industries Inc., Belle Meade, N.J.
Caro-Safe	Carolina Biological Supply Company, 2700 York Road, Burlington, NC
Perfect Solution	Carolina Biological Supply Company, 2700 York Road, Burlington, NC

however, be detoxified with products such as "Formaldetox", "DeTox" and "VYTAC". The formulation of the products is secret but, after detoxification, a 10% solution of formalin usually contains less than 100 ppm (0.01%) of formaldehyde. With the exception of formaldehyde (and any other impurities of the detoxified solution), all the other substances produced by the detoxification reaction have been declared no longer carcinogenic. Care must be taken, however, since the detoxification reaction produces a considerable amount of heat.

4.3.3.3. Preservation (long-term storage)

After the specimens have been fixed, they are extracted from the fixing solution, quickly rinsed and left to drip. The specimens must then be washed by placing them in aqueous solutions of ethanol at progressive concentration (30%-50%-70%) until they are transferred to the final preservation solution composed of 70% ethanol + 5% glycerine, which is still found to be the best preservative solution. Many alternatives are being tested; preservation in propylene glycol seems to have the property of reducing the toxic risk but does not appear to give satisfactory results with large organisms over a long period.

4.3.3.4. Coloration

Coloring the material contained in the sample may help the sorting procedures, by making identification of the smallest organisms easier during this phase. One of the most common colorants used for this purpose is Rose Bengal (see paragraph 4.3.4.3), which should however be used carefully and sparingly because, as with other colorants, it is considered a carcinogenic substance by the IARC. Moreover, its use may alter the color pattern of some organisms, for example polychaetes, making identification during the subsequent phase more difficult. Where a large quantity of vegetal debris is present, Rose Bengal may cause confusion as it could color part of the detritus.

For further discussion of this issue, readers are advised to consult the specialized bibliography (Holme and McIntyre, 1984).

If a paler color is preferred, methylene blue may be used, a substance that is far less toxic. However, it should be used only at the time of identification, and it is almost completely reversible.

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- The use of a relaxant is recommended to prevent the fragmentation of organisms in the sample during fixation.
- The sample should be left in contact with the relaxing solution for about half an hour, inverting the container several times.
- Different colored adhesive labels or strips of sticky tape of different colors should be used to mark the containers that contain samples in relaxing solution (yellow) and those that contain samples in fixative solution (black).
- Containers must not be filled with more than 40% of the sieved matter.
- The fixative solution must be roughly twice the volume of the sample.
- Many colorants used in biology, such as Rose Bengal, "Chlorazol black E", "Rhodamine B" etc., are considered carcinogenic by the International Agency for Research on Cancer (IARC, Lyons, France); it is therefore better to avoid contact by taking all necessary precautions to reduce the quantity used.
- Many of the colorants used may highlight non-useful components (vegetal

fragments) and may irreversibly alter the color of some organisms, thereby making their identification difficult.

For safety reasons, and in particular for the rapid identification of the hazard of the content, all the containers that contain different solutions must be marked with an adhesive strip.

The colors that can be proposed to differentiate the strips are those shown in Tab. 4; in addition, the label must bear the written indication of the name of the liquid and the skull and crossbones mark for toxic substances.

Tab. 4 - Color code for containers holding different solutions.

Liquid	Color
Water	Blue
Sea water	Green
Alcohol	Red
Formalin	Black
Narcotic	Yellow

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- When a container has been used for formalin, it must always be marked with a black strip.

4.3.3.5. Transport and storage of samples

Transportation of the samples should be carried out with the maximum care. If at all possible, the containers should be stored in sealed, insulated crates or at least in strong crates with trays placed at the bottom to collect any spillage.

The crates must be strong enough and of a suitable shape and weight such as to allow them to be handled without causing strains or jeopardizing the safety of workers and contents.

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- Vehicles must have suitable, adequately ventilated compartments to prevent possible spread of toxic substances and inhalation by workers.
- The containers holding preserved material should be kept in special airtight, ventilated cabinets or, when these are not available, they should be stored in the open air on ventilated shelves and protected from light, heat and atmospheric pollution.
- A special delivery record (Loading and Unloading cards) of the samples must be prepared, indicating the origin, delivery date, type of transport and the names of the persons in charge of loading, transport and unloading. The record must enable materials and persons in charge to be traced (“Chain-Of-Custody-Form”).

4.3.4. Formulation of solutions

Shown below is the formulation of some of the main solutions indicated for the procedures mentioned above.

Each time it is desired to obtained a solution at a known concentration starting

from a stock solution instead of from a pure substance, the following equation may be used:

$$Concentration_1 \; x \; Volume_1 = Concentration_2 \; x \; Volume_2$$

where: $Concentration_1$ and $Volume_1$ = concentration and volume of "stock" solution;

$Concentration_2$ and $Volume_2$ = concentration and volume of the solution at the desired concentration;

e.g.: to obtain a 4% solution of formaldehyde (solution at desired concentration), the procedure makes use of 40% commercial formalin (stock solution), diluted 1:10 with seawater (1 part formalin and 9 seawater).

4.3.4.1. Narcotic solutions

- 7% Magnesium Chloride Solution: 0.70 kg Magnesium Chloride ($MgCl_2$) in 10 liters of seawater (Source: Marine Monitoring Handbook - Davies *et al.*, 2001).
- 7.5% Magnesium Sulphate Solution: 0.75 kg Magnesium Sulphate (MgSO4•7H2O) in 10 liters of seawater (Source: SCBFMC - AAVV, 2002). Magnesium Sulphate is known commercially as Epsom Salts and is also used as bath salts.
- Solution of Propylene phenoxetol (1-phenoxy-2-propanol): 15 ml propylene phenoxetol in 10 liters of seawater (Source: SCBFMC - AAVV, 2002)
- 10% Ethanol Sulphate Solution: 100 ml of Ethanol (also denatured) in 1 liter of seawater. The solution of 10% Ethanol has often proven to be the most efficient and economic of the narcotics, especially for polychaetes.

4.3.4.2. Fixative solutions

- 4% formaldehyde solution: to obtain 10 liters of 10% formalin solution (4% formaldehyde) 9 liters of seawater is added to 1 liter of buffered formalin. This solution should always be expressed as a percentage of formaldehyde and not of formalin.
- Formalin solution (40% formaldehyde) buffered with sodium tetraborate (Borax, $Na_2B_4O_7$) [Neutral Buffered Formalin (pH 7.0) (NBF)]: 50 g sodium tetraborate ($Na_2B_4O_7$) in 1 liter of commercial formalin (Source: SCBFMC - AAVV, 2002). As previously mentioned, formalin may also be buffered with calcium carbonate in saturation.
  Formalin solutions buffered with sodium tetraborate or calcium carbonate should not be used for long-term preservation since their buffer effect tends to deteriorate and sodium tetraborate may cause lysis of tissue and depigmentation. Phosphate buffers such as Sörensen's Buffer, described below, are preferable for long-term preservation.
- Phosphate Buffer (Phosphate Buffered Formalin, Sörensen's Buffer): 40 g Monobasic Sodium Phosphate $NaH_2PO_4 \bullet H_2O$, 60 g Dibasic Sodium Phosphate (anhydrate) Na_2HPO_4 in 1 liter commercial formalin (40% formaldehyde) [Source: "A Manual of Histotechniques" 3rd ed. Ann Preece, 1972, and Surgical Pathology - Histology Staining Manual No. 1:4 Rev. 1 National Museum of Natural History (NMNH), Smithsonian Institution].
  Formaldehyde may also be buffered with hexamine.

– Hexamine Buffer: 100 g hexamethylenetetramine (Hexamine = Urotropin) in 1 liter of commercial formalin (Source: Manual for Marine Monitoring in the COMBINE Programme of HELCOM for the Baltic).

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- Formaldehyde is included amongst carcinogenic substances in the "National Toxicology Program's (NTP) Fourth Annual Report on Carcinogens", and among potentially carcinogenic substances by the American OSHA "Occupational Safety and Health Administration".
- Formaldehyde may cause severe irritation to the eyes and skin; it is corrosive, toxic when ingested or inhaled, with specific effects on the respiratory system, causing sensitization on respiration and on contact.

4.3.4.3. Colorant solutions

Rose Bengal: 4 gl^{-1} in formalin (see Holme and Mc Intyre, 1984).

4.3.4.4. Preservation solutions

– Alcohol and glycerine: Ethanol 700 ml; Glycerine 50 ml; Water 250 ml (see Holme and Mc Intyre, 1984).

While in practice, treatment often cannot be separated according to organisms, some solutions are more suitable for some taxa than others. See Table 5 below [modified by United States Antarctic Program (USAP)/ National Museum of Natural History (NMNH), Smithsonian Institution Department of Invertebrate Zoology, NMNH/ NSF Cooperative Agreement United States Antarctic Program, Museum Collection Management Terms and Invertebrate Specimen Processing Procedures: Methods of Fixation and Preservation, http://nmnhwww.si.edu/iz/usap/usapspec.html.

4.4. Laboratory treatment of samples

4.4.1 Instruments and materials required

Sink with fume hood; workstations with fume hoods; illuminated worktable; illuminated bench lens; stereomicroscope (zoom 1-7×); optic fiber illuminator; 250-μm mesh sieves of various diameters; variously sized funnels adjustable for buckets and sieves; variously sized dust funnels; washing solutions; preservative solution; containers for spent solutions; large plastic or glazed enameled steel tanks (approx. 50 x 40 x 15 cm); small plastic or glazed enameled steel tanks (approx. 30 × 20 × 5 cm); 3- or 5-liter plastic beaker; 20-ml spark vial or similar sized test tubes suitable for containing collected organisms; plastic spoons and spatulas; variously sized plastic tanks; wash bottles and spray nozzles for alcohol and water; permanent marker pens; 8-10 cm diameter Petri dishes marked with parallel lines; standard 6 cm diameter Petri dishes; counters; dissection set; fine stainless steel Dumont 5 tweezers; large tweezers; flat tweezers; handled needles; fine-tipped scissors; scalpels; elastic bands; pencils/Indian ink pens; labels; filing cards.

4.4.2 Sorting

The following sequence of activities is to be observed during laboratory sorting: removal of fixing solution, sample washing, division into sub-samples (if necessary),

Tab. 5

General Taxa	Specific Taxa	Relaxing Agent	Fixative	Wash	Preservative	Dangers
Bulk Macrofauna	General bulk processing	$MgCl_2$ isotonic to sea water (approx. 7%)	5-10% phosphate buffered formalin	30%, 50%, 70% ETOH	70-80% ETOH	remove CRT, ECH, and shelled MOL from formalin solution ASAP
Annelida	Hirudinea, Oligochaeta, Polychaeta	$MgCl_2$ isotonic to sea water (approx. 7%)	10% phosphate buffered formalin in sea water	30%, 50%, 70% ETOH	70% ETOH	
Arthropoda	Decapoda & other larger crustaceans	$MgCl_2$ isotonic to sea water (approx. 7%) or oil of cloves	5-10% phosphate buffered formalin in sea water or 75% ETOH	50%, 70% ETOH	70% ETOH	legs will separate from the body if specimen is over-fixed (too much time in formalin solution)
	Branchiopoda, Ostracoda, Copepoda, Cumacea, Tanaidacea, Amphipoda Isopoda	$MgCl_2$ isotonic to sea water (approx. 7%)	4-10% phosphate buffered formalin in sea water or 70% ETOH (Ostracoda, Cumacea)	------	70% ETOH	
	Mictacea	$MgCl_2$ isotonic to sea water (approx. 7%)	4-10% phosphate buffered formalin in sea water or 70% ETOH	------	70% ETOH	
	Mysidacea	$MgCl_2$ isotonic to sea water (approx. 7%)	2-4% phosphate buffered formalin	------	2% phosphate buffered formalin or 70-80% ETOH	
	Pycnogonida	$MgCl_2$ isotonic to sea water (approx. 7%) or 70% ETOH dropwise	10% phosphate buffered formalin	70% ETOH	70% ETOH	
Echinodermata	Crinoidea	$MgCl_2$ isotonic to sea water (approx. 7%)	90% ETOH (hold arms downward)	------	70% ETOH	prolonged contact with formalin destroys the echinoderm skeleton
	Holothuroidea, Asteroidea, Echinoidea	$MgCl_2$ isotonic to sea water (approx. 7%)	70-75% ETOH	------	70% ETOH	prolonged contact with formalin destroys the echinoderm skeleton
	Ophiuroidea	$MgCl_2$ isotonic to sea water (approx. 7%)	70-75% ETOH	------	70% ETOH	prolonged contact with formalin destroys the echinoderm skeleton
Mollusca	Bivalvia	$MgCl_2$ isotonic to sea water (approx. 7%)	10% phosphate buffered formalin or 70% ETOH	30%, 50%, 70% ETOH	70% ETOH	
	Gastropoda	$MgCl_2$ isotonic to sea water (approx. 7%)	10% phosphate buffered formalin	30%, 50%, 70% ETOH	70% ETOH	
	Monoplacophora/ Polyplacophora	$MgCl_2$ isotonic to sea water (approx. 7%)	10% phosphate buffered formalin	30%, 50%, 70% ETOH	70% ETOH	
Nematoda	Nematoda	------	5% formalin-sea water	------	5% phosphate buffered formalin	Avoid using ETOH

follows

(*follows*) Tab. 5

Nematomorpha	Nematomorpha (Nectonema sp.)	$MgCl_2$ isotonic to sea water (approx. 7%)	4-10% phosphate buffered formalin	------	3-5% phosphate buffered formalin or 70% ETOH	
Nemertea	Nemertea	$MgCl_2$ isotonic to sea water (approx. 7%)	5-10% phosphate buffered formalin	------	5-10% phosphate buffered formalin	
Platyhelminthes	Turbellaria	10% phosphate buffered formalin in sea water and freeze in flat container. Float or coax specimen onto filter paper and place latter on frozen mix.		70% ETOH	70% ETOH	
Porifera	Porifera	------	10% formalin-sea water buffered by methenamine	70-80% ETOH change twice	70-80% ETOH	Long-term storage in formalin will macerate tissue and impede ID
Priapulida	Priapulida	$MgCl_2$ isotonic to sea water (approx. 7%)	5-10% phosphate buffered formalin	------	5% phosphate buffered formalin or 70% ETOH	
Sipuncula	Sipuncula	rock dwelling specimens in 10% ETOH & sand dwelling ones in $MgCl_2$ isotonic to sea water (approx. 7%)	10% formalin in 70% ETOH or 5% formalin	70% ETOH	70% ETOH	
Urochordata	Ascidiacea	$MgCl_2$ isotonic to sea water (approx. 7%)	10% phosphate buffered formalin	------	70% ETOH	

preliminary selection using a lens, fine selection with a stereomicroscope, separation of the specimens belonging to the different higher taxa.

Although sorting of fresh material is more advantageous, it is usually difficult to perform and is therefore carried out on preserved material after rising away the fixing solution in a smaller mesh sieve than the mesh size generally used for sieving.

It is very important that sorting procedures are performed under table fume hoods to prevent the release of toxic fumes, especially during removal of the fixing solution. This should be done by pouring it into a suitably sized beaker (or into a plastic tank) through a sieve with a smaller sized mesh than that used for sieving the "fresh" sample (e.g. 250 μm). The sieve should be placed over a wide-necked funnel that is firmly supported by the beaker. As an alternative to the sieve-funnel combination, a funnel equipped with a mesh may be used. The container must then be rinsed well with a spray nozzle so that not even the slightest trace of the sample is left; the rinsing water itself must also be sieved. After inspecting and, if necessary, washing the sieve again, the container with the preservative solution must be emptied into the appropriate laboratory (recycling) container that is kept for used preservative.

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- During solution changes and storage of the solutions in the special containers, it is essential to avoid mixing them.

If the sample contains shells (or shell fragments) or coarse detritus, these must be stored in a different container, rinsing every valve, seaweed, leaf or detritus with a spray nozzle. The following procedures are then recommended: pour the content of the sieve into a 3- or 5-liter beaker and fill it three-quarters full of water; carefully inspect the sieve; suspend the sample in the beaker again, stirring the water; allow sand and coarse material to settle for about ten seconds; pour the supernatant material floating on the surface into the sieve. This sequence can be repeated several times until there are no further organisms or detritus left in the supernatant. At this stage, the material left at the bottom of the container must be carefully inspected in order to recover any organisms present.

An alternative to this technique, useful mainly for coarse sediments (coarse or bioclastic sand, gravels), consists in the preparation of a dense solution of water and sugar (1 kg of sugar diluted in 1 l of fresh water), to operate a more efficient separation between organism of low density (e.g., polychaetes, small crustaceans) which float on the surface of the solution, and those heavier (shelled molluscs, echinoderms, organic detritus) which remain on the bottom of the container. Only small quantities (3-4 cooking spoons) of substrate are processed each time, taking care to rinse the residual, collected in the sieve, from the sugar solution. Also in this case the material left on the bottom of the container must be carefully inspected to remove possible organisms present in the residual sediment.

Sometimes it is useful to divide the sample into sub-samples to be sorted in sequence, preferably by the same operator. For this reason, it advisable to immerse the sieve containing the sample in a small tank full of water, making it "float" so as to evenly distribute the material across the mesh. The material may then be divided into a different number of parts, of roughly equal size. It is generally preferable to divide it into quarters; the parts thus obtained can be placed into separate containers containing 70% Ethanol, labeled like the original sample with the percentage of the total sample recorded, together with a 'split number' (1/4 "one out of four", 2/4, 3/4, 4/4).

The material that is to be sorted should be placed in a small white plastic or enameled tank. An initial examination can then be carried out with the naked eye or with the aid of a bench lens. Tanks made of transparent material may also be used. The advantage of these is that a black, white (or colored) background can be placed under their base, increasing as desired the contrast with the animals to be identified. The lens body must be connected to a stable moveable articulated arm with an integrated internal cable.

The lens should be fitted with a round white neon (approx. 20 W) light-bulb to ensure optimal illumination of the field of vision. The lens must be aspheric, have a diameter of about 20 cm and approximately 2-3× magnification to permit a working distance: object-lens and lens-eye of about 20-25 cm.

Large organisms thus identified must be placed in the appropriate containers after making sure that no other smaller specimen is attached to their surface.

The subsequent sorting phase is performed with a stereoscopic dissection microscope. For this purpose, a small quantity of the sample is placed in an 8-10 cm glass Petri dish with sufficient water to cover it; it is then evenly distributed and examined to identify the organisms. To aid this procedure the Petri dish may be prepared by dividing it graphically into sections, tracing for example some parallel lines spaced one centimeter apart on the exterior of the base. Examination of the sample is performed by means of a methodical scansion of the area within the parallel lines using a 5-10× magnifying lens (or even stronger if necessary). It is advisable to

use a 40-cm optic fiber illuminator (cold light source with 20-W halogen light bulb, complete with light conductor).

During this initial sorting phase the organisms are separated according to their main taxonomic groups, usually in the following easily identifiable categories: polychaetes, other worms (oligochaetes, nematodes, nemertines…), bivalves, gastropods, amphipods, other crustaceans, insects (present in brackish environments), cnidarians, porifers and other animals (a.c. = *animalia cetera*). However, the groups most representative of soft bottom macrofauna are usually the polychaetes and bivalves.

As containers, roughly 20-ml glass or High Density Polythene Cylindrical vials of the type used in liquid scintillation with watertight screw plugs can be used. Alternatively, the most suitable containers for the specific study may be chosen, provided that they are watertight.

For cross checking, the animals can be counted even in this first phase: every time an animal is placed in the container the corresponding counter is updated, suitably labeled, or a bar is drawn on a special record card.

Containers are labeled by placing a waterproof paper label inside them. The best type of paper is glossy transparency paper, on which the information must be written in Indian ink (left to dry) or in pencil. At least the following data must be recorded on the label: sample code; operator's initials; sorting date; sampling date; systematic group; any split number where samples are stored in several containers (e.g. 1/3).

It is advisable to take a note of the time taken to sort the sample, of the sorting date, and of all other useful information, on a special filing card. The remaining material, consisting of the sample with no animals, is stored and suitably disposed of.

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- Quality control in sorting consists of checking that the animals have been satisfactorily extracted from the detritus. Extraction of more than 95% of the animals is considered satisfactory. When the percent of effectiveness (PE) is between 90 and 95%, technical staff should receive further training.
- A minimum of 10% of the samples sorted by each operator should be inspected for quality control during a project, and results recorded in a special notebook.
- Quality control in sorting is performed as follows:
  1. All the samples already sorted by one operator are gathered into groups of 10 samples.
  2. One sample is taken from each group.
  3. The detritus is sorted again by a second skilled operator who did not assist with the sorting.
  4. Individuals that were still present in the detritus are counted.
  5. The following simple equation is applied: [number of animals found in the first sorting / (number of animals found in the first sorting + number of animals found in the detritus)] × 100. That is to say, the percentage of animals found during the first sorting out of the total of animals found in the two sortings.
  6. If the percentage of effectiveness is less than 90%, a recount must be performed on all the samples in the group of 10 from which the examined sample comes.

Once the Quality Control has been performed, the detritus may be disposed of. Appendix 4.1. shows a list of instruments and materials necessary for the disposal process.

4.4.3 Taxonomic determination and quantification

Once sorted, the animals are identified up to the required taxonomic level of the study. If the specimens are to be identified at the lowest possible taxonomic level (LPT= *Lowest Possible Taxon*), this level will depend on the organisms' maturity and on the state of conservation. LPT almost always corresponds to the species level.

S&Q

- Quality control at this phase consists in comparison with a Reference Collection and the re-identification of 10% of the samples by a second identifier (analogously to the procedure for sorting).

4.4.3.1. Reference collection

Each laboratory should organize its own "Reference Collection". This collection is composed of various "Reference Series", i.e. a certain number of specimens that refer to a single taxon in sufficient quantity to include the morphological variability presented by that taxon in all or most of the habitats under study.

If the laboratory is of local interest, the reference series may refer to the geographic area concerned, without necessarily recording the morphological variations on a regional scale.

The reference series must be prepared, even if it is not possible to put a name to the species, by labeling it simply as "species A" or "species B". Taxa identified at higher than species level, for example genus or family, may also form part of the collection provided they are suitably labeled. Wherever possible (or appropriate), juvenile and adult forms of the same species of both sexes as well as ovigerous females should be included in the reference series. It is advisable to label reference series specimens adequately, indicating the family to which the species belongs, genus, author, collector (with date and place of collection), identifier, and any inspector, as well as the Checklist reference code of the species of Italian fauna (Minelli *et al.*, 1995). The set of reference series forms the 'reference collection'.

It is recommended that a reference collection be prepared at the start of every study by those who will later be in charge of identifying the taxa. In this initial phase the identifiers should work in conjunction with experts.

The reference collections pertaining to each study should be added to the laboratory reference collection. Each collection must have its own file where all the characteristics of the specimens and the history of the collection itself are recorded

To organize species, names, synonyms, and their numeric codes correctly, it is strongly recommended that the Checklist of species of Italian fauna is used. This contains the complete list of all species of Italian fauna updated to 1995, together with additional information including their geographical distribution by macro-area. The Checklist has been compiled by specialists in each taxonomic group and is composed of 110 pocket-sized booklets (Minelli *et al.*, 1995).

4.4.3.2 Instruments and devices useful for specimen identification

Stereomicroscope (zoom 1-7×); compound microscope preferably fitted with phase contrast (zooms 4, 10, 20, 40, 100×); optic fiber illuminator; fume hoods on work stations; dissection set; fine Dumont 5 stainless steel tweezers; large tweezers; flat tweezers; handled needles; fine-pointed scissors; scalpels; small approx. 10-cm diameter 250-μm sieves; plastic or stainless steel trays (approx. 20 × 15 × 3 cm); spray

nozzles for alcohol and water; permanent marker pens; pencils/Indian ink pens; 5- and 10-cm Petri dishes; various 5- and 10-ml glass or plastic test tubes with watertight lid; scintillation vial with cap; Eppendorf test tubes; 1.5-ml vials with cap; labels.

Dichotomic analytical keys are usually used for organism identification. This is performed by answering a series of questions either positively or negatively (that is dichotomously), observing and identifying their features, leading gradually, where possible, to the identification of the species. Dichotomic keys are usually accompanied by detailed illustrations to make identification easier. During a study, all specimens should be identified at the same Lowest Practical Taxonomic Level (LPTL).

4.4.3.3. Determination procedures

Taxonomic identification should be performed using a dissecting stereomicroscope together with a compound microscope when observation of fine details is required.

To observe an anatomical detail with the compound microscope, put a few drops of water or, better, of a glycerine/ethanol solution (1:1) on a microscope slide, dip the animal, or the part of it that it is to be examined, into the solution and finally cover it with a cover slip. To avoid the formation of bubbles, the cover slip should be placed at a slanting angle on one side and then gently lowered onto the specimen. If the organism examined was not colored at the time of fixation it can be colored prior to observation in order to increase contrast in the structures to be analyzed (see paragraph 4.3.3.4).

All the animals present in a sample must be counted; division into sub-samples is not recommended even when large numbers of individuals are also present.

When damaged organisms are present, only cephalic parts (anterior fragments) can be taken into consideration for counting purposes. When animals with shells (e.g. bivalve molluscs or gastropods) or animals living in calcareous tubes (e.g. some sedentary polychaetes) are present, it is necessary to prove the presence of soft parts.

When samples come from a zone that is familiar to the identifier, it is best to identify specimens completely, to the lowest taxonomic level. In contrast, when samples come from an unknown area, it is best to proceed with caution (e. g. carefully identifying all specimens first to family level, then to genus level and finally to species level). For correct identification, the most recent analytical keys for the geographic region from which the samples derive should be used.

S&Q

- Great care should be taken to ensure correct use of the dichotomic keys, to avert the risk of severe mistakes. The risk is particularly high when it becomes necessary to use keys pertaining to areas beyond the Mediterranean (which are in effect the only keys available for certain taxonomic groups). For example, although species lists are generally used for predominantly ecological purposes, they are invariably used in biogeographic and systematic work since they are often the only data available. Since at ecological level it is the species that supplies the most detailed clues, the importance of the training of staff specialized in the organism identification phase should be emphasized.

Organisms that belong to the same species must be counted by recording their number using a mechanical counter (clicker) or by marking, with a tick, a counting list prepared at the start of the identification process. These organisms must then be stored together in a test tube containing the preservative solution. The specimens that

will form part of the project reference collection should be counted and their removal must be indicated in a note on the datasheet. At the end of the identification of each container, both the Petri dishes and the strainers should be carefully examined and then washed. Once all the datasheets have been completed, they should be handed to the head of the laboratory who will check that all the groups have been examined. All the datasheets of the systematic groups and of the replications referring to a sample should be stapled together and then transferred to an electronic spreadsheet. It is vital that the datasheets are promptly transferred to electronic support. This should be performed by two people: the reader who must dictate the content of the datasheets (it is recommended that this person is also the identifier) and the compiler who inserts the data into a preset matrix of a spreadsheet. As soon as the compiler has written the data he/she must repeat the dictated data aloud. Two (or more) photocopies of the completed datasheets must be made and two copies must also be printed of each electronic data sheet; in addition, two computer back-up copies must be made. The copies must be kept in suitable ring or flat-bag folders and stored in separate places.

The head of the laboratory or a person specially appointed by him or her is responsible for keeping the originals and the copies.

S&Q

- The reference collections form an important stage in quality control: periodically some samples are to be sent for identification to the taxonomic experts of the group, or, preferably, the laboratory identifiers themselves must take part of the reference collection to the experts and carry out the task of identification jointly with them.
- If corrections need to be made to the identification of specimens belonging to the reference collection, such alterations must inevitably also be reflected in the identification of specimens not belonging to the collection.
- Quality control during identification consists in checking that specimens have been counted and identified correctly.
- A minimum of 10% of the samples identified by each operator should be examined for quality control during a project and the results recorded in a special notebook.
- Two sorts of error are common during identification:
  a) counting errors: thus counting 34 *Hediste diversicolor* instead of 30 is the equivalent to making 4 errors;
  b) identification errors: for example, the identification of a specimen of *Hediste diversicolor* as *Neanthes succinea* is an error even if the final count is not changed. This type of error may correspond to various degrees of severity depending on the taxonomic level where the error has been made and, above all, depending on the differences between the ecological requirements of the wrongly identified species.
- Quality control during identification is performed in a similar manner to that applied for sorting: samples already sorted by a given operator are divided into groups of 10 samples (ideally the 10 samples of each group should come from the same environment).
  1. A sample is taken from each group of 10.
  2. The sample is identified by a second skilled operator who did not contribute to the identification of that sample.
  3. Errors are counted as explained above, summing the two types of error. It may be assumed that the identifiers have received good technical training, and therefore

the different severity of the taxonomic identification errors may be disregarded.

4. The percentage of errors is calculated by applying the following simple equation: [total number of errors/(total number of individuals in re-identification)] × 100, i.e., the percentage of errors per individual.
5. If the percentage of errors exceeds 10%, all the samples of the group of 10 from which the examined sample was taken must be checked again. If the error percentage ranges between 10 and 15%, the technical staff must receive further training.
6. Whatever the error percentage, errors must always be recorded in the appropriate notebook and then communicated to the taxonomic identifier who made them and to the laboratory head.
7. All variations both in counting and identification must be recorded on the sample datasheet by marking the incorrect data with a single red line and indicating the correct data in the interlinear space above it, again using a red pen. In addition, the date and initials of the operator who made the corrections must be inserted. This should be the same person who performed the recount and re-identification during the Quality Control.

- Identification standardization is achieved as follows:
  a) by comparison of the identifications with laboratory reference collections;
  b) by making periodic visits to museums and/or comparing the laboratory identifications with those of experts;
  c) by attending training courses if necessary.

4.4.4 Biomass measurement

The biomass is the weight of the living matter. The weight of an organism is given by the sum of the weight of its soft body parts, plus the mineralized structures. The water present in the organism is highly variable and is therefore excluded from the estimate of the biomass through the calculation of the dry weight. The metabolically inactive mineralized parts can exert considerable influence on the weight of an organism and should therefore also be subtracted from the organism weight.

The most practical method for obtaining an estimate of the metabolically active biomass is therefore the calculation of the Ash-Free-Dry-Weight (AFDW), which is the value of the dry weight from which the ashes are subtracted.

To determine the biomass, organisms are therefore weighed after drying at 80 °C for 24-48 h when reaching the constant weight; higher temperatures can lead to a significant loss of the most volatile compounds of the organic matter.

Organisms are then ignited in a muffle furnace at 450 °C for 4-5 hours to estimate ash content. Higher temperature can lead to a significant carbonate loss. AFDW is calculated including shells. Measurements should be performed using an analytical balance with an accuracy of 0.1 mg.

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