National Marine Biological Analytical Quality Control Scheme

Guidelines for processing marine macrobenthic invertebrate samples: a Processing Requirements Protocol

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SUMMARY

Processing requirements are described for marine macrobenthic samples. They are divided into guidelines for sample management, sample processing, blotted dry biomass assessment, and subsampling. Of these, the basic management/processing guideline forms the basis of best practice for all marine macrobenthic samples. It is reduced to a Processing Requirements Protocol (PRP), detailing those aspects that are required, without recommending specific methods. It also describes and discusses some of the issues inherent to sample processing.

Also included is a summary overview of a taxonomic discrimination protocol (TDP). The final TDP, which will detail treatment required for all taxa, will be in database format. An example for Oligochaeta is included here.

INTRODUCTION

This report introduces **standard guidelines for marine macrobenthic sample management and processing.** The purpose is to assist fieldworkers, commissioning organisations, and laboratory operatives with the management, tracking and processing of samples from the point of collection through delivery to the laboratory, sample analysis, quality assurance and the production and archiving of data products with the aim of producing comparable data.

There is currently no publication that provides processing requirements for macrobenthic invertebrate samples in sufficient detail for confident data comparability between laboratories. Monitoring handbooks (Holme & McIntyre, 1984, Baker & Wolff, 1987 Davies *et al*., 2001) give only very broad specifications for macrobenthic surveys including short notes on laboratory methods within guidelines for sampling. More guidance for processing macrobenthic samples is available in Rees *et al*. (1990) and Rumohr (1990). The Proceedings of the Humber Benthic Field Methods Workshop (Proudfoot *et al*., 2003) includes a review of laboratory subsampling and biomass measurement but little on laboratory processing. The UK Clean Seas Environment Monitoring Programme (CSEMP - formerly the National Marine Monitoring Programme or NMMP) presents advice for macrobenthic samples in the CSEMP Green Book, Appendix 10 (Cefas, 2009), but includes only a single paragraph on sample processing and a few paragraphs on biomass measurement. The International Standard 16665:2005 (EN ISO, 2005) offers the most comprehensive overview to date of the requirements for processing macrobenthic samples.

However, the specifications found in these documents can still be interpreted differently by different laboratories to the point of compromising direct comparison between data from different sources. Reviews of laboratory methods for the NMBAQC Scheme (Worsfold & Hall, 2001; Hall & Worsfold, 2002, Cooper & Rees, 2002) have shown significant differences in basic practices between laboratories. Working methods and skills vary widely between laboratories and they identify taxa to varying levels of accuracy. There may also be variation between staff at one laboratory, though some standardise through constant communication or in-house protocols.

Macrobenthic sample analysis is subject to many errors. The most significant relate to inadequate extraction of biological material from the sediment. Extraction errors are also impossible to correct where there has been discard of residues. Identification and enumeration discrepancies are also important sources of error and differences in extraction and recording policy may compound all errors

Data comparability is currently best achieved by use of a single analyst (impractical for national projects), through continuous comparison between analysts / laboratories, or through extensive data truncation (subject to inaccuracies and resulting in loss of information).

The aim here is to produce a Processing Requirements Protocol (PRP): a detailed standard document that outlines requirements for macrobenthic sample management and processing, from the point of collection to the final storage of data and sample material. The PRP is intended to augment the International Standard with the provision of more clarity on the detail of processing specifications; the TDP will supersede the International Standard in terms of taxon recording policy. Throughout the document a distinction has been made between actions that that are **imperative** and **must** be undertaken and those less stringent

requirements that are **recommended** as good practice and **should** be undertaken. The purpose of the PRP is to provide clear, unambiguous, and comprehensive instructions to facilitate the efficient management and processing of samples and the consistent production of good quality data which are comparable between different laboratories at a national level. The processes can be divided into different tasks which might potentially be completed by different organisations or laboratories. It is essential that the whole protocol is effectively managed to ensure the integrity of sampling and analytical information and the PRP includes guidelines on management aspects along with detailed laboratory procedural requirements. **For each task the key procedural requirements are outlined within text boxes; imperative actions that must be undertaken appear in red text**.

Although the PRP is intended as a standard document, it is likely that some details of the guidelines will be subject to review by the NMBAQC committee. The committee would be grateful for notice of any text requiring further specification or clarification. Each laboratory will have its own detailed Standard Operating Procedure (SOP) outlining exactly how procedures are carried at that specific lab. These SOPs may vary from lab to lab but provided that the different SOPs include the key sample processing requirements from this PRP then they should produce comparable results.

The implementation of appropriate health and safety requirements (*e.g.* CoSHH assessments for preservatives and other reagents) is an essential part of laboratory management. However, health and safety issues are not included in this PRP as they do not constitute processing requirements.

A Taxonomic Discrimination Protocol (TDP) is under development, alongside the PRP. It will detail how different taxa should be quantified and recorded and the taxonomic level at which they should be identified. The aim is to standardise and improve taxonomic resolution wherever possible. Taxonomic workshops and improved taxonomic literature may allow more precise identifications in future. An overview TDP and the TDP for Oligochaeta are included with this document (Appendices 3 and 4).

Abbreviations:

CMA	Competent Monitoring Authority
CoSHH	Control of Substances Hazardous to Health
CSEMP	Clean Seas Environmental Monitoring Programme
DGN	Dangerous Goods Note
H & S	Health and Safety
IDA	Industrial Denatured Alcohol, formerly Industrial Methylated Spirit (IMS)
LPM	Laboratory Project Manager
NMBAQC	National Marine Biological Analytical Quality Control
PCM	Primary Contract Manager
PRP	Processing Requirements Protocol
PSA	Particle Size Analysis
QA	Quality Assurance
QC	Quality Control
SDF	Sample Data Form
SPF	Sample Progress Form
SOP	Standard Operating Procedure
SPF	Sample Progress Form
TDP	Taxonomic Discrimination Protocol
TREM	Transport Emergency

PROCEDURAL GUIDELINE A: Management and Processing of Marine Macrobenthic Samples

A.1 Logistics

The manager of the project, Primary Contract Manager (PCM), must oversee the transfer of samples from the field to the analysing laboratory and provide clear instructions to all involved. They are then responsible for the ultimate fate of both data and samples and external quality control.

Sample analysis is typically conducted at a laboratory distant from the survey location, often by a different organisation from that which completed the survey.

A.2 Equipment

An efficient office system is necessary for maintenance of both paper and electronic records. Robust packaging is required for sample transport, particularly for postal dispatch. Vented premises are required for storage.

The sample analyst must have access to a laboratory equipped with washroom facilities and fume cupboard, along with desk space and microscopes of both compound and stereo types, with a range of magnifications e.g. x 10 to x 1000. Other equipment required includes:

- A range of certified standard mesh sieves: *e.g.* 0.5mm (1φ), 1mm (0φ), 2mm (-1φ), 4mm (-2φ), 32mm (-5φ) to separate sample fractions.
- Trays and dishes for sorting.
- Scraping knives and forceps of different sizes and coarseness.
- A range of watertight containers of different sizes for containment of samples and extracted fauna and appropriate alcohol resistant labels.
- Supplies of fixatives and preservatives (formaldehyde solution and Industrial Denatured Alcohol, IDA) must be available.

The premises must be equipped with comprehensive collections of both identification literature and reference specimens.

A.3 Personnel

The staff should include experienced personnel trained in sample management, sample processing and specimen identification, to cover all taxonomic groups encountered in the samples processed. There should be enough fully trained staff to provide adequate supervision for less experienced staff.

A.4 Sample collection

Detailed guidelines for sample collection are not provided here. Some guidelines are available in the CSEMP Green Book (Cefas, 2009). A review of best practice for field procedures for collecting macrofaunal samples was undertaken by Proudfoot *et al*. (2003). However an outline of some of the issues relating to sample collection is presented here as sample treatment during fieldwork may affect subsequent sample processing and quality.

Samples are commonly commissioned, collected and processed by different organisations. It is essential that a clear line of communication and responsibility is maintained throughout.

A single Primary Contract Manager (PCM) from the commissioning organisation should know who is responsible for each stage of the process.

The commissioning organisation PCM must give clear instructions to surveyors regarding procedures for sample collection including collation of field notes and field processing (e.g. sieving and preserving). **The entire sample is important.** It is not uncommon for surveyors to assume that only sediment is important and to discard stones or large animals. Many sources of confusion come from past traditions and terminology. Macrobenthic samples may be described as 'macrofaunal' or even 'infaunal' samples. The terms carry an implication that plants and 'epifauna' can be ignored and there may have been justifications raised for ignoring these components. One reason that all biota should be considered is that all are relevant to the nature of the habitat and biotope definitions. Another is that there are no firm distinctions between concepts such as 'infauna' and 'epifauna'. **Some surveyors are unaware that many animals move to the surface of water collected with the sample and that they will be lost if water is spilt over the side of containers.**

Preservation methods vary and should be clearly specified. Surveyors should also be aware that samples must be preserved quickly, especially in hot weather and that preservative must be thoroughly mixed into each sample. Surveyors must also remember that a 4% solution added to a container over half full with sediment and trapped seawater will no longer be 4%. **Inadequately preserved samples will impact on the physical quality of the preserved fauna and may render it difficult, or impossible, to identify.**

Many organisations routinely add a stain, such as Rose Bengal, to samples during preservation. Alternatively stain may be added later in laboratory prior to sample sorting (extraction). The PCM should specify whether this is necessary or acceptable and consider the requirements of the analysing laboratory, if possible. Some laboratories consider staining to be useful as an aid to extraction. This may be especially so if large volumes of residue require to be sorted. However others see stain as an impediment to identification as it may obscure diagnostic pigmentation patterns which are retained in some fauna. **Excess Rose Bengal leaches into alcohol during identification and obscures visibility; it cannot be removed from specimens reducing their value as reference material.**

Sample collection (PCM responsibilities)
A single Primary Contract Manager (PCM) from the commissioning organisation should know who is responsible for each stage of the process.
Clear instructions must be provided to surveyors to ensure that sample treatment during fieldwork is appropriate and that all required field notes are recorded.
The PCM must ensure that instructions to the fieldwork team are consistent with the requirements of laboratory analysis (*e.g.* with respect to sieving, retention of all material, preservative and staining).
The PCM must take responsibility for ensuring that all subcontractors receive the samples and all relevant information (*e.g.* details from field log) and are aware of the protocols to follow.
Each sample must be in a clearly labelled watertight container (or group of containers clearly identified as representing a single sample). It should be complete (with no loss of material coarser than the required mesh prior to containment) and adequately preserved.

A.5 Transport of samples

Samples are often inadequately packaged and leak chemicals en route. In many instances the legal requirements for labelling are not followed.

Transport of samples
The PCM should ensure that all samples arrive at the analysing laboratory in good condition.
Samples must be transported in fully watertight containers, with at least one other watertight layer surrounding each or all samples.
Containers must be robust and well insulated against damage.
All hazardous substances must be clearly labelled in accordance with the law and with the regulations of the carrier and requirements of all personnel who handle the package.
The PCM should also ensure that the analysing laboratory confirms receipt of the samples, with details of their condition.

A.6 Sample tracking

Samples often arrive at analysing laboratories without documentation or clear instructions. It is important that the origins of samples are clear and that all parties know basic details such as the number of samples to be processed and where they are or should be at any point in time.

Clear labelling is an obvious issue but links to other survey information (*e.g.* PSA data or field photos) are equally important. They are usually not all passed on to the analysing laboratory, either for reasons of confidentiality or difficulty of compilation. It may be considered that only the commissioning organisation needs this information but samples are generally retained at the analysing laboratory and their value may be lost to the future if information links are broken, so it is recommended that all data from field logs is passed on, where possible.

Sample tracking
The PCM must ensure that they obtain a comprehensive list of samples and sampling details from the organisation responsible for fieldwork.
They should produce an electronic document (*e.g.* a spreadsheet) with links to the following information for each sample and provide as much of this as possible (without breach of confidentiality) to the analysing laboratory: • station and sample code, • visual description of sample, • sampling position (with coordinate type and projection specified), • sampling depth (corrected to chart datum), • sampling date and time, • organisation, individuals and vessel involved in sampling (as appropriate), • sampling equipment (including surface area sampled), • details of all treatment of the sample post-collection (*e.g.* field sieving, with mesh, any material removed before preservation, preservative and any other additives used), • details of all other samples or data collected at the same sites or during the same survey (*e.g.* PSA, chemistry, photography, sonar, bathymetry).

Further to the information above the data guidelines for "Sediment sampling by grab or core for benthos" provided by the Marine Environmental Data and Information Network (MEDIN, 2009) should be adhered to by surveyors and laboratory analysts to ensure that all necessary information is collected.

A.7 Instructions for the analysing laboratory

For many years, commissioning organisations have believed that reference to a short line on processing requirements stating, for example 'identify to species where possible' will produce comparable data. In fact, every laboratory interprets such instructions differently and effectively follows its own standard practice. Many routinely ignore certain taxa by tradition without comment, or discard material without note. **Prescriptive instructions are essential and those included here should help, alongside the TDP.**

Instructions to the analysing laboratory
The PCM is responsible for ensuring that a suitable analysing laboratory is chosen and for providing them with all processing requirements. The PCM must communicate all relevant information to the Laboratory Project Manager (LPM).
Details of basic requirement options and basic survey information required by an analysing laboratory are summarised in the Sample PRP checklist in Appendix 1. It is recommended that the PCM complete a form of basic details (such as the Sample PRP checklist) and send it to the analysing laboratory along with the sample list and copies of the relevant processing guidelines.
The PCM must ensure that the analysing laboratory has an appropriate SOP in place, including Internal QA methods. The lab's SOP must be available for inspection and the laboratory must adhere to it.
The PCM must confirm that the analysing laboratory participates in an appropriate external QA scheme and must coordinate submission of relevant samples and data for any external QC exercises.
Samples, including extracted fauna and sorted residues must be retained at least until all internal and external QC is completed.
Disposal of samples and specimens is not recommended; if deemed necessary, they should first be offered to other agencies/organisations with an interest in marine biodiversity (*e.g.* universities or museums.)

A.8 Sample Processing

Processing time for macrobenthic samples may vary widely, depending upon mesh size required, sample type/size, sediment type and location of sampling point. All of these factors ultimately affect the richness of the sample, which, in turn affects time required for processing. Both quantity and diversity of benthos affect processing times, as does the difficulty of extraction from certain substratum types. Processing times vary from 30 minutes to five days per sample. Biomass assessment at species level usually adds about 10% to time costs for sample analysis.

The actual time for completion of a group of samples will depend upon the laboratory's existing workload. A backlog of several months for non-urgent samples is common. Laboratories should assess a subset of samples to gauge their difficulty in order to estimate the completion time for sample sets.

Sample Processing (Laboratory Project Manager responsibilities)
The analysing laboratory should appoint a Laboratory Project Manager (LPM) to assume responsibility for the conduct of the macrobenthic analysis.
The contact details of the LPM should be provided to the PCM.
The LPM should appoint a team to conduct sample analysis, in-house quality control and data management for the project.
The LPM must ensure that all procedures are documented.
All personnel involved in the process should be named and their work detailed by initials.
All documentation should be retained indefinitely and made available to the PCM, as necessary.
The analysing laboratory may work to its own standard operating procedure (SOP) but this must be compliant with the current NMBAQC PRP, be approved (by the PCM) and be made available on request for consultation by other organisations and for reference in reports.
Any procedures that differ significantly from the NMBAQC PRP must be agreed with the PCM before proceeding and documented.
The LPM should prioritise samples to meet deadlines for external quality assurance schemes and data submissions to national databases.

A.9 Sample logging

Sample logging
The analysing laboratory should check all sample containers for signs of external damage or leaks and report any to the PCM.
They should check the external labels against the sample list sent by the client laboratory, report any discrepancies and return an annotated list to the PCM for confirmation prior to sample analysis.
The analysing laboratory should use the list as the basis for a Sample Progress Form (SPF), which should be available in hard copy, retained indefinitely, and contain a log of all processes carried out on each sample.
Each sample must also have its own Sample Data Form (SDF), which should be available in hard copy, retained indefinitely, and include all information from the SPF.

A.10 Sample washing and sieving

The basic requirements for sieving samples in the laboratory are to wash the sample on a standard mesh to remove fixative and ensure that no material is lost over the sides of sieves. Sample washing should take place in a ventilated area. Some suggestions are made on how to divide a sample for extraction of biota but the details would belong to an SOP, rather than the PRP. An example SOP flow diagram for washing and sieving and extraction is provided in Appendix 2.

Some laboratories routinely treat samples with a stain, such as Rose Bengal, as an aid to sorting. There is no evidence to suggest stained samples are more accurately picked than unstained ones but it may increase efficiency (*i.e.* reduce sorting time) if large volumes of residue are to be processed. However analysts should be aware that stain may give a false sense of security that only stained material need be searched for and extracted, whereas many animals, such as mollusc and crustacean shells, do not readily stain and may be easily missed by an analyst in a stain mindset. Stain may also make identification of same taxa more difficult as it obscures pigment patterns. **Hence the use of staining should be regarded as optional, rather than a necessary requirement of sample processing, provided that each laboratory ensures all other measures for accurate extraction are in place.**

The gauge of sieve mesh used for marine macrobenthic surveys varies between sampling programmes. Both 0.5mm and 1mm are widely used. The 0.5 mm sieves are most frequently used in estuarine (transitional) waters and also for offshore oilfield samples, while 1 mm has generally been used for coastal waters. Conservation Agencies tend to use a 0.5 mm mesh for mapping and monitoring coastal waters under the Habitats Directive. For CSEMP and WFD there are specific sieve size requirements which differ between transitional and coastal waters (0.5mm and 1.0mm respectively).
The result of different mesh usage has been poor comparability between data from certain areas and artificial distinctions between sites that have been treated differently in different areas. One solution has been to record data at both mesh sizes but this is the most time-consuming option. This could involve stacking 0.5mm and 1 mm sieves for processing live samples in the field or field collection at 0.5mm and subsequent separation of the 1mm fraction of a fixed sample in the lab. These alternatives may produce different data as some live fauna may actively pass through the 1mm mesh whereas the same fauna, fixed and immobile, may be retained on the same 1mm sieve. Also, field sieving may not be complete and more may pass through with more rigorous sieving in the lab.

It would be useful in the long term, and for new initiatives, if only one mesh were standard but that would always cause problems for comparing with past data at a different mesh.

Sample washing and sieving
For each sample, the individual who carried out the initial sieving should record their name on the SPF.
Appropriate health and safety procedures should be in place for processing samples.
All sieves used for laboratory processing must be certified by the manufacturer and calibrated. Sieves with damaged or distorted mesh must not be used.
For each sample, sieves must be cleaned thoroughly before use to avoid contamination from previous samples.
Note should be made of the appearance or composition of the sample prior to washing/sieving, as features may be seen that would be missed by particle size analysis.
Inform PCM if the sample appears to have been poorly preserved (*e.g.* decomposing fauna and odours).
Decant preservative/fixative over a 250um sieve for recycling/disposal and return retained residue to sample.
Each sample must be sieved over an appropriate square mesh as specified in the initial instructions; the mesh size must be quoted in all documentation that relate to the sample.
Where samples have been pre-sieved in the field, they must be re-sieved in the laboratory at the appropriate mesh size (*i.e.* equal to or larger than mesh size used in field).
If sediment samples arrive from field unsieved and unfixed then they should be washed or sieved with isotonic water as delicate unfixed marine fauna will be damaged (bloated) if exposed to freshwater (tap water).
All material contained within the sample container must be retained until completion of processing unless it passes through the sieve. Notes or photos should be made on the composition and volume of residue after washing.
Samples can be divided into a light and a heavy fraction during sieving. The light fraction ('float') will comprise material that can be poured off the sample after moderate agitation in water.
If coarser sieves are used to subdivide a sample into manageable fractions, they should be placed above either a watertight container or a sieve of the specified mesh size or finer.

All fractions must be clearly labelled at all times.
Samples must be sieved gently at the specified mesh until no particulate material passes through the sieve.
Once washing/sieving is complete the sample should be gently washed into a sorting tray. The sieve mesh must be checked to ensure no fauna is left behind.
Containers that may contain biological material must not be left without adequate preservation for more than 24 hours.

A.11 Sample sorting (extraction)

Sample residues, or portions of residues, should be evenly spread in water in a shallow, flat vessel (*e.g.* white sorting tray for coarse fractions; Petri dish for fine), with good illumination. The residue depth should be sufficient to allow any contained fauna to be visible upon sifting with a spatula or forceps or following gentle agitation of the sample. The residue should be sorted/searched systematically (*e.g.* left to right, in concentric rings) with the aid of forceps or pipettes to extract the fauna. Fine fractions (*e.g.* <2mm) should be sorted with the aid of magnification (*e.g.* illuminated magnifier or using a stereo microscope).

It is best practice for <u>all</u> biological material retained by the sieve that would have been alive at the time of sample collection to be extracted from the sample. This is contrary to the practice of many laboratories, where certain taxa are ignored. The reasons for ignoring taxa usually stem from the idea that only 'infauna' are to be recorded. It is not possible to define 'infauna'. In a mixed substratum sample there will be taxa that live within sediment, some that live on the surface, some that nestle amongst stones, some attached to stones (fixed or motile), some clinging to epibiota and others that move between microhabitats. It makes no sense to ignore any taxon; they are all part of the same community. Similarly, taxa are sometimes ignored because they are considered meiofaunal. The meiofauna/macrofauna distinction is based on size of animals and, during the extraction phase, should be made by the sieve used, not based on taxonomic groups. Proper washing should pass most meiofaunal taxa through the sieve. (Any residual meiofauna should be recorded at the identification stage and, if required, can be removed at a subsequent data truncation stage). Plants and non-countable animals should also be extracted. If any taxon is ignored (not recommended) then this should be clearly stated in all documentation that refers to the sample.

It may be time-consuming to extract everything from samples with large amounts of material, so subsampling and in-situ counts are acceptable, in certain prescribed circumstances. A separate procedural guideline is provided for subsampling.

Sample sorting (extraction)
For each sample, the individual who carried out the sample sorting should record their name on the SPF.
The laboratory SOP must detail quality assurance methods for sorting.
All in-house QC procedures must be documented and the form of documentation approved by the PCM.
All biological material that would have been alive at the time of sample collection should be extracted from the sample, as should all items for which there is doubt as to whether it was alive at the time of sample collection. "If in doubt – pick it out!"
Abundant and easily identifiable taxa are best counted during extraction.
Taxa may be identified more efficiently if first separated into major taxonomic groups.

It will be necessary to break tubes, bored shells and soft rock to extract cryptic fauna.
All biological material must be preserved in industrial denatured alcohol (IDA) (>70%). Glycerol (10%) can be added to the preservative mix to prevent desiccation.
Note should be made of the fixation state of the extracted biota, if inadequate, and passed on to the PCM.
Residues from which all biological material has been removed must be re-preserved and retained until completion of all QC procedures.
Exceptions to the requirements of the above are listed below; **they must be agreed with the PCM** and details documented: • Taxa occurring in very high numbers may be sub-sampled or counted *in situ* (see below), • large volumes of 'float' may be sub-sampled, • residues of fibrous or entangled material (*e.g.* algae, fibrous tubes) containing large numbers of very small organisms may be re-sieved after loosening of the material, • large volumes of coarse substrata may be sub-sampled (see below), • sessile organisms considered to have been small enough to pass through the specified mesh had they been loose may be ignored, • small portions of large or very abundant organisms may be ignored if it is certain that they will have no significant impact on biomass measures, (*e.g.* small fragments of brittle-star legs, or detached tentacles from cirratulid worms), • certain sessile calcareous organisms, such as coralline algae, encrusting bryozoa or barnacles, may be preserved in a dried state, • organisms that clearly represent contamination (*e.g.* insects in offshore samples) may be ignored but should be expressly agreed with the PCM.

A.12 Macrofaunal identification

The requirement for a Taxonomic Discrimination Protocol (TDP) has been born out of varying levels of identification noted between laboratories within the NMBAQC Scheme. This PRP states that the standard requirement for identifying taxa (including 'epibiota') should be to the most accurate taxonomic level practicable, usually species. The aim of the TDP is to standardise identification levels, taxon by taxon. The use of stains or clearing agents is useful for the identification of some taxon groups. This PRP does not include methods for clearing, these can be found in specialist literature. However, where clearing is considered necessary, to improve taxonomic resolution it is recommended that worms are first separated into groups based on gross features before selecting the largest specimens for clearing. Where large abundances of mixed taxa are present that cannot be distinguished without clearing then it is acceptable to mount only a subsample of the specimens (*e.g.* 10% or 100 specimens, whichever greater).

The use of stains and clearing agent is recommended as follows:

- Methyl green stain may be used to aid resolution of certain features, particularly in capitellid, maldanid, or ampharetid polychaetes. It should be used sparingly and cleared before specimens are returned to storage.
- Oligochaetes may be cleared using Poly-vinyl lactophenol to allow a better view of chaetae and reproductive anatomy. The process is time-consuming and permanently alters specimens, such that they must be maintained on slides.

Macrofaunal identification
For each sample, all individuals involved in identification of the biota should record their names on the SPF and details should be included on the sample data sheets.
The laboratory SOP must include details of quality assurance for identification.
All procedures must be documented and the form of documentation approved by the PCM.
All organisms removed from each sample must be identified to the most accurate taxonomic level practicable (follow TDP), usually species. Identifications should be recorded in pencil on the SDF. Any subsequent changes should be initialled and dated.
Biota must be identified using appropriate keys and taxonomic literature and using current nomenclature (see following section for details).
The identifier should divide the identified material into separate vials per recorded taxon, including a reference collection (see below).
If biomass is required at the recorded taxonomic level, all non-countable portions of animals must be identified and added to separate taxon containers as far as is practicable. Where fragments cannot be identified, then they can be apportioned according to the head count.
If a stain such as methyl green is added to aid recognition of features it should be cleared for the long term storage of specimens. Any animals cleared with polyvinyl lactophenol should be retained on clearly labelled slides.
A note should be added to the alcohol preserved specimen vials detailing the number removed and mounted on slides.
The analysing laboratory is responsible for sourcing and obtaining the literature required for identification at the level specified in the TDP (for a summary overview see Appendix 3). They may use the NMBAQC standard identification literature list to source references but should not regard it as comprehensive. They should submit additional literature citations to the list as they find them; in this way, all laboratories will be informed of new literature as soon as possible.
The NMBAQC Scheme will provide unpublished workshop guides as they become available.
The analysing laboratory must follow a specified and transparent in-house quality control procedure for identifications. External QC is detailed elsewhere.

A.13 Taxonomic literature and nomenclature

The NMBAQC Scheme has three methods of relaying literature to participating laboratories:

- Through the development of the NMBAQC Taxonomic Literature Database (v107) listing published literature which can be searched taxonomically.
- Through the organisation of taxonomic workshops which may highlight recent new literature or produce new draft keys for particular taxonomic groups.
- Through notes added to the Ring Test bulletins.

The Scheme resources should not be considered definitive in terms of required literature.

Taxonomic literature and nomenclature
Each laboratory should take responsibility for developing its own resources and should maintain an inventory of their literature collection.
Labs should undertake literature searches on relevant taxonomic groups on a regular basis (*e.g.* annually - using internet sites such as British Library Direct: http://direct.bl.uk/bld/Home.do).

Older literature should not be ignored as it may provide valuable keys or illustrations. Many older taxonomic works are now available on the internet (*e.g.* via the Biodiversity Heritage Library – www.biodiversitylibrary.org)
Information on useful taxonomic works should be shared. Constant feedback between laboratories within the Scheme will help to improve access to taxonomic resources.
Laboratories should use and maintain checklists of marine species employing current nomenclature and correct spellings. Nomenclature must be taken from the most recent published sources. Published species directories (*e.g.* Howson & Picton, 1997, Costello *et al*. 2001), may be outdated and may contain errors. Species lists should be compliant with the World Register of Marine Species (WoRMS - see www.marinespecies.org), except where there is good evidence that WoRMS is outdated or erroneous.

A.14 Enumeration

Some taxa are easily counted, as they exist as whole, discrete individuals. Most, however, are subject to damage and fragmentation and standard counting protocols are needed. Heads are the usual unit; exceptions are discussed below and would be included in the TDP.

There remain many problems with the recording of sessile taxa. The extreme cases are that they have been ignored by some laboratories (which significantly reduces the value of data), while attempts have been made, at other labs, to quantify by biomass (which is extremely time-consuming for encrusting taxa). Records of non-countable taxa as 'present' can currently be taken as standard but it may be possible to develop a more quantitative method in the future. Details of which taxa are to be considered to exist as discrete individuals or as encrusting or erect colonies will be provided in the TDP. Empty shells or tests or cast skins of crustaceans should not be counted although it may be useful to note the occurrence of unusual or abundant taxa. **Some shells (*e.g. Turritella*) may need to be carefully searched for preserved soft parts.**

Enumeration
Enumeration would normally be carried out during identification, by the identifier.
All taxa that occur as discrete individuals must be counted by heads, or by hinge lines for bivalves, or mouths for echinoderms / Anthozoa.
Fauna should be removed from tubes. Where fauna is tightly bound in tubes and removal would cause excessive damage or time loss (*e.g.* for *Phoronis* or *Galathowenia*) then the empty tube portions should be "topped and tailed" to confirm that a head/anterior portion is present.
Taxa that occur as discrete individuals but for which only non-countable portions are present in a sample should be recorded as 'Fragments' (fr.) (*e.g.* if a single *Chaetopterus* tail occurs but with no head region then the presence of the taxon should be recorded).
Non-countable taxa (e.g. sessile taxa, encrusting taxa, plants) must be recorded (at least as 'present') for each sample in which they were found.
Counts from sub-samples must be detailed on the SDF but calculated as values for whole samples prior to data entry. All identifications and enumerations and calculations must be recorded in full in pencil on the SDF. If tally marks are used the final count should be shown in brackets. Any subsequent data changes or alterations should be initialled and dated.

A.15 Sample storage

The processed sample will comprise three parts – Residues, Extracted fauna and Reference collection. The residues from which all biological material has been removed and the extracted fauna must be retained until all QC procedures are complete. If external QC is required, they will need to be transported to the auditing laboratory.

Analysing laboratories must establish and maintain a reference collection which at least combines representative specimens of all taxa they have recorded from various surveys. In addition reference collections may be specified for all individual surveys (*i.e.* one collection per survey). In some instances duplicate collections may be required per survey with one collection retained by the analytical laboratory and one provided for the survey commissioner.

PCM storage responsibilities
The PCM must specify what should happen to residues, extracted fauna and reference specimens and coordinate any transfer of material between laboratories.
Residues The PCM must provide clear instructions as to whether or when residues may be discarded or returned.
Extracted fauna The extracted fauna must be retained until completion of QC and, should be archived for a number of years thereafter to allow ad hoc taxonomic reviews. The PCM should specify whether the extracted fauna should be retained by the analysing laboratory, returned, or sent elsewhere for archiving. If due for disposal, samples and specimens should be offered to other agencies/organisations with an interest in marine biodiversity (*e.g.* universities or museums). They should also specify any requirements regarding container types or subdivision of fauna (*i.e.* whether stored by recorded taxa, major taxonomic groups or as a single pot per sample).
Reference collections The PCM should specify whether any additional collection should be returned or sent elsewhere.

LPM Storage responsibilities
The LPM must ensure all residues, extracted fauna, or reference specimens are stored properly at the analysing laboratory any subsequent disposal, transfer, or archiving is as agreed with the PCM.
All stored material must include internal labels clearly written or printed with an alcohol-resistant ink and with enough information to identify the sample and its treatment.
The analysing laboratory must store residues from which all biological material has been removed in clearly labelled, watertight containers until completion of QC.
Sediment containing animals counted *in situ* or sub-samples with non-extracted animals must be retained in 70% IDA or formaldehyde solution.
All residue containers to be retained should have external labels detailing the nature and concentration of the preservatives contained, as well as sample/sub-sampling details.
The sample/sub-sampling detail should also be on the internal labels.
Subsample residues must be stored in a separate container to the main sample.
The analysing laboratory must retain all extracted fauna until completion of QC.
Samples should be stored in watertight containers, clearly labelled with sample and survey details and separated by recorded taxon; sub-sampled material should be stored separately.

With the exception of certain encrusting organisms, which may be dried (see above), fauna must be stored in 70% IDA.
An example of each of the taxa recorded by the analysing laboratory must be retained in a separate container, as a reference collection and retained indefinitely. Separate reference collections may be established for each survey.
A record must be kept (*e.g.* on the SDF) of which specimens have been removed from the sample for reference.
Each reference container should include all of the specimens and identifiable portions of that taxon from its sample.
Each reference container must be clearly labelled with the species name, sampling location, sampling date, initials of the identifier and a second confirming analyst, as well as a sample code to link to any information not on the label.
A reasonable effort should be made to ensure that those specimens selected for reference are among the most suitable for that purpose (in terms of condition, size range and numbers of individuals in the reference pot).
Multiple reference lots should be made for rare or taxonomically difficult taxa. Reference lots must be clearly labelled and preserved as for the extracted fauna.
Reference collections must be maintained indefinitely by the analysing laboratory. Laboratories should arrange for reference material to be validated externally by other analysts or recognised experts where possible.
Inventories of all reference collection material held should be maintained.

A.16 Data management

It is important that all data associated with a project are stored at a location from which they can be retrieved at any time and are accessible to appropriate personnel. The information should be passed on in full to any successor.

Data management
The PCM or LPM should ensure that they are always in a position to access the original data in their original form, along with all sample details and associated data.
The commissioning organisation is responsible for ensuring that all data are accessible and that none are lost. Information should be available in full to any successor (or temporary replacement).
All information documented during processing must be written by hand on a series of laboratory forms and retained for later inspection, if necessary.
The nature of the forms would follow the analysing laboratory's SOP but should include, as a minimum: • Sample Progress Forms (SPF) with analysis details and QC. • Sample Data Forms (SDF) with taxa, counts, biomass figures and reference collection selections.
The information from the forms should be transcribed electronically and supplied to the PCM (in spreadsheet or database format), on completion of the project.
The name of the person entering data into an electronic form for each sample should be recorded in the SPF.
The laboratory SOP must specify how quality control is ensured during data entry.
All in-house QC procedures must be documented and approved by the PCM.
There must always be an accessible resource in which the original data are retained in their original form. Later data truncation or data analysis methods will depend upon the objectives of the project and are beyond the scope of this guideline.

A.17 Data products

The basic product of macrobenthic sample analysis is a matrix of taxa recorded and enumerated in each sample. It is best stored in a database and presented in a spreadsheet format (*e.g.* Microsoft Excel). **There should always be an accessible resource in which the original data are retained in their original form.**

Data products

The sample codes must link clearly with sample information obtained from the survey:

- name of organisation that owns data,
- name of PCM,
- organisation, individuals and vessel involved in sampling (as appropriate),
- station and sample code,
- visual description of sample,
- sampling position (with coordinate type and projection specified),
- sampling depth (corrected to chart datum),
- sampling date and time,
- sampling equipment (including surface area sampled),
- details of all treatment of the sample post-collection (*e.g.* field sieving, with mesh, any material removed before preservation, preservative and any other additives used),
- details of all other samples or data collected at the same sites or during the same survey (*e.g.* PSA, chemistry, photography, sonar, bathymetry).

They should also link clearly with processing details:

- names of individuals involved in the different stages of processing the samples (including LCM),
- details of any sub-sampling carried out,
- details of specimens removed for reference collections,
- location of sample components.

The data guideline and templates for "sediment sampling by grab or core for benthos" provided by the Marine Environmental Data and Information Network (MEDIN, 2009) should be followed as far as is possible.

A.18 Quality assurance and quality control

Quality Assurance (QA) is the adoption of practices and procedures aimed at ensuring the products from a laboratory consistently achieve acceptable standards. Quality Control (QC) is the systematic testing of products or samples to determine whether the quality targets are being achieved. QA involves training records and competency assessment, documenting and validating procedures, sample tracking and traceability, calibrating equipment, provision of reference material (voucher collections) and taxonomic literature and implementing a quality management system. QC involves setting appropriate analytical targets for testing via the re-analysis of a randomly selected proportion of samples. **Where samples fail to meet required quality the cause of the failure should be investigated and a suite of remedial actions should be implemented to improve the quality and prevent or minimise re-occurrence of errors.**

Internal QA/QC
Each stage of the laboratory analysis process should be subject to internal Quality Control (QC).
The analysing laboratory must produce or adopt Standard Operating Procedure(s) (SOP) that are fit for purpose and should demonstrate that these have been validated through production of acceptable data.
The analysing laboratory must ensure that all staff adhere to methods described in its SOP and all records relating to the SOP are available for inspection.
The competency (education, training, work experience and/or other demonstrated skills) of staff involved in analysis should be checked and recorded within the laboratory. For less experienced staff undergoing training, appropriate supervision of work should be provided until the required competency in the method is achieved. Competency should be improved / maintained through participation in internal or external training exercise or workshops in all relevant aspects of laboratory analysis.
A voucher/reference collection must be compiled containing examples of all taxa encountered. The samples must be fully labelled stating at least the taxon name, sampling location, and the identifier. Ideally determinations should be confirmed by a second analyst.
The laboratory must maintain a comprehensive and regularly updated library of taxonomic literature.
There must be an internal system of double checking (quality control) for at least 10% of samples for extraction, identification and enumeration.
There should be an internal system of double checking (quality control) for a proportion (*e.g.* 10%) of electronic records (in spreadsheets or databases) of biological data against original handwritten datasheets.
Appropriate quality criteria must be detailed in the SOP indicating acceptable targets for sorting, enumeration, identification, and biomass (if required) and relevant remedial actions where targets are not achieved.
The analysing laboratory should maintain an appropriate quality management system to document its audit trail of checked laboratory samples and spreadsheets/databases. This should include details the re-checked samples, comments on the differences from the original sample and details of any remedial action taken.
All samples (biota and residues) associated with samples which are subject to external QC must be retained until samples are deemed to have passed (or remedial action of failed samples has be completed satisfactorily).
All laboratory equipment should be maintained and calibrated, with remedial action in place to ensure normal functioning. This internal auditing system should also be documented.

External QA/QC
External Quality Assurance (QA) is mandatory for laboratories involved in the analysis of samples collected by Competent Monitoring Agencies for statutory monitoring programmes (*e.g.* WFD and CSEMP) or for projects funded by Government Departments or Agencies.
The analysing laboratory must demonstrate its participation in an external quality assurance scheme *e.g.* the National Marine Biological Analytical Quality Control (NMBAQC) scheme or equivalent. Minimum participation must involve exercises where a random selection of the participant's own samples is audited. The laboratory must achieve the scheme's quality standards and complete any required remedial actions.

All reports and Statement of Performance certificates provided by the external QA scheme must be available for inspection.
The analysing laboratory should participate in training exercises and workshops arranged by the QA scheme (*e.g.* the NMBAQC or equivalent) or other institutions to demonstrate staff member's knowledge of current analytical or taxonomic issues.
The analysing laboratory should seek laboratory certification or accreditation of their operations against recognised national / international guidelines such as Good Laboratory Practice (GLP), International Organisation of Standardisation (ISO), or United Kingdom Accreditation Service (UKAS).

PROCEDURAL GUIDELINE B: Biomass of macrobenthic samples

Biomass data are required for CSEMP macrobenthic samples but is not a requirement for WFD macrobenthic samples. Biomass from samples is also measured for other reasons such as estimating available food resources for populations of fish or birds. Sometimes, biomass may be considered an important attribute of the benthos itself. Biomass data could also potentially be used to measure changes in reproductive cycles or average size of particular taxa.

Advantages

- provides a measure of biological material that may be more relevant than numbers of organisms, which will be of varying sizes.

Disadvantages

- additional time and cost per sample,
- some damage to material, making quality control of data difficult,
- difficult to apply biomass to taxon groups that are permanently mounted on slides (*e.g.* oligochaetes).

Biomass measures must always be considered subject to considerable error, unless ash free dry weight is used. Ash free biomass destroys the specimens and any possibility of QC, therefore it should be considered only where there is a very specific need for highly accurate biomass measures.

The methods presented here allow for a basic wet weight biomass estimate. Biomass estimates will always be subject to variability due to differing effectiveness of drying methods. There is currently no precise methodology that will provide consistent results for blotting fauna of differing sizes, shapes, or physical consistency ranging from hard shelled molluscs to soft fragile worms. There is little point in adding highly time-consuming methods to standardise preservation time or rinsing, prior to wet biomass measures.

The choice of 'species' versus 'family' or 'phylum' level biomass will be specified by the monitoring programme. It is important to remember, however, that not every taxon can be recorded to species level and that small phyla may be combined and larger ones may be divided. Reference should instead be made to biomass at levels of 'recorded taxon or 'major taxonomic group'. Subdivision between taxonomic groups and taxa excluded from biomass currently vary between laboratories. Standard groups are provided here, more detail will be given in the TDP. Treatment of tubes and shells also currently varies between laboratories, so a standard is provided.

Conversion factors exist for transforming wet weight biomass to ash-free dry weight biomass (see Ricciardi & Bourget, 1998). They are not part of the laboratory procedure. However, conversion values are available only for a limited selection of species and those for major groups must be inaccurate due to the range of animals involved (especially for 'others' and molluscs). At some time, a revised list of factors should be produced.

Where biomass is to be carried out at the 'species' level, it must, in practice be conducted separately for the majority of taxa recorded during sample processing. **There will always be some taxa recorded at higher taxonomic levels.** In addition, many taxa are commonly

excluded from biomass. A standard list of taxa to exclude should be followed. This will be found in the TDP (for a summary overview see Appendix 3).

B.1 Logistics

Biomass is typically conducted at the laboratory that carried out basic sample analysis. These guidelines are for the analysing laboratory;

B.2 Equipment

The sample analyst must have access to a laboratory equipped with a recently calibrated, annually serviced balance, accurate to 0.0001 g. They will need trays and dishes for sorting, forceps and a range of watertight containers of different sizes for containment of samples. Supplies of preservatives (IDA) must be available.

B.3 Biomass by major taxonomic group

Biomass by major taxonomic group

Where biomass is required by phylum, it will, in practice be conducted by major taxonomic groups, with some large phyla divided, certain small phyla combined and others excluded from biomass. Traditionally biomass estimates are focussed on infaunal communities. (Epifaunal communities are assessed by percentage coverage estimates). The distinction between infaunal and epifaunal (or between solitary and colonial) taxa is not always clear cut. Nevertheless, a convention for dividing major infaunal groups for biomass assessment is presented, below:

- Cnidaria (infaunal forms only: Pennatulacea, Ceriantharia, some Actiniaria)
- Polychaeta
- Oligochaeta
- Crustacea (excluding Cirripedia (barnacles) and sessile parasites)
- Mollusca
- Echinodermata
- Other minor phyla (*e.g.* Nemertea, Platyhelminthes, Priapulida, Sipunculida, Phoronida, Chelicerata, Insecta, Hemichordata, Chordata)

Sessile taxa physically attached to the substratum are not weighed. A full list of taxa considered sessile and to be excluded from biomass is included in the TDP but a condensed list is included below.

- Protozoa
- Porifera
- Cnidaria (sessile colonial forms: Hydrozoa, Zoantharia, Alcyonaria)
- Entoprocta
- Cirripedia
- Sessile parasites
- Bryozoa
- Ascidiacea
- Plants and algae,
- Deposited eggs of invertebrates or vertebrates

B.4 Blotted wet weight biomass

Blotted wet weight biomass is very susceptible to recorder variability. Different operators apply varying degrees of pressure when blotting, rolling or puncturing specimens and wait for different periods of evaporation before recording the biomass. While a standard wait time can be adopted, it is difficult to standardise manipulation of specimens during blotting. Large numbers of animals weighed en masse produce different results from single animals weighed individually due to differing relative surface areas exposed.

There may also be differences caused by different chemical (preservative) treatments of samples and the time each sample was left in each substance. Though the findings of different biomass studies have varied a recent investigation by Wetzel, Leuchs, and Koop (2005) suggests that there is no difference between ethanol and formalin preservatives and that biomass loss of preserved specimens is minimal after a storage period of three weeks.

Blotted wet weight biomass
The name of the person weighing each sample should be recorded, along with a unique code for the analytical balance used.
Fragments of organisms must be extracted from the residue, as well as countable parts, as they will constitute a significant proportion of the biomass.
Fauna extracted from the samples must be sorted into individual taxa or the taxonomic groups required for biomass. Faunal fragments should be assigned to respective counted taxa as far as is possible.
Fauna from each biomass group should be removed from IDA with forceps (or sieved out, if necessary).
Fauna must then be placed on absorbent paper and gently dried (blotting with tissue paper is recommended) until no free surface moisture is apparent. Larger fauna should be gently rolled over to ensure moisture is absorbed from all surfaces.
Blotted fauna should be carefully transferred to a plastic or foil boat and placed on an analytical balance (tared with respect to the weighing boat).
Fauna must then be weighed in grams to an accuracy of 4 decimal places. Fauna weighing less than 0.0001 g should be assigned a nominal mass of 0.0001 g. The weight should be recorded once stability of the reading has been reached. It is recommended that a standard wait time is used to achieve stability (*e.g.* 30 seconds) to avoid progressive water loss by evaporation.
Care must be taken to avoid damage to the specimens; particular care must be taken with reference collection material, which would be treated separately from the main part of the sample.
All animals must be weighed intact, including the shells of molluscs and tests of echinoderms. Large specimens of taxa which might retain significant fluid (*e.g.* bivalves, echinoids, ascidians) should be punctured and drained prior to weighing.
Tube dwelling taxa should be removed from their tubes prior to weighing. Where fauna is tightly bound in tubes and removal would cause excessive damage or time loss (*e.g.* for *Phoronis* or *Galathowenia*) then the specimens can be weighed *in situ*. A tubed to un-tubed conversion factor can be created for specific taxa by weighing a subsample comprising a small number of specimens before and after careful removal of the tube. This factor can them be applied to other samples with the same taxon. Where a conversion factor has been applied it should be clearly indicated on the SDF.
Attached fauna (*e.g.* parasites and commensals) should be left attached and weighed with hosts.

B.5 Storage – biomass considerations

Storage – biomass considerations
Biomassed material should be returned as soon as possible to its preservative for storage to avoid excessive drying and damage to the specimens.

B.6 Data management – biomass considerations

Biomass data management
All information documented during biomass must be written by hand on the SDF and retained for later inspection, if necessary.
The information from the form should be transcribed electronically and supplied to the PCM (in spreadsheet or database format), on completion of the project.

B.7 Data products – biomass considerations

Biomass data products
The basic product of biomass analysis is a matrix of taxa recorded and weighed in each sample. It is best stored in a database and presented as a spreadsheet format (*e.g.* Microsoft Excel).
The sample codes must link clearly with other sample information obtained from the survey. Where biomass is recorded per taxon it must be possible to match up abundances and biomasses for each taxon.

B.8 Quality assurance and quality control

Biomass QA/QC
The analysing laboratory must ensure that it adheres to internal QA methods described in its SOP and that these are available for inspection.
Each stage of the process should be subject to QA.
External QC can provide a second estimate of biomass as a measure of recorder variability.

PROCEDURAL GUIDELINE C: Sub-sampling and *in situ* counts for macrobenthic samples

C.1 Introduction

Sub-sampling would be carried out at the laboratory responsible for basic sample analysis. These guidelines are for the analysing laboratory. The purpose of sub-sampling is to reduce the time and costs required for sample processing (which it may do by over 50% per sample) by fully analysing only a proportion of the sample. **The sub-sampling process aims to produce results that are not significantly different from those that would have been achieved had the entire sample been fully analysed.** The time and cost are directly related and affected by the same factors. **It should, be remembered that, although sub-sampling reduces total sample processing time, it will not do so by a factor directly related to the subsample fraction.** The sub-sampling process itself can become quite complicated and will also take some considerable time. Moreover numerical calculations required to convert sub-sample counts from fractions of samples to achieve full sample estimates are potentially prone to errors.

C.2 Equipment

Several techniques were tested at the Humber Benthic Field Methods Workshop (Proudfoot *et al*., 2003) including; marked tray, riffle box, quarteriser, aerated column, fulsom splitter, and magnetic stirrer. **Of these, the quarteriser proved most effective with sub-sample abundance estimates within 10% of the actual full sample value.** This is the method recommended here. Use of other methods must be documented and agreed with the PCM.

The quarteriser comprises a large perspex cylinder sectioned internally for about a third of its length into four equal compartments. The sample is poured in the top and the cylinder is filled with water to about 2cm above the height of the compartment dividers before being inverted to mix the sample and then stood upright to allow the sample material to settle into the four compartments. The sub-sample is obtained by draining one of the four compartments. The quarteriser method works best with light fractions or fine sediment fractions which can be temporarily suspended in water. For heavier coarser fractions the drained material should be tipped into two or four equal sized containers to achieve a similar "depth" and hence volume (this should only be necessary for dry material e.g. Bryozoa).

C.3 When and how to sub-sample

Sub-sampling and *in situ* counts should only be considered where processing times/costs would otherwise be prohibitive, where there would be no significant loss of information through sub-sampling and where agreed by the PCM. The recommendations here apply to sub-sampling of macrobiota samples.

Fractionation of the sample residues is advised, especially with more heterogeneous samples. Residues should be separated into heavy and light (float) fractions which can be further split into different sieve fractions (see Appendix 2). The different fractions may be treated differently from a sub-sampling point of view. Sub-sampling may then be considered where one of these fractions exceeds a particular volume or where a particular taxon group is excessively abundant. In practice most or all of the non-attached fauna will be floated / washed off the larger heavy fractions so sub-sampling of the latter fractions may effectively be estimating the abundance/occurrence of attached fauna only.

It is recommended that the minimum volume of a sub-sample sediment residue (*i.e.* total heavy fraction) should be 0.5 litre and that at least 10% of the total sample sediment residue volume should be processed in full. The minimum volume of a sub-sample of the fine fraction (<4>0.5mm) of the light/float material should be 0.05 litre. Sub-sampling aims to minimise sorting time by reducing picking effort on selected abundant taxa only. Other less abundant taxa should be sorted from the whole sample to gain a proper estimate of diversity.

All of the selected abundant taxa (or taxon group) must be picked from the sub-sample and should comprise a minimum of 100 specimens (or 10% or the total sample estimate, if greater). **Hence sub-sampling should not be considered unless the estimated total count for a taxon exceeds 200, 400, or 800 for 1/2, 1/4, 1/8 sub-sampling respectively. In practice if a taxon count does not exceed 100 in the subsample then that taxon does not qualify as "abundant" and should be picked/counted in the whole sample.** Where abundant taxa in a sub-sample can confidently assigned to particular species *in-situ (e.g. Mytilus edulis* juvs. or *Hydrobia ulvae*) then counting can be undertaken *in-situ*. Where the abundant taxon group is likely to include one than one similar species (*e.g.* for Oligochaeta sp.) then all specimens must be removed for microscopical examination.

When and how to subsample
If the total sample residue volume exceeds 1 litre and estimated counts of some abundant taxa are liable to exceed 200 for the whole sample then sub-sampling can be considered. Less abundant taxa must be counted from the whole sample.
Heterogeneous samples should be separated into light/float and heavy fractions and each of these split into sieve fractions (*e.g.* >31.5mm, <31.5>4mm, <4>2mm, <2>0.5mm).
If the settled volume of the fine fraction (<4>0.5mm) of light/float material in water exceeds 0.2 litre, and estimated counts of some abundant taxa are liable to exceed 200 for the whole sample then sub-sampling can be considered. Light fine fraction sub-samples should be at least 0.05 litre (50ml) and selected abundant taxa should have a minimum of 100 specimens in the sub-sample. Coarser fractions (>4mm) of light /float material should be sorted in full.
If the settled volume of the coarse fraction (<31.5>4 mm) of heavy material exceeds 1 litre, and encrusting biota are present on the majority of stones/shells then sub-sampling can be considered. Heavy coarse fraction sub-samples should be at least 0.5 litre (500ml). Countable fauna must be sorted in full. Coarser fractions (>31.5mm) and finer fractions (<4>2mm, and <2>0.5mm) of heavy material should be sorted in full.
Coarse heavy material retained at 4 mm but passing through 31.5 mm should be: a) sorted in full, if less than 1 litre in volume; b 1/2 sub-sampled if between 1 and 2 litres, b) 1/4 sub-sampled if between 2 and 4 litres, c) 1/8 sub-sampled if over 4 litres.
The procedure to be used for sub-sampling must be agreed with the PCM. Smaller sub-samples may be used with the express agreement of the PCM. Details of sub-sampling must be summarised in the SPF and detailed in the SOP.
Details of any sub-sampling undertaken must be provided on the SDF and all calculations to achieve final whole sample count estimates must be shown on the SDF.
Subsample residues should be stored in a separate container to other parts of the sample and clearly labelled.

C.4 The 'quarteriser' method

The 'quarteriser' method
The 'quarteriser' may be used only for sub-sampling the light fraction of samples.
After extraction of taxa not requiring sub-sampling, the light fraction should be washed into the 'quarteriser' and water added to approximately half the depth of the device.
A bung should be placed into the top and the 'quarteriser' inverted several times to ensure equal division of sediment between the four compartments.
After shaking, any residue left on the bung and the sides of the "quarteriser" should be gently rinsed into the compartments.
The device should then be left to stand undisturbed for several minutes, until all sediment in the sample has settled.
One of the quarter compartments should then be emptied slowly, to prevent disturbance that might cause material to flow between compartments, and rinsed into a watertight container or 0.5 mm sieve.
The fraction may be sub-sampled again, to generate a smaller fraction.

C.5 Subsample storage

Subsample storage
All sub-sampled biota must be retained in a separate container to those collected from the sample as a whole.
The duplicate subsample residues, which contain biota that have not been extracted, should also be preserved and retained.
All subsample components must be clearly labelled.

C.6 Data management

Data management
The sample analyst must enter details of samples that have been sub-sampled, the sub-sampling method and the fraction sorted onto all forms relating to the sample.

C.7 Data analysis

Data analysis
There must always be an accessible resource in which the original data are retained in their original form.
The final data matrix output, however, may record only the calculated estimates of each taxon for the whole sample.

C.8 Quality assurance and quality control

QA/QC
The sub-sampling process should be supervised by an experienced staff member. Calculations should be checked by a second staff member. All other processes must be subject to quality control.

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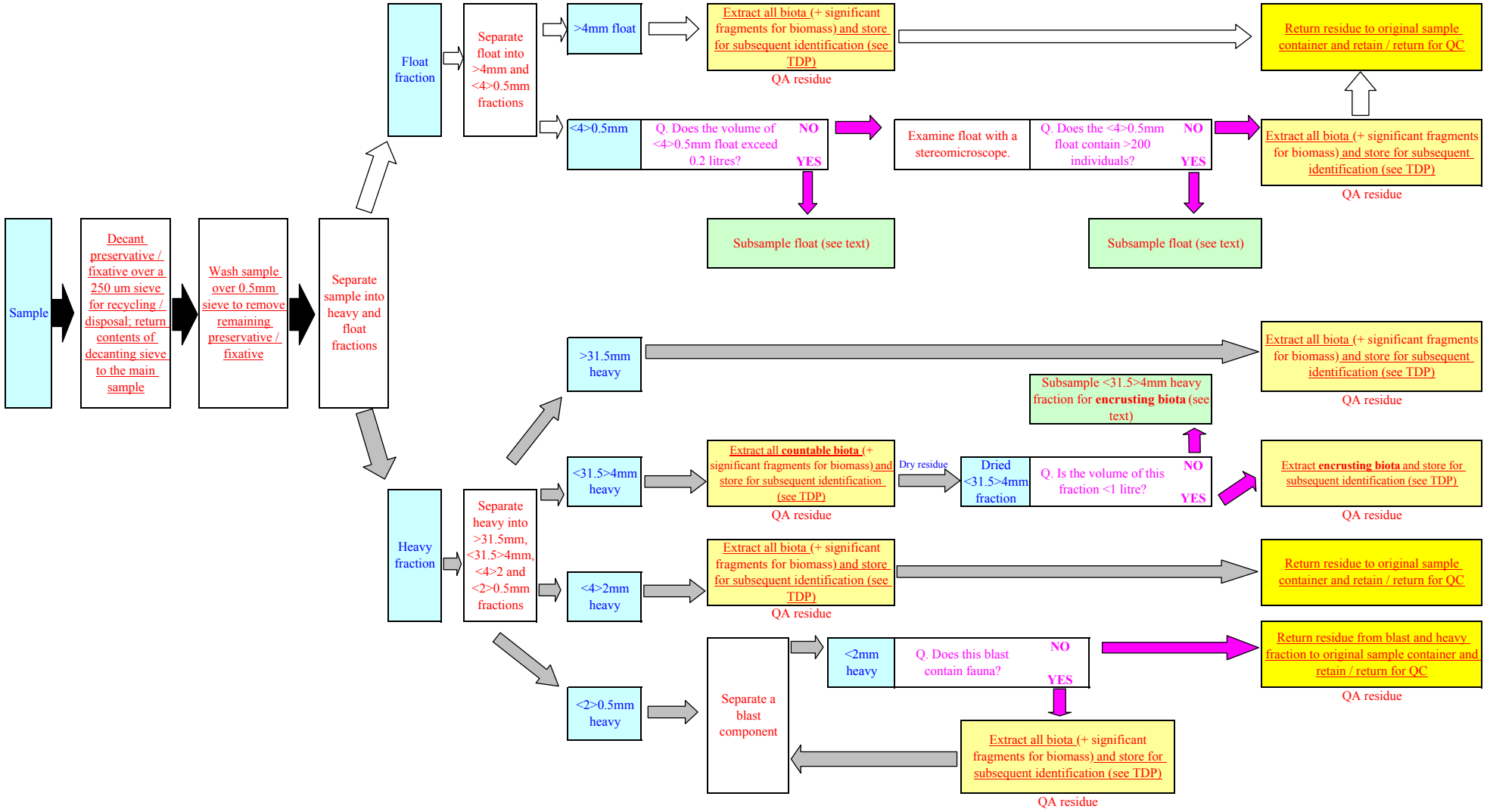
APPENDIX 1 - Macrobenthic Sample Analysis Checklist

Process	Best Practice: NMBAQC Processing Requirements
Survey Design	Not covered in this document.
Sample Collection	An adequate sample is collected using the specified equipment.
	Retain all material collected above 0.5mm (or 1mm) sieve mesh for subsequent processing.
Sample Preservation	All material preserved/fixed adequately with chemical content noted for subsequent handling/processing.
Sample Storage	Storage in air and watertight robust containers.
Sample Logging/Tracking	Unique external and internal reference labels; list all samples on the SPF (sample progress form).
Sample Transportation	Packaging, preparation and transportation to be conducted only by appropriately accredited couriers and/or trained staff.
	Complete/provide TREM card, DGN, TEI documentation and HazChem labels, where applicable.
	Package to minimise impact of damage & potential spills in transportation.
Sample Processing	Provide processing laboratory with SPF, PRP & TDP with samples.
	Any deviation from NMBAQC Guidelines (PRP and TDP) must be approved prior to laboratory sample processing.
Sieving / Faunal Extraction (See sieving and extraction flowchart for an example SOP, Appendix 2)	Conduct Sieving in a ventilated washroom and observe all H&S considerations including CoSHH.
	Decant liquid over a 250µm certified sieve for recycling/appropriate disposal. Rinse retained material over 0.5mm sieve mesh.
	Wash sample over 0.5mm certified sieve mesh (cleaned and checked for debris/defects prior to commencing each sample analysis).
	Separate sample into 0.5-1mm and >1mm fractions and extract biota; use a range of certified sieves, where applicable.
	Extract biota according to PRP and store for subsequent identification. It will be necessary to break tubes, bored shells and soft rock to extract cryptic fauna.
	Return residue to original sample container, with adequate preservative/fixative, and retain/return for QA/QC.
Identification & Enumeration	Identify and enumerate biota according to PRP/TDP and record on the SDF (sample data form).
	Create a survey reference collection including individuals of all taxa recorded. Make multiple reference lots for rare or taxonomically difficult taxa. Maintain reference collections indefinitely.
Biomass	Biomass according to PRP and record on the SDF (sample data form).
	Blotted dry biomass to 0.0001g using certified equipment.
Sample Storage (Post-analysis)	Residue - unique external and internal reference labels; biota stored as specified in PRP with unique reference labels.
	Store samples (residue & extracted biota) until all QC checks are completed.
Data Entry / Storage / Submission	Data from each SDF should be entered (separate 0.5-1mm & >1mm fractions) and stored electronically using a standard taxon list.
	Data should always be accessible in their original form, along with all sample details and associated data.
	Supply abundance and biomass data following PRP.
	Data submitted must detail any deviation from PRP and TDP.
Quality Control	Participate in all necessary AQC checks and undertake fully any prescribed remedial action.
Data Analysis	Not covered in this document.

APPENDIX 2

NMBAQC Scheme Sample Processing: Sieving and Extraction - an example SOP (processing requirements underlined).



Key

Sample = biota and residue material preserved / fixed adequately and stored in a water / air tight container with unique internal and external reference labels.
Sieve = certified mesh sieve; cleaned and checked for debris / defects prior to sample processing.
Float fraction = material poured off the sample after light agitation in water.
Heavy fraction = material remaining after removal of float material.
Blast component = material decanted from heavy fraction during high agitation with a jet of water.
Underlined text denotes that this is a processing requirement.

APPENDIX 3

Summary Overview of Taxonomic Discrimination Protocol (TDP)

Exclusive meiofaunal, freshwater & planktonic groups not shown.

				Recording/Identification			Biomass (significant fragments always included)			
Major Taxonomic Group/Items	**Forms/Subgroups**	**Extraction***	**Preservation**	Enumeration/Presence	Criteria	Tax. level**	Weighed	Major group	Tubes/shells incl.	**Notes**
Protozoa	conspicuous only (*e.g. Lagotia, Astrorhiza*)	In part	Dry or Alcohol	Varies	Complete	Varies	☒	n/a	n/a	
Porifera		In part	Varies	Presence	n/a	Varies	☒	n/a	n/a	
	easily detachible	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
	small encrusting patches	In part	Dry or Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
	boring (*e.g. Cliona*)	In part	Dry or Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Cnidaria		Varies	Varies	Varies	n/a	Varies	Varies	Cnidaria	☒	
Hydrozoa	erect	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Hydrozoa	stolonal or encrusting	In part	Dry or Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Octocorallia	erect (*e.g. Alcyonium*)	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Octocorallia	encrusting (*e.g. Sarcodictyon*)	In part	Dry or Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Ceriantharia	*e.g . Cerianthus*	All	Alcohol	Counted	Mouth	Varies	☑	Cnidaria	☒	
Zoantharia	*e.g. Epizoanthus*	All	Dry or Alcohol	Counted (polyps)	Complete	Varies	☒	n/a	n/a	
Actiniaria	inc. Edwardsiidae	All	Alcohol	Counted	Mouth	Varies	☑	Cnidaria	☒	
Platyhelminthes		All	Alcohol	Counted	Head	Class	☑	Others	n/a	Freshwater taxa to genus/species
Nemertea		All	Alcohol	Counted	Head	Phylum	☑	Others	n/a	Distinctive taxa taken further
Nematoda		All	Alcohol	Counted	Head	Phylum	☑	Others	n/a	Mainly meiofaunal
Priapulida		All	Alcohol	Counted	Head	Species	☑	Others	n/a	
Entoprocta		In part	Alcohol	Presence	n/a	Genus	☒	n/a	n/a	
Chaetognatha		All	Alcohol	Counted	Head	Genus	☑	Others	n/a	Mainly planktonic; benthic sp. to spp.
Sipuncula		All	Alcohol	Counted	Trunk	Species	☑	Others	n/a	
Echiura		All	Alcohol	Counted	Trunk	Species	☑	Others	n/a	
Annelida		All	Alcohol	Counted	Head	Varies	☑	Varies	Varies	See Oligochaeta TDP
Chelicerata		All	Alcohol	Counted	Head	Varies	☑	Others	n/a	
Crustacea		Varies	Varies	Counted	Varies	Varies	Varies	Crustacea	n/a	
	free living (most)	All	Alcohol	Counted	Head	Varies	☑	Crustacea	n/a	
	attached parasites	All	Alcohol, with host	Counted	Head/Attachment	Varies	☒	n/a	n/a	Biomassed with host
	sessile (barnacles)	Varies	Dry or Alcohol	Counted	Head/Cirri	Varies	☒	n/a	n/a	
Myriapoda		All	Alcohol	Counted	Head	Class	☑	Others	n/a	
Hexapoda	*e.g.* insects	All	Alcohol	Counted	Head	Varies	Varies	Others	n/a	
Mollusca		All	Alcohol	Counted	Varies	Varies	☑	Mollusca	☑	
Brachiopoda		All	Alcohol	Counted	Lophophore	Species	☑	Others	☑	
Bryozoa		In part	Varies	Presence	n/a	Varies	☒	n/a	n/a	
	erect (*e.g. Flustra, Bugul* a)	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
	stolonal (*e.g. Nolella, Aetea*)	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
	encrusting (most)	In part	Dry or Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Phoronida		All	Alcohol	Counted	Head	Genus	☑	Others	☑	
Echinodermata		All	Alcohol	Counted	Mouth	Varies	☑	Echinodermata	☑	
Hemichordata		All	Alcohol	Counted	Head/collar	Class	☑	Others	n/a	
Chordata		Varies	Varies	Varies	Varies	Varies	Varies	Varies	n/a	
Tunicata	solitary	All	Alcohol	Counted	Branchial sac	Varies	☒	n/a	n/a	
Tunicata	stolonal (*e.g. Perophora*)	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Tunicata	detachible colonies (*e.g. Botryllus*)	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Tunicata	encrusting (*e.g.* Didemnidae)	In part	Dry or Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Fish and Cephalochordata	-	All	Alcohol	Counted	Head	Varies	☑	Others or Fish	n/a	Biomass requirements project related
Cyanophyta		In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Rhodophycota		In part	Varies	Presence	n/a	Varies	☒	n/a	n/a	Record only if attached
Chromophycota		In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	Record only if attached
Chlorophycota		In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	Record only if attached
Fungi		☒	n/a	☒	n/a	n/a	☒	n/a	n/a	
Tracheophycota	flowering plants	In part	Alcohol	Presence	n/a	Species	☒	n/a	n/a	Angiospermae
Animalia 'eggs'		Varies	Alcohol	Varies	Varies	Varies	☒	n/a	n/a	
Eggs	egg masses	In part	Alcohol	Presence	n/a	Varies	☒	n/a	n/a	
Eggs	discrete eggs (*e.g.* fish)	All	Alcohol	Counted	Complete	Varies	☒	n/a	n/a	
Anthropogenic material	including seeds	☒	n/a	☒	n/a	n/a	☒	n/a	n/a	

* = some may be counted *in situ* / subsampled if present in high numbers

** = minimum level required (good condition given); there may be some exceptions to be detailed in the fully expanded TDP

APPENDIX 4

Taxonomic Discrimination Protocol (TDP) for Oligochaeta

Some meiofaunal, freshwater & planktonic groups not shown.

					Recorded/Identification			Biomass			
Class	**Family**	**Genus**	**Extraction***	**Preservation**	Enumeration/Presence	Tax. level**	Juv. separated	Weighed	Fragments incl.	Tubes/shells incl.	**Notes**
Oligochaeta			All	Alcohol	Counted	Varies	☒	☑	☑	n/a	
	Naididae		All	Alcohol	Counted	Varies	☒	☑	☑	n/a	
		Amphichaeta	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Chaetogaster	All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
		Dero	All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
		Nais	All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
		Paranais	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Stylaria	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Uncinais	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
	Tubificidae		All	Alcohol	Counted	Varies (Family, except where stated below)	☒	☑	☑	n/a	
		Monopylephorus	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Limnodriloides	All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
		Clitellio	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Heterochaeta	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Limnodrilus	All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
		Tubifex	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Tubificoides	All	Alcohol	Counted	Species (except T.brownae, T.crenacoleus, T.diazi and T.pseudogaster, all as T.pseudogaster agg.)	☒	☑	☑	n/a	
		Potamothrix	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Psammoryctides	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
		Quistadrilus	All	Alcohol	Counted	Q. multisetosus to Species	☒	☑	☑	n/a	
		Branchiura	All	Alcohol	Counted	Species	☒	☑	☑	n/a	
	Enchytraeidae		All	Alcohol	Counted	Family (except Grania spp. to genus)	☒	☑	☑	n/a	
		Grania	All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
	Branchiobdellidae		All	Alcohol	Counted	Family	☒	☑	☑	n/a	
	Aeolosomatidae		All	Alcohol	Counted	Genus	☒	☑	☑	n/a	
	Haplotaxidae		All	Alcohol	Counted	Species	☒	☑	☑	n/a	
	Lumbriculidae		All	Alcohol	Counted	Family	☒	☑	☑	n/a	
	Dorydrilidae		All	Alcohol	Counted	Species	☒	☑	☑	n/a	
	Glossoscolecidae		All	Alcohol	Counted	Species	☒	☑	☑	n/a	
	Lumbricidae		All	Alcohol	Counted	Family (except Eiseniella tetraedra to species)	☒	☑	☑	n/a	

* = some may be counted *in situ* / subsampled if present in high numbers

** = minimum level required; occasional specimens may be left at higher taxa if damaged, small or with unusual combinations of features