

SECTION 2.4.

DISEASES OF MOLLUSCS

CHAPTER 2.4.0.

GENERAL INFORMATION

INTRODUCTION¹

The following information and procedures are appropriate for OIE listed pathogens and will provide direction to pathologists investigating mollusc disease outbreaks or conducting disease surveillance procedures. However, experienced shellfish pathologists should be consulted if a disease condition persists or spreads beyond the locations where first detected.

1. Assessing the health status of the epidemiological unit

1.1. Sample material to be used for tests

Sample material depends on the species, life stage and size of animals and the objective of testing (i.e. diagnosis of overt disease, or sampling for targeted surveillance to demonstrate freedom from a specified disease). See individual disease chapters in this *Aquatic Manual* for specific details of sample requirements

1.2. Specifications according to mollusc size

1.2.1. For the listed parasites

1.2.1.1. Juveniles below 1 cm

Sample the entire animals but remove the shell or proceed with a decalcification protocol. Sections should be prepared to include most organs. This may entail taking two sections from each histological block to obtain organs from different areas (e.g. those located near the surface or deeper in the block).

1.2.1.2. Juveniles and adults 1–6 cm

Sample the entire mollusc and cut one or several 3–5 mm cross sections including labial palps, gills, and digestive gland.

1.2.1.3. Molluscs over 6 cm

Take several sections of the body containing individual organs/tissues including mantle, gill, digestive gland, gonad, and kidney.

1.2.2. For withering syndrome of abalone

For abalone ≥ 20 mm, excise several 3–5 mm cross sections containing posterior oesophagus (post-oesophagus), digestive gland, and foot muscle.

¹ NB: Version adopted by the World Assembly of Delegates of the OIE in May 2012.

1.2.3. For abalone herpesvirus infections

Sample as outlined in Section 1.2.2 above with the addition of a cross section of the head to obtain the cerebral ganglion and removal of several sections of the foot and adductor muscle complex including one section 0.25–1.0 cm (distance depends on abalone maximum length) posterior to the head to obtain the pedal ganglion. In addition, a longitudinal section from the anterior pedal ganglion to the posterior portion of the pedal musculature should be taken. For more specific information on sampling, please refer to Section C of the general introduction to this Section of the *Aquatic Manual: Part 2. Recommendations applicable to specific diseases*.

1.3. Specifications according to mollusc populations

For general guidelines, see the OIE *Guide for Aquatic Animal Health Surveillance* (2009), and for specific details of sample requirements for a particular listed disease, see the individual disease chapter in this *Aquatic Manual*.

1. Usually grow-out cultured mollusc populations are reared semi-intensively (in trays, bags, nets, or on ropes, stakes, and using bottom culture methods) and they have extensive interaction with natural environments;
2. Cultured mollusc populations frequently have components of their lifecycle occurring in a wild state (e.g. collection of wild spat for further grow-out) and such populations are not always accessible for observation and sampling;
3. Multiple culture methods for the same species may exist within the same jurisdiction and thus represent different populations with respect to risk characteristics;
4. Extremely large geographic areas may comprise many small contiguous farms or production units with different ownership and management. This can present challenges when designing sampling programmes based on stock ownership;
5. Molluscs can be sessile (e.g. adult oysters) at some points during their lifecycle and very mobile at other stages (e.g. oyster larvae);
6. Mollusc populations, particularly wild molluscs, may be difficult to locate due to their habitat (e.g. buried clams, subtidal populations);
7. Geographical areas frequently contain many species and age classes of molluscs derived from different sources, causing difficulty in identifying separate mollusc populations to which surveillance programmes can be targeted;
8. Wild mollusc populations are very important for the disease status of a country but are not necessarily available for sampling at specific times.

1.4. Specifications according to clinical status

In the case of clinical infection, besides target organs and tissues, organs such as mantle, palps, etc., showing macroscopic abnormalities or lesions should also be sampled. Samples from ten diseased or moribund molluscs should be taken for the pathogen test(s). Parallel samples ($n > 10$) from apparently normal animals in the same production region should also be collected.

2. General processing of samples

All sampled molluscs should be delivered alive to the approved diagnostic laboratory. The laboratory should be informed of the estimated time of arrival of the sample so the required materials to process the molluscs can be prepared before reception of samples.

Mollusc samples should be packed in accordance with current standards in order to keep them alive. If the sampling site is a long distance from the laboratory, moribund animals or those with foul-smelling tissues may be of little use for subsequent examination. Required samples should be shipped as soon as possible after collection from the water so as to reduce air storage and possible mortality during transportation, especially for moribund diseased molluscs. Unless otherwise specified moribund animals should be sent on ice (but not frozen) to reduce sample decomposition.

For samples that cannot be delivered live to the diagnostic laboratory, because of advanced stages of disease, long distance or slow transportation connections, etc., specimens should be fixed on site as recommended in the following sections of this chapter or the individual disease chapters of this *Aquatic Manual*. While this is suitable for, for example, subsequent histology or transmission electron microscopy examination, other techniques, such as fresh smears, tissue imprints, routine bacteriology, mycology or Ray's fluid thioglycollate culture of *Perkinsus* spp., cannot be performed. Diagnostic needs and sample requirements should be discussed with the diagnostic laboratory prior to collection of the sample.

Samples should be accompanied with background information, including the reason for submitting the sample (surveillance, abnormal mortality, abnormal growth, etc.), gross observations and associated environmental parameters, approximate prevalence and patterns of mortality, origin and nature of the molluscs (species, age, whether or not the samples are from local mollusc populations or stocks transferred from another site, date of transfer and source location, etc.). This information should identify possible changes in handling or environmental conditions that could be a factor in mortality in association, or not, with the presence of infectious agents.

2.1. Macroscopic examination

The gross observation of molluscs should target, as far as possible, animal behaviour, shell surface, inner shell and soft tissues.

It is often difficult to observe the behaviour of molluscs in open waters. However, observation of molluscs in certain rearing facilities, such as broodstock in tanks and larvae in hatcheries, can provide useful indications of disease-related behavioural changes. If signs are noted (e.g. pre-settlement of larvae on the bottom, food accumulation in tanks, signs of weakening, etc.), samples may be examined for gross signs, including observation under a dissecting microscope for abnormalities and deformities, fouling organisms, and fixed for further processing as recommended below. For adults and juveniles, signs of weakening may include gaping, accumulation of sand, mud and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, decreased activity (scallop swimming, clam burrowing, abalone grazing), etc. The righting reflex of abalone after being inverted does not occur in weakened animals, and it is a good indicator of weakness. Open-water mortality should be monitored for patterns of losses, and samples should be collected for further analysis. Environmental factors, pre- and post-mortality, should be recorded.

Even under culture conditions, the shells of molluscs may not be clean and fouling organisms are normal colonists of mollusc shell surfaces. Organisms such as barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, etc., do not normally threaten the health of molluscs. Culture systems, such as suspension and shallow water culture, can even increase exposure to fouling organisms and shells may become covered by other animals and plants. This can affect health directly by impeding shell opening and closing or indirectly through competition for food resources. Signs of weakening associated with heavy fouling should be a cause for concern rather than fouling itself. Shell damage by boring organisms, such as sponges and polychaete worms, are usually benign, but under certain conditions may reach proportions that make the shell brittle or pierce through to the soft-tissues. This degree of shell damage can weaken the mollusc and render it susceptible to pathogen infections. Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but may not be indicative of a disease concern. Burrowing epibionts may cause deformities and weaken the shell(s). Abnormal coloration and smell may indicate a possible soft-tissue infection that may need to be examined at a laboratory.

The molluscs should be opened carefully so as not to damage the soft tissues, in particular the mantle, gills, heart and digestive gland. The presence of fouling organisms on the inner shell surface is a clear indication of weakness. The inner surface of the shell is usually smooth and clean because of mantle and gill action. Perforation of the inner surface may occur, but can be sealed off by the deposition of additional conchiolin and nacre. This may result in formation of mud- or water-filled blisters. Blisters may also form over superficial irritants such as foreign bodies. The degree of shell perforation can be determined by holding the shell up to a strong light. Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be brought intact to the laboratory or fixed for subsequent decalcification, as required. The appearance of the soft-tissues is frequently indicative of the physiological condition of the animal. Soft tissues should be examined for the presence of abscess lesions, pustules, tissue discoloration, pearls, oedema, overall transparency or wateriness, gill deformities, etc., and, when found in association with weak or dying animals, these abnormalities should be a cause for concern.

Abnormalities and lesions of the tissues should be noted and recorded, as well as any shell deformities, shell-boring organisms and conspicuous mantle inhabitants. Levels of tissue damage should be recorded and samples of affected and unaffected animals collected for laboratory examination as soon as possible.

2.2. Examination of stocks where abnormal mortality occurs

Abnormal mortality of molluscs is usually recognised as a sudden sizeable mortality that occurs in a short time between two observations or inspections of the stocks (for example, about 15 days in the case of facilities located in an inter-tidal zone). In a hatchery, abnormal mortality results in the failure of successive production of larvae coming from different broodstock. Given the broad spectrum of species, environments and culture conditions, these definitions should be adapted when and where necessary.

Whenever abnormal mortality occurs in stocks of molluscs, an urgent investigation should be carried out to determine the disease determinants.

The samples taken should be collected, preserved or fixed and stored in accordance with the procedures described in this *Aquatic Manual*.

Where and when available, unaffected or control molluscs should also be fixed for histological comparison with abnormal tissues. Whatever the fixative, it is essential that the shell be removed to allow easy ingress of the fixative. Bivalves and operculated gastropods can keep the shell shut against fixative until autolysis begins.

2.3. Diagnostic methods

Techniques applicable to mollusc disease agents are limited to direct detection of the causative agent. Classic serological methods cannot be used for diagnostic purposes because molluscs do not produce antibodies. In addition to histology and cytology, immunoassays using monoclonal antibodies or nucleic acid probes can be used for the detection of listed disease agents. From this point of view, the development of DNA-based diagnostic techniques for mollusc disease agents has certainly been the most significant advancement in recent years. Given the development and potential for widespread application of these diagnostic techniques and the inherent problems currently associated with their use, the issue of validation is of the utmost importance.

Three levels of examination procedures are proposed in the following sections. Histology is recommended as a standard screening method because it provides a large amount of information. It is particularly important because macroscopic examination usually gives no pathognomonic signs or solid indicative information. Also, mortality may be caused by several disease agents or physiological problems, such as loss of condition following spawning, and this can only be determined using histology. Screening (surveillance) is also routinely performed by histology. However, according to each epidemiological situation, and when it is justified, targeted surveillance may rely on other techniques.

When abnormal mortality outbreaks occur, histology is also recommended. Various presumptive diagnostic methods can be used in addition to histology, among which, tissue imprints, Ray's fluid thioglycollate medium (RFTM) culture or the polymerase chain reaction (PCR) may be used, as recommended in the individual disease chapters. Such methods may provide advantages of quick and/or cheap procedures as an answer to suspicion of infection with a given disease agent.

When a disease agent is encountered during screening or mortality outbreaks, molecular methods are increasingly being used, in addition to electron microscopy, for specific identification. Some of the OIE listed diseases for molluscs are caused by disease agents belonging to genera encompassing closely related species. Specific protocols designed to detect certain listed agents are recommended in the following chapters, to be used to confirm histological examination results and/or give a species-specific diagnosis.

2.4. Histological techniques

As there is generic use of histology in diagnostic procedures for diseases of molluscs, a detailed technical guideline is provided in this chapter.

Histology is a technique that is used to study the structure of cells and tissues under light microscopy. Tissue preparation involves different steps, including tissue fixation, dehydration, impregnation and embedding of samples, preparation of sections, staining and mounting of slides.

Live moribund animals or freshly dead (within minutes) animals provide the optimum conditions under which to collect tissues. Frozen samples should be avoided because of tissue lysis that occurs during the freeze-thaw cycle. Should a delay between animal mortality and sampling occur, it is recommended that animals be stored intact on ice or in a refrigerator. A standard section should be taken through the digestive gland, to include the gills, mantle and palps, where possible. Alternatively for large specimens, several sections should be taken to include all the important tissues.

2.4.1. Tissue fixture

The role of the fixative is to maintain the morphology of the tissues as close to *in-vivo* morphology as possible and to prevent post-sampling necrosis. Recommended fixatives used for the study of marine molluscs are Davidson's solution and Carson's solution for large specimens. For smaller specimens, glutaraldehyde fixatives may be used and are compatible for electron microscopy use. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation. Non-formaldehyde preservatives may be used following the manufacturer's instructions. These should be used with caution until the user is satisfied with the results. Whether the formaldehyde substitute can be used in the same manner as formaldehyde needs to be tested by the investigator.

Davidson's solution:

Sea water	1200 ml
95% alcohol	1200 ml
36–40% formaldehyde ¹	800 ml
Glycerol	400 ml
Glacial acetic acid	10% (add extemporaneously)

Carson's solution:

NaH ₂ PO ₄ ·2H ₂ O	23.8 g
Sodium hydroxide (NaOH)	5.2 g
Distilled water	900 ml
40% formaldehyde ¹	100 ml
Adjust the pH to 7.2–7.4	

**Stock 1G4F solution(may be held at 4°C for up to 3 months):*

37–40% buffered formalin solution**	120 ml
50% glutaraldehyde	20 ml
Tap water	360 ml

***Buffered formalin solution:*

37–40% formaldehyde	1 litre
Disodium phosphate (Na ₂ HPO ₄)	15 g
NaOH	0.06 g
Phenol red (pH indicator)	0.03 g

Working solution(should be prepared immediately prior to use):

Filtered ambient seawater	500 ml
Stock 1G4F solution*	500 ml

¹A saturated 37–39% aqueous solution of formaldehyde gas

There is no universal fixative and choice should be made taking into account later use of fixed material as well as practical aspects of fixative use (price, component availability, etc.). Davidson's solution is an excellent choice for preserving the structure of the tissues. In addition, tissue sections fixed in Davidson's solution can be stained later by different histochemical methods, as well as *in-situ* hybridisation with DNA probes. For this purpose, over-fixation (over 24–48 hours) should be avoided. Carson's solution may not be as good as Davidson's solution for histological analysis. Nevertheless, it does allow good preservation of the ultrastructure and may be used to preserve samples for later study by electron microscopy. 1G4F also provides the flexibility of preserving tissues for both histology and electron microscopy, but tissue optimum thickness is 2–3 mm. 1G4F produces high quality histological slides and good electron micrographs. As electron microscopy may be a valuable adjunct in diagnosing or confirming infections in molluscs, fixation of some samples (especially smaller samples) using glutaraldehyde, as described in Section 2.5.1 of this chapter, may be considered and will provide the highest quality electron micrographs. Otherwise, material fixed in Carson's solution, and shown to contain adequate levels of targeted disease agents or abnormalities, can be refixed in glutaraldehyde. It is recommended that part of the mollusc be fixed in Davidson's solution while the other part is fixed in Carson's solution or 1G4F for further investigation. This should be done in order to ensure fixation of all tissues/organs in the two fixatives. If neither is available, 10% formalin buffered with filtered seawater is adequate. Within each country, the mollusc aquaculture industry should agree on the most effective way of ensuring adequate fixation.

2.4.2. Dehydration, impregnation and embedding of the samples

The embedding of the samples in paraffin requires several steps during which the water contained in the tissues is progressively replaced, first by alcohol, then by xylene or equivalent less toxic clearing solution, and lastly by paraffin.

After having fixed the samples in Davidson's, Carson's or 1G4F solution, they are transferred through graded alcohols (70–95 [v/v]) before final dehydration in absolute ethanol. The alcohol contained in the tissues is next eliminated by immersing them in xylene. The tissues are then impregnated with paraffin, which is soluble in xylene, at 60°C. These steps may be all carried out automatically using a tissue processing machine. Should processing be delayed, preserved tissues may be stored in 70% ethanol.

Blocks are produced by letting the tissues cool in moulds filled with paraffin on a cooling table; cooling and moisturising are essential to section cutting.

2.4.3. Preparation of the sections

After the blocks have been cooled on a cold plate, which allows the paraffin to solidify, histological sections of about 2–5 µm are cut using a microtome. The sections are recovered on histological slides, drained and dried for up to 1 hour at 40–42°C or overnight at room temperature. Drying the samples allows the excess moisture to be eliminated and thus the sections adhere to the slides.

2.4.4. Staining and mounting the slides

Before staining, the paraffin is removed from the sections by immersing them in xylene or equivalent less toxic clearing solution for 10–20 minutes. This is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10-minute periods each, and they are then rehydrated through a descending series of ethanol baths (for example 95%, 70%, 50%, 30%, 10 minutes each) with a final immersion in a bath of tap water for 10 minutes. Different topographical or histochemical staining techniques can then be performed.

When haematoxylin-eosin (H&E) stain is used (haematoxylin or equivalent), nuclear and basophilic structures stain a blue to dark purple colour, the endoplasmic reticulum stains blue, while the cytoplasm takes on a grey colour. The acid dye eosin stains the other structures pink. This staining technique is simple and reproducible and, although it only allows a limited differentiation of cell structures, it is possible to detect any abnormalities in tissue and cellular structure. Other techniques may be applied to demonstrate particular structures or features as required (e.g. trichrome for connective tissue and cytoplasmic granules).

2.5. Transmission electron microscopy methods

As transmission electron microscopy is very frequently used for confirmatory identification of disease agents in diagnostic procedures for diseases of molluscs, detailed technical guidelines are provided in this chapter.

Fixation for electron microscopy should be done immediately after the animal has been killed, before fixation for histology. Only samples taken rapidly from live animals will be of any use. The preparation of samples for electron

microscopy involves the following steps: tissue fixation, decalcification of the samples (when necessary), dehydration, impregnation and embedding of the samples, preparation and counterstaining of the sections.

2.5.1. Tissue fixation

For tissues that are to be examined by electron microscopy, it is important that the fixation be performed correctly in order to cause as little damage as possible to the ultrastructure. The specimens are cut such that their dimensions do not exceed 1–2 mm. This small size allows the various solutions to penetrate rapidly into the sample.

Fixation of the samples is carried out directly in 3% glutaraldehyde for 1–4 hours. The samples are washed in buffer three times, then fixed in 1% osmic acid (aqueous OsO_4) and washed twice again in buffer. Various formulations of glutaraldehyde fixative and buffers work equally well.

In order to cause as little damage as possible to the ultrastructure, the samples are treated with solutions that have an osmolarity close to that of the tissues. Thus, mollusc tissues are treated with solutions with an osmolarity of approximately 1000 mOsm. The osmolarity of the solutions is adjusted with artificial sea salts or NaCl. As mollusc tissues are nearly iso-osmotic with seawater, it is possible to make the glutaraldehyde up with 0.22 μm filtered seawater, and use the filtered seawater for subsequent washes.

Sodium cacodylate	0.4 M: 8.6 g in 100 ml of distilled water
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Sodium chloride	10% in distilled water
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Cacodylate buffer, pH 7.4:

1000 mOsm

Sodium cacodylate	50 ml from 0.4 M stock solution
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NaCl	20 ml from 10% stock solution
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Distilled water	30 ml
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Adjust the pH to 7.4

3% Glutaraldehyde:

1000 mOsm

25% glutaraldehyde	2.5 ml
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0.4 M sodium cacodylate	5 ml
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10% NaCl	3.5 ml
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Distilled water	9 ml
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1% Osmic acid:

1000 mOsm

4% Osmic acid	1 volume
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0.4 M sodium cacodylate	1 volume
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NaCl	1 volume from 10% stock
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Distilled water	1 volume
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5% ethylene diamine tetra-acetic acid(EDTA):

Disodium EDTA	5 g
Cacodylate buffer	100 ml

EDTA dissolves when the pH is above 8. When the solution becomes clear adjust the pH to 7.4 by adding concentrated HCl.

If the samples have been previously fixed and stored in Carson's solution, they should be washed several times in a bath of buffer before fixation with 3% glutaraldehyde. Tissues preserved in 1G4F may be directly post-fixed in 1% osmic acid solution.

2.5.2. Dehydration, impregnation and embedding of the samples

The samples are dehydrated in successive baths of ethanol: 70% ethanol once, 95% ethanol twice, absolute ethanol three times. The dehydration is completed by two baths of propylene oxide, which allows the subsequent impregnation with Epon or other resin.

The samples are impregnated progressively. After a first bath in a mixture of polypropylene oxide-Epon (50/50), the samples are placed in a bath of Epon. The longer the incubation, the better the impregnation of the tissues.

Embedding is carried out by placing the samples in moulds filled with Epon resin. A label identifying the sample is included in each block and the blocks are then placed at 60°C (the temperature at which Epon resin polymerises) for 48 hours.

2.5.3. Preparation of the sections and the counterstaining

The blocks are cut to appropriate sizes with a razor blade and the sections are then cut using an ultra microtome. Semi-thin sections (0.5–1 µm) are cut and placed on glass slides. These will be used to control the quality of the samples by light microscopy and to find the areas of interest on the section.

The semi-thin sections are stained at 90–100°C with 1% toluidine blue solution. After drying, the slides are mounted under cover-slips with a drop of synthetic resin and observed under the light microscope.

Ultra-thin sections 80–100 nm thick are placed on mesh copper grids for electron microscopy analysis. Uranyl acetate and lead citrate are used to counterstain the ultra-thin sections.

2.6. Molecular methods

Molecular techniques usually offer an advantage in sensitivity that is frequently offset by technical problems. PCR is particularly dependent on the conditions under which it is run, and can yield false-positive or false-negative results. Whenever molecular techniques are used, they should be performed with caution and with special attention to the inclusion of adequate positive and negative controls in order to overcome the possible lack of robustness, as well as to maintain adequate accuracy. It is important to recognise that PCR and sequence-based assays only detect pathogen nucleic acid and do not indicate the presence of a living parasite or presence of infection and disease. However, use of a RNA-based PCR assay may indicate the presence of a living parasite but will still not confirm presence of infection or disease.

PCR, PCR-RFLP (restriction fragment length polymorphism), sequencing, *in-situ* hybridisation and immuno-histochemistry are increasingly used in confirmatory identification of disease agents. For these techniques, samples should be prepared to preserve the DNA of the pathogen. Likewise, samples intended for testing with antibody-based methods should be preserved to retain the reactive antigenic sites for the antibodies used.

2.6.1. Sample preparation

Samples selected for DNA-based or antibody-based diagnostic tests should be handled and packaged with the greatest care to minimise the potential for cross contamination among the samples or degradation before the assay can be performed. To prevent contamination, new containers (plastic sample bags or bottles) should be used. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set. Use of household permanent markers (e.g. Sharpies) should be

avoided as their ink dissolves in ethanol, which is used in molecular methods and may result in loss of the sample label. Use pencil or histology pens only to label vials or jars.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

2.6.1.1. Live iced specimens or chilled specimens

For specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in an insulated box and ship to the laboratory

2.6.1.2. Frozen whole specimens

Select live specimens according to the purpose of sampling, quick freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at -20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory

2.6.1.3. Alcohol-preserved samples

In regions where the storage and shipment of frozen samples is problematic, 90–100% non-denatured ethanol (i.e. methanol-free ethanol) may be used to preserve, store, and transport certain types of samples. For instance, whole molluscs (when the specimen is small) and excised tissues from larger molluscs. Pack for shipment according to the methods described above.

2.6.1.4. Fixed tissues for *in-situ* hybridisation and immuno-histochemistry

For this purpose, classic methods for preservation of the tissues are adequate. Davidson's solution is usually a good choice for later use of molecular probes. For DNA, specifically, over-fixation (over 24–48 hours) should be avoided.

2.7. DNA extraction

For DNA extraction, homogenise tissues in 9 volumes of extraction buffer (NaCl [100 mM], EDTA [25 mM], pH 8, sodium dodecyl sulphate [SDS, 0.5%]) are added with proteinase K ($100\ \mu\text{g ml}^{-1}$). Following overnight incubation at 50°C , DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol.

Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternatives. Use of commercial kits should be validated by comparison with a standard phenol/chloroform protocol prior to their routine use in diagnostic laboratories.

For RNA extraction, use 1 ml Tri Reagent (Trizol) per 50 mg of tissue, $5\text{--}10 \times 10^6$ cells or $10\ \text{cm}^2$ of culture plate. Homogenise samples then let set at room temperature for 5 minutes to allow for dissociation of nucleoprotein complexes. Add 200 μl chloroform, shake vigorously and let stand for 15 minutes at room temperature. Centrifuge at 12,000 g for 15 minutes at 4°C . Transfer the aqueous phase to a fresh tube and precipitate RNA from the aqueous phase by gently mixing with 0.5 ml of isopropanol per 1 ml of Tri Reagent. Store samples at room temperature for 10 minutes and centrifuge at 12,000 g for 8 minutes at 4°C . Remove the supernatant and wash the RNA pellet with 75% ethanol and subsequent centrifugation at 7500 g for 5 minutes at 4°C . RNA can be solubilised in water or other appropriate solution.

2.8. Preparation of slides for *in-situ* hybridisation

For *in-situ* hybridisation, molluscs are fixed in Davidson's fixative for approximately 24 hours and then embedded in paraffin, according to the methods described above for histology. Sections are cut at $5\ \mu\text{m}$ thick and placed on aminoalkylsilane-coated slides, which are then dried overnight at room temperature or in an oven at 40°C . The sections are dewaxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in a descending ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K ($100\ \mu\text{g ml}^{-1}$) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 10–30 minutes.

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