Supplementary file 3. A primer on analysis methods

To investigate the potential causes of MMEs, the preserved samples can be stored as explained and later analysed with multiple diagnostic methods, in collaboration with laboratories experienced in each method. The following gives some examples of the kinds of analysis methods that could be considered; however, this is not an exhaustive list, and appropriate analysis methods should be discussed with specialists (before sample collection, if possible).

From tissues frozen at -80°C (e.g., the posterior end of the mussel), small biopsies of key organs (for example, the gut or gonads) could be taken for molecular diagnostic methods. Frozen haemolymph and non-lethaly collected tissue biopsies can also be used for molecular diagnostic methods. Nucleic acids can be extracted from these tissues and analysed with methods such as metabarcoding, which targets specific genes, for example, the 16S or 18S rRNA gene, to investigate the role of certain groups of potential pathogens (Alfjorden et al., 2023; Da Silva Neto et al., 2024; Richard et al., 2021). Broader, untargeted metagenomic methods, which sequence everything in the sample, can also be used. This can be done with prefiltration steps to enrich potential pathogens of interest, such as viruses (Richard et al., 2020, 2022, 2023).

After taking biopsies for molecular analysis from the frozen posterior end of the mussel, the remaining tissue could be homogenised and used for some chemical analysis methods. If targeting harmful chemicals, a specialist should be consulted because whole mussels may be necessary (Section 4.4.4). Etiologies related to harmful chemicals are challenging to identify, partly because there are numerous possible toxins, with a diverse range of analysis methods for investigating each, many of which are poorly studied in mussels. Many methods also require a large amount of tissue (0.5-1 g), making it impossible to preserve enough material from small mussels to address every potentially harmful chemical. It may be necessary to pool samples to get sufficient material for the analysis of a range of chemicals. Ensure to save some tissue for molecular methods before pooling.

In many chemical analysis methods, the preserved tissue is freeze-dried and homogenised before the creation of aliquots for different analyses. For samples collected from the environment, pore water for analysis can be obtained using procedures such as those used by Keimowitz et al. (2016). The remaining sediments can be freeze-dried for 72 h (US EPA, 2015) and then sieved to collect the fine fraction (<63 μm). For both environmental and mussel tissue samples, the appropriate methods for each target analyte should be applied to the sample after appropriate method validation. Example methods include inductively coupled plasma atomic emission spectrometry (ICP-AES), gas chromatography-mass spectrometry (GC/MS), enzyme-linked immunosorbent (ELISA) assays, and colourimetric methods (Campisano et al., 2017; E. Metzger et al., 2019; Wu et al., 2017). Interpreting the results of chemical analyses can be difficult due to a lack of established thresholds for healthy levels of many chemicals in most freshwater mussel species. Toxicity tests for some chemicals have been completed in limited species (Farris & Van Hassel, 2006; Grabarkiewicz & Davis, 2008).However, further research will likely be needed to assess the potential impacts of any discovered toxic chemicals associated with an MME (more information can be found in Southwick and Loftus (2017)).

Samples preserved for histology can be trimmed, sectioned onto slides, stained, and analysed by a pathologist familiar with bivalves. Details of histology sample analysis and interpretation specifically for freshwater mussel MMEs have been provided by Knowles et al. (2023). For a detailed guide with representative images of normal freshwater mussel histology see the work of McElwain and Bullard (2014). Disseminated neoplasia (DN) can also be detected with histology. Although lacking in freshwater mussels, there is much information about the application of histology in the study of DN in marine bivalves (Barber, 2004; Bower, 1989; Burioli et al., 2019; Carballal et al., 2015; Ciocan & Sunila, 2005; M. J. Metzger et al., 2016).

Molecular and chemical analysis methods can be undertaken on histological slides as well if samples are preserved correctly by washing out of formalin into ethanol before 48 hours. Such methods include *in situ* hybridization, immunohistochemistry, and/or PCR (Alfjorden et al., 2023). Transmission electron microscopy (TEM) can also be undertaken on formalin-fixed paraffin-embedded tissues to provide higher magnification and resolution images. However, if planning to use TEM, consider fixing some material in glutaraldehyde, which is more suitable for TEM (Alfjorden et al., 2023; Knowles et al., 2022; Zhang et al., 1986; Zhong et al., 2016).

If sufficient haemolymph and tissue are collected, additional tests using samples frozen at -80⁰C to assess biomarkers that evaluate the general health status of the mussels could also be undertaken. Several biomarker assays have been used in freshwater mussels, including clinical chemistry measurements such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, and protein concentrations (Fritts et al., 2015; Gustafson et al., 2005). Since genotoxic contaminants can induce a plethora of health disorders, assays to evaluate genotoxic endpoints such as the incidence of micronuclei (MN) could be undertaken (Klobučar et al., 2003); however, genotoxicity assays must be done immediately. Recent studies have also demonstrated the application of metabolomics in freshwater mussels as a useful biomarker assay for health assessment during an MME and an indicator of environmental quality. (Legrand et al., 2023; Putnam et al., 2023). Reference ranges specific to the ecosystem and species under study should be established. It may also be possible to adapt similar assays used in marine bivalves (Binelli et al., 2015; Faria et al., 2014; Gagne, 2014; Martínez-Gómez et al., 2017) to freshwater species.

In the case that mussels were sent to a specialist lab for a comprehensive post-mortem examination and sample preservation procedure, the following accillary analysis methods could also be considered.

Several studies have utilised culture techniques when studying freshwater mussel MMEs to compare the bacteria associated with mussels of varying health status by picking unique colonies for molecular identification (Leis, Dziki, Richard, et al., 2023; Leis, Dziki, Standish, et al., 2023; Leis et al., 2019). These cultured samples can also be stored for use in a later experimental infection. If metagenomic samples have been analysed using short-read sequencing technologies, then individual colonies could be sequenced using Sanger sequencing to provide longer reads. However, with the improved accuracy of long-read next-generation sequencing technologies such as Oxford Nanopore Technologies (Oxford, United Kingdom), this may be unnecessary.

Samples collected for haemocytological methods following similar procedures to Burioli et al. (2019) with the addition of N-ethylmaleimide for the preservation of freshwater bivalve haemocytes (Hinzmann et al., 2013) can be searched for neoplastic cells or otherwise abnormal haemocyte morphologies and parasites. There are many reference materials regarding the identification of neoplastic cells in marine bivalves (Barber, 2004; Bower, 1989; Burioli et al., 2019; Carballal et al., 2015; Carella et al., 2013; Ciocan & Sunila, 2005; M. J. Metzger et al., 2016) and the typical morphology of freshwater mussel haemocytes (Burkhard et al., 2009; Evariste et al., 2016; Hinzmann et al., 2013; Salimi et al., 2009; Soares-da-Silva et al., 2002). Haemolymph for this analysis must be processed within 24 hours before haemocytes die.

Cytological methods of tissues can also be used. Examining whole tissues or wet mounts with a dissecting microscope, if available, may help identify tissue abnormalities and infectious organisms in advance of histology. Ectoparasites, such as trematodes, mites, or ciliate protists, may be lost during sampling or fixation for histology. Their identification can be facilitated by examining tissues with a dissecting scope (Brian & Aldridge, 2021), or by microscopic examination of wet-mounted tissue samples (Sharkey et al., 2020). For the latter, fine gill clippings and light scrapings of the integument surface of the foot, mantle, or visceral mass can be transferred to a slide with a drop of saline or freshwater under a coverslip and viewed with a compound microscope. Avoid previously scraped areas when collecting samples for histology. Similar wet-mount methods specific for the quantification of trematodes in the gonads can also be used following the procedures of (Brian & Aldridge, 2020b). The application of this method should not compromise the collection of a full set of samples using the core procedure (Section 4.4.1).

In cases where not enough material is available for both wet-mount trematode quantification and histology or where only non-lethal sample collection is required, gonadal fluid can be analysed according to the methods of Brian & Aldridge (2020a).

Microscopic examination of Diff Quik® stained impression smears of the cut surface of the visceral mass (Sharkey et al., 2020) can provide early identification of microorganisms, especially encysted digenean larvae or intracellular protists. Wet-mounted preparations must be examined immediately, whereas Diff Quik® stained preparations can be stored in a slide box for later review.

Supplementary File 3 References

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