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Critical Review

Review and Development of Best Practices for Toxicity Tests with Dreissenid Mussels

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Abstract: Since their introduction to North America in the 1980s, research to develop effective control tools for invasive mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) has been ongoing across various research institutions using a range of testing methods. Inconsistencies in experimental methods and reporting present challenges for comparing data, repeating experiments, and applying results. The Invasive Mussel Collaborative established the Toxicity Testing Work Group (TTWG) in 2019 to identify "best practices" and guide development of a standard framework for dreissenid mussel toxicity testing protocols. We reviewed the literature related to laboratory-based dreissenid mussel toxicity tests and determined the degree to which standard guidelines have been used and their applicability to dreissenid mussel testing. We extracted detailed methodology from 99 studies from the peer-reviewed and gray literature and conducted a separate analysis for studies using presettlement and postsettlement mussels. We identified specific components of methods and approaches that could be refined or standardized for dreissenid mussels. These components included species identification, collection methods, size/age class distinction, maintenance practices, testing criteria, sample size, response measures, reporting parameters, exposure methods, and mortality criteria. We consulted experts in the field of aquatic toxicology and dreissenid mussel biology on our proposed. The final recommendations contained in the present review are based on published standard guidelines, methods reported in the published and gray literature, and the expertise of TTWG members and an external panel. In addition, our review identifies research needs for dreissenid mussel testing including improved methods for early–life stage testing, comparative data on life stages and between dreissenid mussel species, inclusion of a reference toxicant, and additional testing of nontarget species (i.e., other aquatic organisms). *Environ Toxicol Chem* 2023;42:1649–1666. © 2023 His Majesty the King in Right of Canada. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC. Reproduced with the permission of the Minister of Environment and Climate Change Canada. This article has been contributed to by U.S. Government employees and their work is in the public domain in the USA.

Keywords: *Dreissena*; Toxicant control; Standard methods; Aquatic invasive species; Aquatic invertebrates; Mollusk toxicology

INTRODUCTION

The zebra mussel (*Dreissena polymorpha*, Pallas 1771) and the quagga mussel (*Dreissena rostriformis bugensis*, Andrusov 1897) are freshwater mollusks that have spread from their native range in the Ponto-Caspian region across Europe and North

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America (Son, 2007), causing economic and environmental harm to these newly invaded ecosystems. The adverse effects of dreissenid mussels on freshwater systems have led to their ranking as one of the world's most invasive species (IUCN Invasive Species Specialist Group, 2022; Simberloff & Rejmanek, 2011). The early spread of *D. polymorpha* in Europe (1800s–1960s) is attributed to trade routes and canals that connected many freshwater systems throughout Europe but was limited by several strong barriers (e.g., the Alps that shielded Italy from introduction and the Irish Sea that isolated Ireland), which were eventually breached as a result of enhanced transportation capabilities and reduced water pollution (Aldridge et al., 2004; Van der Velde et al., 2010). Range expansion of *D. r. bugensis*, on the other hand, was delayed and much slower in comparison (after 1940), typically following *D. polymorpha* into infested waters (Karatayev et al., 2015). Dreissenid mussels first arrived in the Laurentian Great Lakes via transoceanic vessels through the St. Lawrence Seaway sometime in the mid- to late 1980s (Benson et al., 2022; Carlton, 2008; Hebert et al., 1989) and subsequently established populations across the basin in both the United States and Canada. From the Great Lakes, overland dispersal of dreissenid mussels is largely attributed to anthropogenic vectors (e.g., transported watercraft) and occurred much faster than the initial spread throughout Europe (Johnson et al., 2001; Karatayev et al., 2015). Dreissenid mussels have spread to large navigable rivers and reservoirs in the eastern United States and Canada (Benson et al., 2022; Mellina & Rasmussen, 1994); westward to the Colorado River system and waters in California, Arizona, and Utah (Benson et al., 2022; Stokstad, 2007); and northward into Manitoba (Fisheries and Oceans Canada, 2014). Global climate change is likely to expand the range of dreissenid mussels into geographical regions previously unsuitable to the species (Gallardo et al., 2013).

Zebra and quagga mussels can coexist in a water body (Karatayev et al., 2015), although quagga mussels have often displaced zebra mussels in many systems they cohabitate (Karatayev et al., 2011; Marescaux et al., 2015; Peyer et al., 2009; Stoeckmann, 2003; Strayer et al., 2019). In a cross-system analysis of dreissenid mussel invasions, zebra mussels most often appeared first at a site and rapidly increased in density. The later arrival of quagga mussels commonly led to a decrease, but not extirpation, of zebra mussel populations, with an overall increase in *Dreissena* biomass at the site (Strayer et al., 2019).

The invasion success of dreissenids is due in part to their high fecundity and free-living planktonic larval (veliger) stage. Dreissenid mussels are broad-cast spawners, releasing sperm and eggs into the water column for external fertilization. A single adult female mussel can produce one million eggs per reproductive cycle (Sprung, 1989, 1991). Typically, reproduction is seasonal, occurring at water temperatures at or above 12 °C; however, reproduction can occur year-round in some systems (e.g., *D. r. bugensis* in the Colorado River; Gerstenberger et al., 2011). Quagga mussels spawn in colder water than zebra mussels (9–10 °C), which may contribute to their higher densities in profundal zones (Claxton & Mackie, 1998; Ram et al., 2012; Stoeckmann, 2003). The microscopic veliger develops from the trochophore and proceeds through several developmental stages (D-hinge, umbonal, pediveliger) and remains in the water column for 18–90 days, depending on water temperature. The pediveliger settles and changes to the benthic plantigrade, which begins the postsettlement stage of the life cycle (Ackerman et al., 1994). Settled mussels secrete byssal threads to maintain attachment to a substrate including aquatic vegetation, rock, native mussels, other dreissenids, and water infrastructure (Ackerman et al., 1994; Gillis & Mackie, 1994; Schloesser et al., 1996, 2006). Quagga mussels are more often found colonizing softer sediments and hypolimnetic zones, compared to zebra mussels, which reach higher densities on solid substrates in littoral zones of a water body (Karatayev et al., 2015; Nalepa et al., 2010). Densities of 800 000–1 700 000 mussels/m^2 have been reported in North America and Europe (Griffiths et al., 1989; Stanczykowska & Nalepa, 1993). More recently, a density of 10 945 056 mussels/m^2 was recorded in Lake Mead, the largest reservoir (in volume) in the United States (Wong et al., 2012), causing complete coverage of benthic habitat and in-water structures.

Dreissenid mussels have few predators outside of their native range that could eliminate a dreissenid population or prevent their establishment in a water body (Culver et al., 2021; Morrison et al., 1997; Naddafi & Rudstam, 2014a, 2014b; Watzin et al., 2008). Once established in newly invaded waters, dreissenid mussels can have substantial adverse effects to ecosystems and economies. They are highly efficient filter-feeders and selectively remove plankton between 1.5 and 100 μm over other sestons (Dionisio Pires et al., 2003; Lei et al., 1996). Whole populations can filter the equivalent of the entire water column daily in some systems (Roditi et al., 1996; Strayer et al., 1999). Dreissenid mussel feeding efficiency has altered food web and energy pathways and shifted otherwise pelagic food webs into a littoral-benthic pathway (Colvin et al., 2015; Fahnenstiel et al., 1995; McEachran et al., 2019; Nalepa et al., 2009). These shifts typically include reduction of phytoplankton production, increased cyanobacteria and filamentous algae abundance, decreased biodiversity, increased benthic biomass, and increased water clarity with concomitant increased littoral production (Limburg et al., 2010; Raikow et al., 2004; Vanderploeg et al., 2010). The selective grazing and filtration efficiency of dreissenid mussels has promoted harmful algal blooms and nuisance *Cladophora* in some systems (Francoeur et al., 2017; Howell, 2018; Raikow et al., 2004; Vanderploeg et al., 2001). Dreissenid mussels can also alter the physical and chemical properties of a water body (Reynolds & Aldridge, 2021) including nutrient cycling and availability (Bruesewitz et al., 2006, 2008, 2009; Marzocchi et al., 2021; Naddafi et al., 2008) and the partitioning and fate of contaminants (Bruner et al., 1994a, 1994b; Cho et al., 2004; Cope et al., 1999; Endicott et al., 1998).

Globally, dreissenid mussels are listed in the top 10 of most costly aquatic invasive species (Cuthbert et al., 2021). In the United States, the economic cost to mitigate biofouling by dreissenid mussels at industrial and public water facilities, docks, and marinas is estimated to be from $100 million up to $1 billion per year (Pimentel et al., 2005; Strayer, 2009). Data from 2014 to 2016 estimated the cost to prevent dreissenid mussel introduction in Pacific Northwest provinces (three

provinces) and states (eight states) at $13.2 million annually, while failure to prevent introduction was estimated to cost over $500 million annually (Pacific Northwest Economic Region & Pacific States Marine Fisheries Commission, 2015). More recent cost estimates that account for the increased number of infested facilities and inflation are lacking. Moreover, estimates do not consider the lost value in altered or removed ecosystem services (Pejchar & Mooney, 2009). Recent estimated costs of quagga mussels on future recreational fishing in the Great Lakes ranged from $0 (status quo) to upward of $128 million dollars per year (worst case, increase in population; Lauber et al., 2020).

Efforts to manage dreissenid mussel populations to reduce their ecological and economic effects have been ongoing since their North American invasion in the 1980s. In 2015, the Invasive Mussel Collaborative (IMC) was established to "advance scientifically sound technology for invasive dreissenid mussel control to produce measurable ecological and economic benefits" (Invasive Mussel Collaborative, n.d.). The IMC includes representatives from federal, state, provincial, tribal, and private agencies. Management goals within the IMC strategy include the development and evaluation of control methods for dreissenid mussels. Control tool development relies on the use of "best practices" for generating data and consistency in test methodology and reporting. Various state and federal agencies, universities, consultants, and others have contributed to dreissenid mussel control research by testing a variety of potential control tools under various scenarios. However, inconsistencies in experimental design and reporting, discussed in the present review, present challenges for comparing data, repeating experiments, and applying results. The IMC established the Toxicity Testing Work Group (TTWG) in 2019, tasked with advancing consistency and coordination in dreissenid mussel toxicity testing methods. Members of the TTWG represent the United States and Canada (US Bureau of Reclamation, US Geological Survey, International Joint Commission), states (Minnesota Department of Natural Resources, Michigan Department of Environment, Great Lakes, and Energy), and the University of Minnesota. The goal of the TTWG is to identify "best practices" and guide development of a standard framework for dreissenid mussel toxicity testing protocols by identifying components of methods/approaches that could be refined or standardized.

Standard toxicity testing methods commonly aim to evaluate the risk of a chemical, contaminant, or effluent to an indicator species. The test species may be chosen because of its ecological, commercial, or recreational importance, as well as its availability and conduciveness to laboratory culturing and testing (ASTM International, 2023). In contrast, the goal of toxicity testing for aquatic invasive species (AIS) management is to develop lethal or efficacious treatments of a control agent to eradicate or suppress the invader, that is, dreissenid mussels. For example, control research studies may be designed to determine relative and selective toxicity of a test article to dreissenid mussels, determine effective treatment concentration and exposure duration of a test article, or develop decontamination protocols. Modification to the test methods may be required to address the objectives of the study and the unique features of dreissenid mussels; however, published standard methods for conducting toxicity tests warrant application in AIS control research wherever appropriate.

Currently no published standard guidelines are avilable for toxicity testing on dreissenid mussels, such as those developed for unionid mussels (ASTM International, 2022). The specific objectives of the TTWG project were to review the literature related to laboratory-based dreissenid toxicity tests; determine the degree to which standard protocols and guidelines have been used and their applicability to dreissenid testing; develop "best practices" for dreissenid testing; identify areas of weakness, knowledge gaps, and future research needs; and establish a publicly available database of dreissenid toxicity test methods and references. A summary spreadsheet of studies is available (IMC, 2022) and is planned to be updated annually.

METHODS

The first stage in the process was to search for existing standard methods and guidelines for conducting toxicity tests with aquatic organisms that could be applied to dreissenid mussels (Table 1).

Next, we conducted a search of published books, papers (peer-review publications and gray literature) and reviewed published bibliographies for all relevant studies on dreissenid mussel toxicity research. We extracted specific information on the study design, collection, handling, test methods, and reporting parameters from each source. Detailed methods from all studies were entered into a main database, including a digital object identified (doi) and/or link to access each paper. For our purposes, we omitted studies from the review and methods analysis that did not measure whole-organism response (e.g., only cytotoxicity or cellular enzyme response) or measured only bioaccumulation or effects of a contaminant. The early life stages (presettlement: trochophore and veliger stages) of dreissenid mussels are microscopic, in contrast to the juvenile and adult stages (postsettlement), and require vastly different methods in some aspects of testing. Therefore, we distinguished between studies that tested presettlement and those that tested postsettlement mussels, or both, and created separate data extraction files for each.

We determined the degree to which standard methods were followed for specific aspects of dreissenid testing, such as holding conditions and the acclimation process; we further identified aspects of testing that were unique to dreissenid mussels and had no current standard recommendation.

For the latter, we identified test methods that have been developed or modified for dreissenid mussels and considered how broadly the method had been applied and reported in the literature. Based on standard guidelines, the literature review, and the experience of TTWG members, we developed a list of 14 outstanding questions about recommended methods for testing dreissenid mussels and solicited input from an external panel of researchers. The panel included eight researchers with expertise in aquatic toxicology and/or dreissenid mussels.

TABLE 1: Examples of standard guides for toxicity testing of aquatic organisms

Standard guide	Citation
ASTM E729-23e1 (2023)	ASTM International. (2023). Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. *Guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates and amphibians* (ASTM E729-23e1).
ASTM E2455-22 (2022)	ASTM International. (2022). Standard guide for conducting laboratory toxicity tests with freshwater mussels. In *Guide for conducting laboratory toxicity tests with freshwater mussels* (ASTM E2455-22).
USEPA (2002)	US Environmental Protection Agency. (2002). *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms* (EPA-821-R-02-012, 5th ed.).
USEPA (2016)	US Environmental Protection Agency. (2016). Bivalve acute toxicity test (embryo-larval). In *Ecological effects test guidelines* (OCSPP 850.1055).
USEPA (1975)	US Environmental Protection Agency. (1975). *Methods for acute toxicity tests with fish, macroinvertebrates and amphibians* (EPA660/3-75-009; PB-242 105/5).
OECD (2019)	Organisation for Economic Co-operation and Development. (1992). Test No. 203: Fish, acute toxicity test. *OECD guidelines for the testing of chemicals*.
SETAC (2019)	Society of Environmental Toxicology and Chemistry. (2019). *Recommended minimum reporting information for environmental toxicity studies* [Technical Issue Paper].
Environment Canada (1990)	Environment Canada. (1990). *Biological test method: Acute lethality test using* Daphnia *spp.* (EPS 1/RM/11).

For each outstanding question, panelists were provided with recommended methods and asked to rank the options or select only the options that were judged acceptable. Most importantly, panelists were asked to provide additional input based on their own research experience. We developed final recommendations from a combination of standard methods, accepted published methods, and agreement of experienced researchers.

RESULTS

We reviewed 120 laboratory-based studies with dreissenid mussels, conducted in the 30 years between 1991 and 2021. Most studies (54.3%) were conducted from 2012 to 2021, followed by 28.6% from 1991 to 2001 and 17.2% in the intermediate period from 2002 to 2011. Gray literature represented 11.4% of studies, and the remaining 89.6% were found in published journals or books. After filtering studies that fell outside of the area of a "control" study (as described above), 99 studies were included in the methods review. We further sorted studies by pre- and postsettlement life stages. Presettled stages, or planktonic stages, include trochophore and veliger stages (D-hinge, umbonal, pediveliger). Postsettled stages include plantigrade through adult stages. Presettled stages were tested in 27.3% (27 of 99) of studies, and postsettled mussels were tested in 86.9% (87 of 99) of studies. Studies that tested multiple life stages (14.1%) were included in both categories.

A standard protocol or method was cited in 26.3% of studies; the most cited method was that of the US Environmental Protection Agency (USEPA; 2002; 16.2%). Most studies (73.7%) did not cite a standard method or guideline that was followed for overall testing; however, in many cases, a standard reference was cited for specific aspects of the experiment (e.g., reconstituted water recipe, lethal concentration determination).

Test organism

Species identification/verification. Both quagga mussels and zebra mussels were tested in 9.1% of studies, 14.1% tested only quagga mussels, and the remainder tested only zebra mussels. A mix of quagga and zebra mussel veligers was tested in 3.0% of studies but determined to be mostly quagga mussels based on the adult population in the source water.

Sources. Toxicity tests with dreissenid mussels have been conducted from sources across the globe (e.g., Italy, The Netherlands, Ireland, Turkey, Bulgaria), with most reported from the Great Lakes Basin in North America (28.3%), followed by the Colorado River Basin in the western United States (13.1%). The source of test animals was not reported in 4.0% of studies.

Presettlement. Veligers were produced by laboratory spawning of adults in 14.8% of studies and commonly collected from surface waters during reproductive periods by drawing water directly from a natural source (29.6%) or by collection with a plankton net (50- to 63-μm mesh net; 55.6%).

Postsettlement. Collection methods include placement and retrieval of artificial substrates and collection from natural substrates. Most studies (65.5%) reported collecting mussels from natural substrates; placement and retrieval of artificial substrates were used in 15.5% of studies. Method of collection was not reported in the remaining studies (19%).

Size/age classes

Presettlement. Veliger stage was reported by size and physical features in 22.2% and 51.9% of studies, respectively. The remaining studies did not distinguish among veliger stages.

Postsettlement. The size range of mussels used in tests ranged from 1 to 35 mm shell length. Test animal size was not reported in 18.6% of studies. Most studies tested adult mussels (94.0%). Juvenile-stage mussels were used in 14.3% of studies. Both juveniles and adults were tested in 8.7% of studies. The designation of juvenile and adult stages was determined by shell length and was inconsistent among studies. Generally, juvenile mussels were defined as <8 to 10 mm shell length.

Holding and maintenance

Presettlement. A holding period for veligers before testing was not reported in 11% of studies; the remaining studies reported holding for ≤24 h. An acclimation period was rare for presettlement stages because testing was commonly initiated within 24 h of collection.

Postsettlement. The duration of the laboratory holding time before testing ranged from <1 day (21%) to >30 days (54%). The duration of the holding period was not reported in 33% of studies. Details on holding conditions varied among studies and ranged from only water temperature to a suite of water chemistry variables. Holding conditions were not reported in 10.7% of studies. Feeding mussels during the holding period was reported in 27.4% of studies. Acclimation procedures were reported in most studies; 20% of studies did not provide acclimation details or reported no acclimation period.

Suitability for testing

Presettlement. Most studies (57.1%) did not report how suitability for testing was determined in early life stages. Veligers that were produced in the laboratory were considered suitable for testing at a specific stage/age in development. Viability of veligers from surface water was determined by estimating percentage alive from a subsample. Flow-through tests that measured settlement did not assess the viability of veligers in the surface water supply but used settlement in control treatments to verify viability.

Postsettlement. The most common criterion used to determine suitability for testing was attachment of byssal threads to a substrate (37% studies), followed by adductor muscle resistance to valve opening (4.7%), no visible physical abnormality (4.7%), and valve closure in response to probing (4.7%). Other criteria included minimal holding mortality, observations of filtering behavior, and response to serotonin (to stimulate spawning). In 5% of studies, more than one criterion was used to determine mussel viability. Criteria for determining the suitability of test organisms were not reported in 49.4% of studies.

TEST METHODS

Sample size and replication

Presettlement. Replication and sample size varied widely in presettlement tests depending on the exposure method (static vs. flow-through) and the source of veligers. Three to five replicates were used in 62.9% of all studies. Static exposures (85.2% of studies) commonly tested three to five (60.9%) replicates with >100 per replicate (50.0%). In flow-through studies that used a surface water supply, sample size varied over time. Water samples were examined to verify presence of veligers in the source water and to estimate veliger density in the source water.

Postsettlement. Most studies (33.3%) tested 3–5 replicates per experimental treatment and used 10–50 mussels per sample. Only 2.4% of studies did not report number of replicates, and 4.8% did not report the number of mussels per experimental unit.

Reporting constituents and exposure methods.

Characteristics of the source water (e.g., temperature, pH, specific conductance) from which mussels were collected were reported in few studies (18.7%), and reports were more frequent when the source water was used as the dilution (test) water. Temperature of source water was reported most often (15% of studies); 7% of studies reported two or more additional characteristics (e.g., alkalinity, hardness, pH, specific conductance, salinity, or total suspended solids). Studies that used source water as dilution water or as a source of veligers (63.0%) reported measurement of two to 17 water chemistry characteristics.

Presettlement. The type of dilution water used in tests was reported in 100% of studies and included source water (70.4%), reconstituted water (22.2%), well water (7.4%), and dechlorinated tap water (3.7%). Of those studies that used source water as the dilution water, 36.4% reported water quality constituents. Most studies exposed veligers in static or static-renewal systems (85.2%). Flow-through chambers were used in 14.8% of studies and included assessment of settlement. Most studies (77.7%) reported a nominal toxicant test chemical concentration. Measured concentration was reported in 18.5% of studies, and 7.4% did not report how concentration was determined. More than one exposure duration was tested in 77.8% of studies; the exposure duration was <48 h in 51.9% of studies, whereas exposures of weeks to months were used in studies (22.2%) that measured settlement response under different conditions or chemical exposures. A postexposure period was not included in 63.1% of veliger studies. In studies that included a postexposure period, veligers were held for 24–96 h (18.5%) or >96 h (9.5%).

Postsettlement. The dilution water used in toxicity studies included source water (34.5%), reconstituted water (19.0%), well water (9.5%), and dechlorinated tap water (21.4%). The type of dilution water was not reported in 12.9% of studies. Most studies exposed mussels in static (45.2%) or static-renewal (33.3%) systems. Flow-through or recirculating systems were used in 20.2% of studies. Concentrations of toxicant were reported as nominal concentration (58.3%) or measured concentration (36.9%). The determination of test agent concentration (nominal or measured) was not reported in 7.1% of studies. The exposure duration was <30 days in 61.6% of studies. Most studies (61.9%) did not include a postexposure period. In studies that included a postexposure period, mussels were held for 24 to 96 h (29.8%) or >96 h (9.5%).

Endpoints/response measures

Presettlement. Mortality was assessed in 81.5% of studies (22 of 27) and was reported as a percentage of mortality (70.4%) or lethal concentration (14.8%); one study measured pesticide-induced apoptosis in embryos and early larval stages (Perry & Lynn, 2009). The only sublethal response reported for veligers was settlement to the plantigrade stage, and it was measured in 22.2% of studies. The criteria used to define settlement or the methods used to quantify settlement were not reported in 11.1% of studies. The remaining studies quantified settlement by enumerating the number of postveliger mussels on artificial

substrates and calculating density per area. Of the 22.2% of studies that measured settlement, 33.3% assessed settlement after prolonged maintenance of veligers in a static system. The remaining studies (66.7%) assessed settlement on substrates in a flow-through system.

Postsettlement. Mortality was the most common endpoint and was reported as percentage of mortality (53%), lethal concentration for a percentage of organisms (LC; e.g., 10%/50%/99% LC [LC10/LC50/LC99]; 39%), or lethal time to mortality for a percentage of organisms (LT; e.g., 99% LT [LT99]; 10%). Sublethal responses were measured in 57% of studies. The most frequently measured sublethal responses were attachment, filtration rate, and valve movement (65.0%). A range of molecular, biochemical, and physiological responses were measured (26.2%) depending on the study goals.

Eight studies evaluated toxicant effects on byssal attachment. Two approaches for assessing byssal attachment were (1) detach mussels from substrate and evaluate byssal thread formation during test agent exposure (75%), and (2) expose attached mussels to the test agent and measure detachment (75%). Attachment was measured as a binary response (attached, not attached; 75%), count of byssal threads (12.5%, $n = 1$), and a byssogenesis index (foot extensions and byssal threads/time; 12.5%, $n = 1$).

Mortality criteria

Presettlement. Mortality assessment commonly included observation under a compound microscope to determine ciliary (63%) and internal organ (40.7%) movement (e.g., gut, foot, velum movement). A vital stain was used in five studies (18.5%). Most studies (54.5%) used more than one criterion to define mortality.

Postsettlement. The criteria used to define mortality included no response to probing (62.1%), gaping (46.6%), no resistance when pressure was applied to open valves (12.1%), and detachment (3.4%). More than one criterion was used in 10.7% of studies. The criteria for determining mortality were not reported in 25.9% of studies.

DISCUSSION AND RECOMMENDATIONS

The goals and objectives of dreissenid toxicant-based studies vary widely and will dictate which specific design and test methods are used. Yet, the importance of following standard and consistent methods whenever possible is also foremost for ensuring repeatable and robust results. The following recommendations aim to outline best practices for dreissenid testing similar to those developed for other aquatic organisms (Table 1) while recognizing that modifications in approaches are necessary to meet specific study objectives. Comprehensive and consistent reporting of study parameters would improve the repeatability of studies, within and among affect laboratories. The following recommendations are based on published standard methods and guidelines, methods reported in the published literature and reports, and the expertise of TTWG members and an external panel of experts.

Test organisms

Species identification/verification. Although zebra and quagga mussels are considered close relatives, differences in their physiology and metabolism can produce variation in their sensitivity to a toxicant (Claudi et al., 2014; Kerambrun et al., 2018; Nowicki & Kashian, 2018; Peyer et al., 2009; Ram et al., 2012); therefore, verifying species identification and reporting the criteria used for identification are important. When a trial is conducted solely for management of a localized population, species identification may be less critical to the study objective but is still important for transferability of results to others.

For all life stages of mussels, the first preference is to choose a location for test organism collection that only has one species (zebra or quagga mussels) present. When the location has both species present, use an adequate subsample to extrapolate the proportion of each species. Report the estimated proportion of each species in the test population.

Presettlement. Distinguishing species by morphological examination of veligers is laborious and time-consuming and requires specialized expertise. Analysis of DNA can be used to verify species but may be impractical for large numbers of individuals. A more practical and recommended approach is to select veliger collection locations with one known species, as stated previously, or use propagated veligers from identified adults. Verify species identification by examination with a compound microscope, and use morphological characteristics, as described in Nichols & Black (1994), to distinguish between quagga and zebra mussel veligers. If using animals collected from mixed populations cannot be avoided, subsample to extrapolate the proportion of each species in the test population and report the method(s) used for identification.

Postsettlement. Shell morphology is suitable for confirming identification of zebra and quagga mussels in locations with one species present. Refer to Claxton & Mackie (1998; early juvenile stages), Mackie & Schloesser (1996), May & Marsden (1992), Pathy & Mackie (1993), and Ram et al. (2012) for descriptions of morphological features. Intermediate morphs can occur in water bodies where ranges overlap (Beggel et al., 2015; Kerambrun et al., 2018). Analysis of DNA is recommended when there is any doubt about shell morphology or when the source is a mix of species (Beggel et al., 2015; Hoy et al., 2010; Marescaux & Van Doninck, 2013; Voroshilova, 2016; Voroshilova et al., 2010) but may be impractical for large numbers of individuals. In a laboratory setting, confirm identification of all individuals, and report the method(s) used for identification. In a field setting, identification may be limited to a subsample of the population.

Sources. Researchers conducting work with dreissenid mussels are responsible for obtaining the required permits for collection and transport of invasive species and following

procedures to minimize risk of transferring invasive species between water bodies and into water supplies. Before field collection of mussels or source water, develop a biosecurity plan that identifies potential AIS hazards in field and laboratory activities and details the procedures for minimizing risk. Follow the biosecurity plan during all aspects of the study.

Presettlement mussels. Laboratory-reared veligers provide test organisms of a known species, age, and environmental exposure and can be produced by inducing adult mussels to spawn in the laboratory. Production and maintenance of early life stages in the laboratory can be laborious, and success depends on culture methods (e.g., pH and alkalinity of culture water; Fisher et al., 1994) and the ability to maintain adults in spawning condition (Stoeckel et al., 2004). For laboratory spawning and culture methods, see Fisher et al. (1994); Nichols (1992); Ram & Nichols (1992); Ram et al. (1996); Wright, Setzler-Hamilton, Magee, Kennedy, & McInnch (1996); Wright, Setzler-Hamilton, Magee, & Harvey (1996), Stoeckel et al. (2004), and Boegehold, Alame, et al. (2018); and Boegehold, Johnson, et al. (2018).

Field collection is the most common method to obtain veligers but presents several challenges. Veliger availability is limited to adult spawning peaks, which can be unpredictable and localized. Development of veligers varies with environmental conditions, and developmental stage may not correlate to age or size (Sprung, 1993). Seasonal variation in environmental conditions may also affect the viability and sensitivity of veligers to a test agent. A field veliger collection will contain other biotic material, including nontarget zooplankton, phytoplankton, and organic debris, which may interfere with the test agent action on veligers. Nevertheless, field collection of veligers may be the most practical method for obtaining an adequate number of test organisms and can be successful when handling and the holding period are minimized. For static tests, veligers can be collected from a water body by plankton nets (53- to 63-μm mesh) or by filtering surface water through a similar-size mesh screen. Extraneous organic debris and nontarget organisms may be removed by size-selective filtering or removal of material with a pipette. Characterize the test population by collecting three or four 1-ml subsamples. Enumerate total number of veligers and number by life stage (D-hinge, umbonal, and pediveliger) in each subsample, and extrapolate densities to the whole sample volume.

A flow-through system that draws directly from an infested water body provides a continuous source of veligers for studies that measure settlement rate (Pucherelli & Claudi, 2017; Waller et al., 2021). Veliger density in the experimental units will vary over time, depending on natural reproduction in the source water. Estimate veliger density and viability in the source water before the start of the test period to ensure that veligers are present. Repeat estimates of veliger density and viability at the midpoint and end of the exposure, at a minimum. To estimate veliger density and viability, filter a known volume of water from the inflow, and enumerate total number of veligers and number by life stage (D-hinge, umbonal, and pediveliger).

Postsettlement. Collection from natural substrates is suitable for experiments on a short time frame and those requiring a range of size classes. Placement and retrieval of artificial substrates are recommended to obtain test animals that are of a consistent age and source, with the same environmental exposure. Depending on the location and method of placement, artificial substrates may also reduce time and effort for collection and handling stress on the mussels. Substrates must be deployed several weeks or months before collection to ensure adequate colonization by mussels. Dreissenids preferentially settle on substrates with rough surfaces, such as wood, concrete, and limestone, and polyvinyl chloride over plexiglass and glass (Kilgour & Mackie, 1993; Marsden & Lansky, 2000). Avoid substrates made from aluminum, galvanized iron, or steel and copper, which are potentially toxic (Kilgour & Mackie, 1993; Marsden & Lansky, 2000). To maximize settlement, place substrates in horizontal orientation, out of direct sunlight, and in areas of minimal wave action. Predation of mussels on exposed surfaces can be minimized by stacking substrate plates separated by 2-cm spacers or by encasing the substrate with mesh to exclude fish.

At all collection sites, ensure that the source water has not been treated recently with a pesticide or is not in an area with inputs of industrial/municipal effluents or contaminants. Report the site of collection and information about the method of mussel collection and transport. At a minimum, measure and report water chemistry and water quality data of the source water at the time of collection including temperature, pH, dissolved oxygen, alkalinity, hardness, and specific conductance.

The study objectives and response measures will determine whether mussels remain attached during collection and transport or are detached from a substrate or clump for transport. Detaching mussels can be stressful and increase metabolic demand for byssogenesis and should be avoided when study objectives include physiological/biochemical response measures or when mussels are tested without a laboratory acclimation period. Yet, transporting mussels attached to a substrate increases the risk of shell damage and transfer of other organisms and organic material into the laboratory. For most acute toxicity tests, mussels should be removed from the substrate in the field and transferred to a container of temperature-acclimated water for transport. Refer to the AIS permit for requirements on the transfer of infested water with the mussels. Remove mussels carefully by severing or cutting the byssus with scissors or a sharpened blade (e.g., scalpel). Avoid pulling on byssal threads. Separate "clumps" of mussels to ensure that moribund, damaged, and dead animals are discarded before transport. Rinse mussels lightly with water to remove organic material and other organisms before placement in the transport vessel.

Size/age class

Presettlement. The planktonic, presettlement stage of dreissenids begins with the early trochophore and ends when the pediveliger settles and transforms to the plantigrade form (Ackerman et al., 1994; Conn et al., 1993). Development is temperature-dependent, lasting from 3 to 6 weeks or more (Nichols, 1996). The sensitivity of each developmental stage may differ (Fisher et al., 1994) and may be critical to the interpretation

and comparability of test results. To determine the composition of presettled mussels in a test, examine a subsample, and estimate the proportion of each presettlement stage. Classify larval stage based on physical features, rather than shell size, including hinge development, shell shape, and the presence or absence of a foot and velum (Ackerman et al., 1994; Nichols & Black, 1994). When size is included with larval features, measure height and length of shell. Height is defined as maximum axis from hinge to ventral shell margin; length is defined as maximum anterior–posterior axis, parallel to hinge.

The following features are used to define larval stages (Ackerman et al., 1994; He et al., 2015):

Trochophore (57–121 μm height)—No velum or shell. The trochophore is not commonly used in tests unless produced in the laboratory from spawning adults.

Straight or D-hinge (70–160 μm height)—Shell has a straight hinge and is shaped as a "D."

Umbonal or veliconcha (120–280 μm height)—Shell has a pronounced umbone near the hinge and is round.

Pediveliger (167–300 μm height)—Shell has a well-developed and prominent umbone and is slightly asymmetrical; a foot is present, has rudimentary gill filaments; may be seen swimming near the bottom and crawling.

Postsettled plantigrade (>158–500 μm height)—Not planktonic, settled mussel; microscopic; shell is triangular.

Postsettlement. The growth rate and size of reproductively mature mussels vary within and among water bodies and geographic regions (Delmott & Edds, 2014; Elgin et al., 2022; Glyshaw et al., 2015). Therefore, no standard shell length can be recommended for separating juvenile and adult mussels of either zebra or quagga mussels. Use test organisms that are of uniform size (e.g., 5-mm range in shell length) and from the same source population to reduce variation in condition and sensitivity to a toxicant (ASTM International, 2023). When the comparative response of juvenile (postsettlement, reproductively immature) and adult (reproductively mature) mussels is needed, determine the age of maturity of the test population by gonadal examination (Ram et al., 1993). To obtain a cohort of juveniles, settlement plates can be placed at a locality and collected after the settlement period (see sections *Discussion and Recommendations*, and *Sources*). Measure shell length (maximum anterior–posterior axis), and report average, standard deviation, minimum and maximum shell length, and number measured in test group. When it is necessary to minimize handling before test exposure, measure mussels at the end of the trial.

Holding, maintenance, and acclimation

Presettlement. Veliger stages should not be held for longer than 24–48 h before testing. Maintain temperature within 3 °C during the holding and acclimation period. When dilution water chemistry differs substantially from the source water, acclimate veligers for 2–24 h, depending on the magnitude of difference between holding and dilution waters. Acclimate by partial water exchange of 10%–20% holding water until veligers are in 100% dilution water. Monitor mortality during the acclimation period, and discard veligers if mortality exceeds 10% during the acclimation period.

Postsettlement. The conditions in which mussels are held in the laboratory and the duration of holding time before testing may have a considerable influence on the viability of test organisms and their response to a test agent (Costa et al., 2008; Kilgour & Baker, 1994). Hold test organisms for at least 1 week before testing to monitor postcollection mortality and behavior (i.e., attachment, filtration, responsiveness) and for acclimation to test temperature and dilution water. Compare source and laboratory holding water temperature and chemistry to determine an appropriate acclimation period (ASTM International, 2023). Provide artificial or clean natural substrate for mussel attachment. Place substrates in horizontal orientation, and reduce light intensity to promote mussel attachment (Kilgour & Mackie, 1993; Marsden & Lansky, 2000).

Observe mussels daily during feeding, cleaning, and water quality monitoring. Remove dead, crushed, and abnormal mussels, including those with open valves that do not respond to disturbance. The number of mussels, size of holding vessels, water volume, and temperature will determine the frequency of water exchange, vessel cleaning, and water quality monitoring. Supply adequate water exchange with continuous flow or periodic water exchanges to maintain dissolved oxygen >5 mg/L (60%–100% saturation) and un-ionized ammonia <35 μg/L during the holding period (ASTM International, 2023). Clean holding tanks as needed by agitating or brushing the tanks to flush out waste. Measure and report daily water quality of holding tanks including water temperature, pH, and dissolved oxygen, at a minimum (ASTM International, 2023). Include weekly measures of alkalinity, hardness, ammonia, and specific conductance, at a minimum.

For extended holding periods (>30 days), maintain mussels at lower temperatures (<12 °C) to reduce metabolic rate and reproductive activity (D. Molloy, Molloy and Associates, personal communication, 2021). The condition and suitability for testing of mussels should be monitored in conjunction with each toxicity test. Mussel condition may be measured by several different condition indices, based on tissue and shell weights, including dry tissue weight/shell length (Luoma et al., 2018), dry tissue weight/(dry tissue weight + dry shell wt; Davenport & Chen, 1987), dry tissue weight/shell cavity volume (Ciparis et al., 2019), and whole-body dry weight/shell length (Claudi et al., 2012). Relative changes in glycogen concentration may also be used as an indicator of mussel condition (Lauer & Spacie, 2000; Palais et al., 2011; Pilotto et al., 2016). Response to a reference toxicant (see section *Use of a reference toxicant*) can also be used as an indicator of relative mussel sensitivity.

When mussels are held in the laboratory for extended periods (>30 days) before testing, provide sufficient quantity and quality of food, and maintain water quality to ensure that mussels are in suitable condition for testing. Mussels held in a

natural unfiltered source water may receive adequate food without supplemental feeding. Their condition can be monitored, as previously described, by measuring condition and/or response to a reference toxicant in conjunction with each experiment. Mussels held in laboratory water without a natural food source may require supplemental feeding. Food ration is dependent on water temperature (based on metabolic requirements) and mussel size. At lower water temperatures (<15 °C), reduce feeding to prevent accumulation of waste. At higher water temperatures (>15 °C), feed mussels daily at 2.0–6.0 mg/L dry weight (ASTM International, 2022; Luoma et al., 2018), and adjust ration for biomass of mussels (Wacker & Von Elert, 2008). Provide aeration to keep food suspended in the water column. The polyunsaturated fat docosahexaenoic acid (DHA) may be an essential and limiting factor for dreissenid mussels. The alga *Isochrysis* aff. *galbana* contains high amounts of DHA and other polyunsaturated fatty acids (PUFAs; Wacker et al., 2002). Feed live or prepared algal diets that include *Isochrysis* supplemented with other algae (e.g., *Nannochloropsis limnetica*, *Pavlova*, *Thalassiosira* sp., *Cyclotella meneghiniana*, and *Chaetoceros calcitrans*) to provide additional fatty acids and minerals (Wacker & Von Elert, 2002, 2008; Wacker et al., 2002; Wright, Setzler-Hamilton, Magee, & Harvey, 1996). For example, a ratio of 1:3:3:5 *Thalassiosira pseudonana*, *Nannochloropsis*, *Thalassiosira weissflogii*, and *Isochrysis* can provide a variety of PUFAs to support mussel maintenance. Report the method (source water or supplemental feeding) that is fed to mussels during the holding period.

Acclimate mussels to the test temperature and dilution water for at least 48 h before testing and longer, if possible (ASTM International, 2022, 2023). Acclimate to temperature at a maximum of 0.5 °C/h or 3 °C/12 h, preferably no more than 3 °C/72 h (ASTM International, 2023). Monitor mortality of mussels in dilution water during the acclimation period. Suspend feeding for 24–48 h before testing to reduce biofouling of test vessels.

Suitability for testing

Presettlement. Veliger stages are more likely to be vigorous and viable if used within 24 h of collection (Edwards et al., 2000, 2002; Pucherelli et al., 2014; Stockton-Fiti & Claudi, 2017; Sykes, 2009). Measure viability before initiating an exposure by examining a subsample of veligers at ×40 to ×100 to determine percentage alive (Stockton-Fiti & Claudi, 2017). The percentage of live veligers should exceed 90%. Include additional vessels (i.e., controls) to monitor natural mortality over the entire duration of the study.

Postsettlement. The condition of mussels used in tests will depend on a range of factors including handling stress, holding conditions and duration, reproductive condition, and nutritional status, among others. Determination of test animal condition is critical for obtaining reliable response measures of a test agent effect.

Hold mussels for at least 1 week before testing to monitor mortality and acclimate to test conditions. Observe mussels daily for signs of feeding activity, feces production, and filtration. Discard mussels that do not close when touched, never open, or do not deposit feces. Do not use for testing if mortality exceeds 0.2%/day for 3 consecutive days before test (ASTM International, 2023). In addition, include at least one of the following criteria to evaluate suitability for testing:

1. Select mussels that are filtering and close the valves when disturbed (Coughlan et al., 2020).
2. Select mussels that resist opening when gentle pressure is applied to open the valves. Discard mussels that can be opened with minimal effort (Luoma et al., 2018; Waller & Bartsch, 2018).
3. Cut the byssal thread 24 h before testing. Select mussels that reattach overnight (Costa et al., 2008; Kennedy et al., 2006; Molloy, Mayer, Gaylo, et al., 2013; Waller & Bartsch, 2018).

When mussels are held for an extended period (>30 days) before testing, consider inclusion of a reference toxicant with each experiment (see section *Use of a reference toxicant*). Substantial shifts in the response measure (LC50, percentage of mortality) to the reference toxicant can indicate a change in mussel condition and sensitivity.

Use of a reference toxicant. A reference toxicant can be useful for assessing intra- and interlaboratory differences in test organisms (ASTM International, 2023), especially when mussels are held in the laboratory for an extended period. In addition, a reference toxicant can be used to compare seasonal, spatial, and genetic differences in sensitivity among mussel populations. The frequency of reference toxicant trials will vary with the duration of a project and study objectives. At a minimum, reference trials may be conducted with the first and last trials of a project or every 2–3 months during an extended holding period. Test the reference toxicant alongside the toxicant(s) of interest. The reference toxicant is most useful when the mode of action is similar to that of the test agent (ASTM International, 2023); however, use of the same reference toxicant across tests with mixed toxicants can demonstrate changes in mussel sensitivity over time and allow for broader comparisons among laboratories and across source populations. Generate full concentration–response curves for inter- and intralaboratory comparisons. The development and compilation of concentration-response data will enable laboratories to construct control charts as an additional acceptance criterion for testing suitability (ASTM International, 2022).

Test methods

Sample size and replication. The number of treatments, replicates, and organisms per treatment will depend on the study objectives (ASTM International, 2023), test system, and mussel life stage. For example, when the study objective is to determine an acute LC50, the design will require a geometric series of at least five concentrations and a control, with a dilution factor of 0.6 between consecutive concentrations (ASTM International, 2023). Proper selection of a concentration range should produce <10%, partial, and complete mortality and enable calculation of an LC50 value. Alternatively, when the

study objective is to determine the percentage of mortality produced by a select concentration or a lethal time to death, only that concentration and a control are needed. Refer to ASTM International (2023), Section 11.1.3, for recommendations on determining the number of treatments, replicates, and mussels per treatment.

Presettlement. Early life stages experience high rates of mortality in natural conditions that can confound results and the ability to detect a treatment effect from a toxicant. To increase statistical power, include additional replicates and control units and organisms per experimental unit to account for natural mortality. In acute toxicity trials, use 100–500 individuals per experimental unit (Davis et al., 2018; Genco & Wong, 2014; Moffitt et al., 2016; Pucherelli et al., 2014; Stockton-Fiti & Claudi, 2017) and a minimum of three replicates per treatment. In settlement rate studies, the surface area of settlement substrates will vary with the test objectives and the test system. Use a minimum of three settlement substrates per experimental unit to account for flow differences within the unit. Include a minimum of three replicates per treatment, including a control.

Postsettlement. Use a minimum of 10–50 individuals per experimental unit and three replicates per treatment. However, dreissenid mussels can detect some chemicals in the water and avoid exposure by closing their valves for an extended period. This toxicant avoidance behavior can produce wide variability in test results. Generally, researchers recognize the variability in dreissenid responses and test sample sizes of at least 25 mussels per experimental unit. The response of mussels to a specific test agent can be determined in a pilot study to observe mussel valve and filtration behavior. If valve closure is observed, increase the number of test animals and replicates to reduce variation within and between experimental units.

Testing conditions and reporting parameters. Environmental conditions can affect mussel sensitivity to stressors, including a toxicant (Choi et al., 2013; Costa et al., 2008; Kilgour & Baker, 1994). Information on the source of mussel stock, date of collection, and source water quality/chemistry should be reported. When possible, collect test animals from the same location and on the same date to minimize environmental influences on mussel response.

Dilution Water. Dilution water can be constructed to a desired chemistry following ASTM International (2023) guidelines for reconstituted freshwater or obtained from a natural source. Do not use dechlorinated water unless there is no other viable option. If source water is from surface water, ensure that the water body has not been treated recently with a pesticide or is in an area with inputs of industrial/municipal effluents or contaminants, as described in section *Test organisms*.

Collection methods. Report the site of collection, and measure and report water chemistry parameters, as described below. Likewise, if well water is used as the dilution water, measure and report water chemistry parameters. Test organisms should demonstrate satisfactory survival and show no abnormal signs in the dilution water if different from natural source or collected at a different time from veliger collection (ASTM International, 2023; see section *Holding, maintenance, and acclimation*). Submit source water samples for chemical analysis if constituents may be present that will affect mussel sensitivity to the test agent (e.g., potassium, copper, chloride).

Tests with presettlement-stage mussels are considerably more challenging than those with postsettled mussels, and test methods for early life stages are less established. Their microscopic size requires additional equipment, expertise, and labor. ASTM International (2022) guidelines for testing glochidia/juveniles of freshwater mussels may be modified for dreissenid mussel early life stages. The study objectives will determine the type of dilution water that is used in the test; however, veligers may be sensitive to significant and/or acute shifts in water chemistry. When the dilution water chemistry varies from that of the source water, acclimate veligers to the dilution water for up to 24 h. Monitor mortality during the acclimation period, and discard veligers if mortality exceeds 10% during the acclimation period (see section *Holding, maintenance, and acclimation*).

For comparability among studies, the source and chemistry of the dilution water used in testing should be measured and reported. At a minimum, report temperature, dissolved oxygen, pH, alkalinity, hardness, and specific conductance. Additional constituents in the dilution water that influence test agent toxicity may be needed, depending on the test agent (e.g., dissolved organic carbon for metal toxicity; Santore et al., 2001; USEPA, 2016).

Water quality measurements during testing. Water quality characteristics (pH, ammonia, hardness, alkalinity, and specific conductance) should be measured in at least one test vessel per treatment at the start and end of acute tests (≤96 h; ASTM International, 2023) and at least weekly in longer-duration tests. Measure dissolved oxygen at the start of a test and at least every 48 h in each test vessel. Measurements should occur when water quality is lowest (e.g., low dissolved oxygen, high ammonia). For example, in static-renewal exposures, measure water quality immediately before each water exchange. Refer to ASTM International (2023) for recommendations on selection of test temperature. To minimize stress and the acclimation period, use a temperature within 5 °C of the source water. Select a standard test temperature from the series 7, 12, 22, and 27 °C, within the 5 °C of source water; temperature >27 °C is not recommended because it approaches the upper thermal limit for dreissenid mussels.

The concentration of the test material in test chambers should be determined by ASTM International or standard analytical methods when possible. Collect a water sample from the midpoint of the test chamber. In acute static and renewal tests, measure test concentration in the control, low, medium, and high treatments at the beginning of the test, at a minimum. An additional time period is advised when the exposure period is >48 h and/or degradation of the toxicant is expected. In flow-through systems, measure the test concentration in all test

chambers at the beginning of the test and at least one additional time (ASTM International, 2023).

Exposure methods

Presettlement. Use test vessels, such as glass beakers or deep Petri plates, which do not crowd veligers or cause turbulence. Veliger test vessels can be constructed by removing the bottom from a small plastic tube (e.g., 50-ml centrifuge tube) and covering it with a nylon mesh screen (<53 μm). These allow immersion into a larger vessel for toxicant exposure and transfer to recovery water (Davis et al., 2016) or fresh exposure solutions. The size of the test vessel should allow for measurement of water quality and toxicant concentration in the test vessel. Limit exposure time to 48–96 h, maximum 120 h, to avoid excess control mortality. Exchange water every 24 h in static tests. Alternatively, ASTM E2455-22 (2022) recommends limiting the duration of an acute toxicity test to <50% of the time that 90% of organisms survive in the test water under test conditions.

Postsettlement. The size of the test vessel or tank will vary with study objectives and the laboratory system. The volume of the test vessel should ensure that minimum water quality standards (e.g., dissolved oxygen, ammonia) are maintained throughout the test. Refer to ASTM International (2023) for loading guidelines in static and flow-through tests. Dreissenid mussels demonstrate reduced tolerance to a toxicant when detached (Kilgour & Baker, 1994; Rajagopal et al., 2002). Therefore, use only mussels that are attached to a substratum for toxicant exposure, except when de novo byssal thread formation is the response of interest. Use clean substrates for attachment of test organisms to reduce organic material in test chambers, which could reduce water quality (e.g., dissolved oxygen and ammonia) and the bioavailability of some test agents. However, when attachment is a response measure, place substrates in holding tanks for 3–7 days to promote biofilm formation and mussel attachment (Kavouras & Maki, 2003; Wainman et al., 1996). Report the type of substrate (e.g., material, size, texture) used for mussel attachment. Mussels are negatively phototactic and preferentially settle and attach in shaded conditions (Kobak, 2001; Marsden & Lansky, 2000). Minimize light intensity by covering test vessels and/or maintaining an ambient light: dark cycle.

Endpoints/response measures

Mortality. Mortality endpoints include lethal concentrations reported by the time of exposure (e.g., LC50, LC99), lethal time to death (e.g., LT50, LT99), and percentage of mortality. Lethal concentrations are generated by exposure to sequentially diluted concentrations of the test agent. The range should include concentrations that produce 10%, partial, and 100% mortality, at a minimum. Lethal concentration values can be compared among test agents, among test organisms, and across laboratories when generated under standard conditions (i.e., exposure duration, dilution water, water temperature). Lethal time to death is generated by exposure to one concentration of the test agent. Exposure durations should include time periods that produce 10%, partial, and 100% mortality. Similar to LC values, LT values are comparable when generated under standard conditions. Percentage of mortality can be compared among treatments in varying concentrations and exposure durations.

Presettlement. Mortality assessment of early life stages should be conducted under a stereomicroscope, at a minimum, or a compound microscope by trained observers. When possible, use the same observer(s) for all readings, and conduct replicate counts on 10%–20% of samples. When more than one observer conducts counts, verify 10%–20% samples between observers (modified from Barbour et al., 1999). If using multiple observers, ensure consistent training, and use quality control checks between observers. Report the number of observers and methods for verifying counts.

Methods for assessment include the following:

Stereomicroscope method (McCartney, 2016): Stain veligers in fast green (Stockton-Fiti & Claudi, 2017) for 20–60 min. Use a stereomicroscope with cross-polarized light (Johnson, 1995) to locate veligers and then examine at ×40 to ×50 without a polarizing filter.

Scoring criteria: mussel

- Live: Larvae are motile; if immotile on first encounter, observe for 2 min, and examine staining pattern.
- Live: Ciliary movement or internal organ movement; green "gut spot" is evident.
- Dead: Immotile and no ciliary movement for 2 min.
- Dead: Diffuse partial staining of internal organs.
- Dead: Diffuse, complete staining of internal organs.

Compound microscope method (Pucherelli & Claudi, 2017; Pucherelli et al., 2014; Stockton-Fiti & Claudi, 2017).

Unstained:

Place 1-ml sample on a gridded Sedgewick-Rafter counting cell, and examine at ×40 to ×100 or examine every veliger in the test at highest magnification during each time point.

Scoring criteria:

- Live: Ciliary movement and/or organ activity; locomotory movement; not moving but organs are intact.
- Dead: No ciliary movement and/or organ activity and abnormal appearance of internal organs and possibly open shells.

Stained:

Stain with fast green solution (Stockton-Fiti & Claudi, 2017) for 20–30 min. Rinse to remove excess stain.

Scoring criteria:

- Live: Nonstained with mantle intact; green "gut spot" is evident.

- Dead: Stained mantle, open shells or nonstained but with no mantle intact; diffuse staining of internal organs (Moffitt et al., 2016; Stockton-Fiti & Claudi, 2017; Whitledge et al., 2015).

Control mortality. Veliger mortality is relatively high in the wild, and collection and handling can further increase mortality of animals used for testing. The ASTM International guidelines (2022, 2023) invalidate results if control mortality exceeds 10% at the conclusion of a test. This guideline may be applied to acute (24-h) toxicity tests but may be impractical for tests >24 h when the daily natural mortality rate may exceed 10%. When the test duration is >24 h, include additional vessels (i.e., controls) for assessment of natural mortality across the entire test period, and assess survival in both controls and treatment replicates at each selected time point, including the postexposure period. Use an adequate sample size of veligers (minimum of 100) in each test vessel to provide a robust measure of natural mortality. Alternative approaches to account for high rates of natural mortality include the following:

1. Use an appropriate statistical model (chi-squared, probit regression of mortality) to compare survival in reference and toxicant treatments (Britton & Dingman, 2011; Moffitt et al., 2016).
2. Use Abbot's formula to correct for spontaneous mortality (unrelated to treatment; Kennedy et al., 2006).
3. Calculate treatment-related mortality as a percentage reduction from control mortality.

Postsettlement. The valves of dreissenids can remain closed after death or sealed when empty, confounding mortality assessments that are solely based on observation and do not include handling individual mussels. Byssal threads can remain attached for days after mussel death; thus, attachment is not a reliable measure of mortality. Cut the byssal attachment, and separate mussels from the substrate or clump to effectively assess mortality. Recommended definitions of mortality are (1) closed valves do not resist opening when slight pressure is applied, and (2) open valves do not close in response to probing.

Other response measures

Presettlement. Preventing settlement of veligers to the plantigrade stage is often the goal of mitigation efforts in water-use facilities. Test agents that aim to prevent settlement should be evaluated in a flow-through system that supplies a viable source of veligers. Settled mussels are defined as plantigrade-stage mussels, approximately 500 µm shell length or more. Assessment of life stage and settlement should be conducted under a stereomicroscope, particularly when mussels are <1 mm shell length. Report settlement as number of mussels per area.

Postsettlement. Test agents that cause detachment at nonlethal concentrations may be effective for preventing biofouling by dreissenids. The effects of a test agent on byssal attachment may be evaluated on detached or attached mussels. Accepted methods for assessing byssal attachment include the following:

1. Detach mussels before exposure to a test agent. Score reattachment during exposure as yes or no (Angarano, 2007; Cope et al., 1997; Rajagopal et al., 2002; Waller & Bartsch, 2018).
2. Detach mussels before exposure to a test agent. Count the number of byssal threads produced during exposure (Giamberini et al., 1995).
3. Expose attached mussels to a test agent. Count the number of mussels that fully detach during toxicant exposure (Waller & Bartsch, 2018).
4. Expose attached mussels to a test agent. Calculate a byssogenesis index: (number of foot extensions × number of byssus threads produced)/time (Rajagopal et al., 2002).

Postexposure period assessment. The inclusion of a postexposure period will depend on the test agent and study objectives. Certain toxicants, such as carbon dioxide and potassium chloride, induce narcosis in dreissenid mussels, producing the appearance of mortality (Fisher et al., 1991; Waller & Bartsch, 2018); the valves gape, the foot extends, and the mussel is unresponsive to probing. Mortality can be overestimated when mussels are evaluated in this condition. Alternatively, lethality of some toxicants can be delayed (e.g., Zequanox; Molloy, Mayer, Giamberini, & Gaylo, 2013), especially in cool water (Luoma et al., 2018). Mortality can be underestimated when mussels are evaluated immediately after exposure to a toxicant that causes delayed mortality.

Presettlement. Because of the high rate of natural mortality in early life stages, limit a postexposure period to 96 h or less. Transfer veligers from test water into recovery vessels with untreated water. To avoid handling mortality, use containers that confine veligers and can be transferred from the test vessel to the recovery vessel (Davis et al., 2016). In a static exposure, the container and veligers should be rinsed several times. In a flow-through system, the container with veligers can be immersed into the recovery vessel to wash and dilute the toxicant (Davis et al., 2018). Examine veligers every 24 h for up to 96 h, and adjust for decreased survival of control animals during the recovery period (e.g., Abbot's formula; see section *Control mortality*).

Postsettlement. Transfer mussels to untreated water for at least 48-h postexposure to monitor delayed mortality and allow recovery from narcotizing toxicants. A longer postexposure period may be needed and should be determined based on the expected mode of action of the toxicant and the water temperature. For example, Zequanox is a biopesticide that causes atrophy of the digestive epithelium (Molloy, Mayer, Giamberini, & Gaylo, 2013). In cold water (e.g., 13 °C), mortality may occur over an extended period, up to 30–60 days (Molloy, Mayer, Gaylo, et al., 2013; Molloy, Mayer, Giamberini, & Gaylo, 2013). Monitor mortality of mussels in

the control treatments during the postexposure period. Test results are acceptable if mortality of mussels in control chambers is <10%.

KNOWLEDGE GAPS AND FURTHER RESEARCH NEEDS

Dreissenid control research has a broad scope with widely ranging goals and objectives.

Studies may aim to identify new control agents by screening candidate toxicants against both dreissenid mussels and nontarget species (i.e., other aquatic organisms) under the same standardized conditions in dilution water with a prescribed water chemistry. In contrast, other studies may aim to determine the efficacy of a registered pesticide for preventing settlement of veligers in a specific water body. Each study will necessarily be designed to address the unique questions posed by the research while also recognizing the importance of following standardized methodology, reporting parameters, and generating comparable endpoints. For example, it may be possible to identify and quantify variables that affect toxicant efficacy such as mussel source and water chemistries. Conducting toxicant trials in standardized conditions will improve the comparability of toxicant efficacy and our ability to identify new candidate toxicants with quantitative structure–activity relationship (QSAR) modeling (Cherkasov et al., 2014). A major barrier to the proper application of QSAR modeling is equivocal endpoints (e.g., 24-h LC50 versus 96-h LC90), which could be rectified by researchers adhering to a standardized method of examining toxicants and reporting common endpoints. A Web of Science search for the application of QSAR modeling on zebra and quagga mussels yielded no return, likely because of a lack of data with similar methods and endpoints to inputs despite the many publications of lethal and sublethal effects of substances, as summarized in the present review.

The major inconsistency found in our review of published dreissenid toxicity studies was the level of detail reported. Regardless of the project goal, inclusion of key details on methods, organisms, environmental parameters, and so on provides context for interpretation of results and practical application of the test agent. These details can also inform modifications or improvements in research methods. Adhering to minimum reporting guidelines outlined in standardized methods would provide important information.

Further research needs

The present review unveiled several areas of research that have received less attention and could produce meaningful advances in dreissenid mussel control efforts.

Expand research efforts on early life stages of dreissenids. Early life stages of dreissenids are a challenge to obtain and maintain in a laboratory setting, as evidenced by the small number of veliger studies. A primary research need in this area is to refine methods for laboratory propagation and maintenance of early life stages. Studies with presettled stages have generally used trochophore- to D-hinge-stage larvae. Methods for rearing veligers to the settled stage (plantigrade) are not well established and could provide a uniform population of organisms for testing. In addition, concurrent trials with laboratory-reared and wild-caught veligers are needed to determine differences in sensitivity between the two sources. A second need in early–life stage research is a cost-efficient method to assess life stages and mortality. Current methods to assess live/dead status of early life stages can substantially limit the testing capability of many laboratories. Development of flow cytometer–like systems (see Chowdhury & Hamerly, 2022; Hassett et al., 2021) with image recognition software that distinguishes live/dead status could decrease time and labor for early–life stage testing and enable testing of greater numbers of samples and individual animals.

Compare responses of early life, juvenile, and adult life stages. Generally, early life stages of dreissenids are considered more sensitive to stressors, including chemical agents. However, few studies have directly compared (within or between laboratories) the sensitivity of pre- and postsettled stages to a test agent and considered how differences may be affected by the mode of action of a toxicant or type of stressor.

Determine sensitivity of adults pre- and post-spawning. Zebra mussels show seasonal variation in sensitivity to several molluscicides, which was explained by water temperature, physical condition, and filtration rate (Costa et al., 2008). Similar studies with both quagga and zebra mussels across a broad geographic range would be beneficial. These data would expand understanding of seasonal influences on dreissenid mussel sensitivity and inform the timing of control actions.

Expand comparative testing of zebra and quagga mussels. Few studies have tested quagga mussels and zebra mussels in comparable exposures to the same test agent. Differences in their physiology, behavior, and reproduction have been documented; but less is known about how these translate to differences in approaches for control actions. Does the mode of action of a test agent influence interspecies differences?

Establish a database for reference toxicants. The database would facilitate intra- and interlaboratory comparisons of results and expand knowledge of spatial, temporal, and genetic influences on dreissenid mussel sensitivity.

Determine the relative influence of intrinsic and extrinsic factors on the sensitivity of dreissenid mussels to a test agent. Standard test methods provide the opportunity to generate comparative data to understand factors that influence sensitivity or resistance of a mussel population to a control tool (e.g., season/reproductive status, sex, genotype, parental exposure, laboratory holding conditions).

Determine the influence of laboratory holding conditions and duration on sensitivity of dreissenid mussels to a test agent. Mussel condition is generally expected to decline during extended holding in a laboratory setting. However, holding conditions may provide higher-quality food and water than that encountered in a natural water body and should be documented and reported with study methods. Comparative tests to compare the sensitivity of mussels in the field (across seasons) with those held in the laboratory under different conditions (12 °C vs. 20 °C, fed vs. unfed) would be informative.

Expand testing of nontarget species in conjunction with dreissenid mussel testing. Selectivity of a test agent and its safety to nontarget species in the early stages of research on a control agent can determine the scope of its use (i.e., closed or open water) and whether further evaluation is warranted. Nontarget data are required for the registration process of new pesticides by the USEPA and Organisation for Economic Co-operation and Development countries and are considered by regulatory agencies when approving permit applications.

CONCLUSIONS

The products of our review are available on the IMC website and include a searchable table of studies, an Excel file with metadata from each study for download, and a summary table of recommended "best practices" for dreissenid testing. The recommendations contained in the present review are based on accepted standard methods, current knowledge, and published methods. The database of dreissenid control studies are planned to be updated annually, and concomitantly modifications or improvements to the "best practices" are planned.

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