



Detection of Apoptosis in Early Life Stages as a Tool to Evaluate Chemical Control of Invasive Species

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PURPOSE: Traditionally, the efficacy of chemical control strategies for aquatic nuisance species (ANS) has been assessed using indices of acute mortality. However, such lethality endpoints may be overly conservative estimates of control. Lower doses of chemicals may significantly compromise organisms at the cellular level and eventually lead to mortality or suspension of development. These effects are likely to occur prior to any observable mortality. The use of cell integrity assays may help to further optimize control strategies for ANS by assessing chemical sensitivity at substantially lower concentrations than traditional acute toxicity studies revealed in a companion technical note, “Application of life stage sensitivity data in chemical control strategies for invasive animal species” (Millward et al. 2007). This technical note focuses on one particularly sensitive assay that may provide an alternate measure of efficacy to acute lethality. While it is intuitive that chemical control strategies designed for the more sensitive life stages might require lower treatment doses, absolute mortality may not be required for effective population control of a target species. Many benefits, including lowered cost, reduced risk for nontarget species, and increased effectiveness of control strategies could be realized by understanding how lethality is induced. In addition, detecting sublethal concentrations that may impair or inhibit reproduction, recruitment to the population, and the continuation of the life cycle could further enhance our understanding of control efficacy. For these studies, sensitivity at different life stages was assayed by a suite of cell bioassays that convey information regarding the health and vitality of individual cells in embryos and larval stages and the developmental competence of early life stages.

BACKGROUND: Global transportation has transformed normally innocuous species in their native ranges into potential nuisance species by moving them into new environments favorable for expeditious reproduction and growth. Although most transported species probably pose little threat, occasional species manage to compete locally and thus impact native ecosystems. Successful invaders are typically characterized by high fecundity, short generation times, with relatively broad feeding and habitat preferences, and considerable physiological tolerances (Erlich 1989, Williams and Meffe 1999).

Control measures for invasive species such as bivalve molluscs frequently rely on chemical treatments and are often easy to implement and cost-effective (Sprecher and Getsinger 2000). One challenge, however, is to provide an adequate margin of safety for non-target species in compliance with environmental regulations. One approach for effective control and reducing environmental impact is to focus on the most sensitive life stages. In general, aquatic species pass through several developmental life stages. Several studies have demonstrated that life stages (gametes, larvae, post larvae and adult) have very different sensitivities to chemicals in their environment. For example, the larval stages of the zebra mussel, *Dreissena polymorpha*, are usually significantly more sensitive than the adults (Sprung 1993, Stoeckel and Garton 1993, Mackie and Kilgore 1994, Steevens et al. 2004). Analysis of chemical controls on early life stages continues to focus on more conservative mortality parameters such as LC50 (50 percent mortality) and LC99 (99 percent mortality) values.

Toxicological studies have also indicated that individual organisms show variability in their response to chemical stressors, and that population extinction or survival is a direct response to this inherent variability. Variability would be expected to be greater, however, in adult organisms than earlier life stages. Larval bioassays for monitoring environmental pollutants have established sensitivity of early life history stages and in particular the differential variability in sensitivity between species (His et al. 1999). This study presents a complementary approach to traditional toxicological studies that evaluates effects at the cellular level to determine concentrations that would induce cell death, thereby interrupting progression to the next developmental stage in lieu of acute mortality. As a comparative approach to evaluate sensitivity, these cellular indices were measured in parallel with traditional lethality studies (see companion tech note, Millward et al. (2007)).

Early life stages of bivalve molluscs are typically comprised of: (1) gametes that are released freely into the environment, (2) a series of rapid divisions (the cleavage stage), (3) a hatched embryo called a swimming blastula, and (4) a swimming trochophore stage that metamorphoses into a D-shell stage larva. The developmental pattern in molluscs is referred to as determinative development. Extensive studies over the past 100 years have established that individual cells of these embryos have a fixed fate even at the two-cell stage. If a single cell is eliminated during early development, the successive larval stages are missing specific tissues that would have been derived from that blastomere (Wilson 1904, Charby 1887, Render 1997). Such classical studies were able to demonstrate that the abnormal larvae developing after blastomere deletion would rarely metamorphose into reproducing adults.

MATERIALS AND METHODS:

Test organisms. Adult *Mytilus galloprovincialis* were obtained from Carlsbad Aquafarms (Carlsbad, CA) or Penn Cove Shellfish Inc. (Penn Cove, WA). Spawning induction followed the methods of Millward et al. (2007) and gametes were pooled for the effects of the toxicants on life stages. Water quality was also monitored according to Millward et al. (2007).

Exposure chemicals. The toxicity experiments on gametes and early life history stages used copper (Cu), chlorine (Cl₂) and the proprietary molluscicide, Bayluscide[®] (Bayer CropScience, Research Triangle Park, NC). Chemical concentrations and dosing strategies were based on experimental procedures of Millward et al. (2007) that were run in parallel with these studies. In the post-fertilization tests, eggs and sperm were combined for 30 min to allow fertilization to occur before addition of chemicals. In addition, some post-fertilization tests were continued to 168 hr to assess delayed effect. Tests were conducted at 16 ± 1 °C, an appropriate temperature for *Mytilus* species (American Society for Testing and Materials (ASTM) 2004). Controls in artificial seawater (ASW) were conducted in parallel with mussels for each stage.

Sub-lethal cell bioassays. The sub-lethal toxicity trials consisted of three fluorescence-based assays: a Live/Dead[®] Sperm Viability Assay, a mitochondrial activity assay, and an APO-BrdU[™] TUNEL Assay (Invitrogen, Carlsbad, CA) for fragmented DNA.

The Live/Dead[®] Sperm Viability Assay (L-7011, Molecular Probes, Eugene, Oregon) was used for testing the viability and fertilizing potential of sperm. Sperm were collected within 30 min of initial spawning at concentrations of approximately 10⁷ to 10⁸ sperm/ml. Aliquots of sperm were treated in

2.0-ml microcentrifuge tubes for 15 min and labeled with SYBR® 14 dye (L-7011, Molecular Probes, Eugene, Oregon) and propidium iodide (L-7011, Molecular Probes, Eugene, Oregon). SYBR® 14 dye, a membrane-permeant nucleic acid stain used to label live cells with intact membranes, fluoresces bright green. The propidium iodide dye, which labels sperm with damaged cell membranes, fluoresces red. Samples were placed onto slides and scored for a minimum of 100 sperm cells.

The JC-1 Mitochondrial Potential Sensor Assay (T-3168, Molecular Probes, Eugene, Oregon) was used to detect mitochondrial depolarization, reflecting a reduced rate of respiration that occurs in cells metabolically inactive or experiencing impending cell death. Aliquots of sperm were treated in 2.0-ml microcentrifuge tubes with JC-1 dye, a cationic dye that exhibits potential-dependent accumulation in mitochondria. In healthy cells, the JC-1 molecules aggregate into a polymer called j-aggregates that fluoresce red. After incubating 10 min in the absence of light, sperm were exposed in each chemical treatment 20 min. Samples were placed onto slides and 100 sperm were scored. Mitochondrial depolarization was indicated by a green fluorescence and more active mitochondria fluoresced red.

The APO-BrdU™ TUNEL Assay (A-23210, Molecular Probes, Eugene, Oregon) was used to detect DNA fragmentation in apoptotic cells in the embryos and larvae. As a cell's DNA becomes fragmented, the 3'-hydroxyl ends are exposed and deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) can then be enzymatically coupled to the break sites. Once incorporated into DNA, an anti-BrdU antibody conjugated to Alexa Fluor® 488 (A-23210, Molecular Probes, Eugene, Oregon) binds to BrdU and the nucleus fluoresces green. Sub-acute toxicity was tested at 4 hr after initial exposure of post-fertilization, 24-hr, 48-hr, and 72-hr old larvae. Additional samples were taken at 24 hr after exposure for each time step to provide toxicity information comparable to the studies of Millward et al. (2007). Samples of fertilized eggs were placed into 2.0-ml microcentrifuge tubes, fixed in 1-percent paraformaldehyde (#15700, Electron Microscopy Sciences, Hatfield, PA), washed in seawater twice, and stored in 70-percent ethanol at -20 °C overnight. The remaining procedures follow the Molecular Probes (Eugene, Oregon) protocol. Hoescht 33342 (#B2261, Sigma-Aldrich, St. Louis, MO) was added to each sample for nuclear co-localization to ensure that BrdU labeling was constrained to the nucleus. Scoring was categorized as the life stage of each larva (for the Carlsbad population¹) and the number of nuclei in each embryo that fluoresced. Green fluorescence localized to the nucleus was indicative of fragmented DNA heralding apoptosis.

Data analysis. An apoptotic index (API) was established by calculating the percentage of embryos in each life stage with greater than five apoptotic cells as determined by the TUNEL assay. Each concentration was compared to the control embryos per chemical and time point. Abbott's correction was used to account for control mortality (Abbott 1925). Apoptotic indices were interpreted as EC50 values, or the concentration at which 50 percent of the test organisms exhibited an effect. Thus, the API based EC50 value was the calculated dosage concentration at which 50 percent of the embryos or larvae had more than five apoptotic cells (determined by fluorescence). These EC50 values were then compared with LC50 values generated from Millward et al. (2007) to compare the relative sensitivities of the cell assays compared to the lethality assays.

¹ The Carlsbad population was selected for this analysis because the developmental stages of the Penn Cove larvae were more asynchronous than originally anticipated.

RESULTS: In these studies, effects of chemical treatments on either the gametes or the embryos/larvae were limited to Cu and Bayluscide for the Penn Cove population and Cu, Bayluscide, and chlorine for the Carlsbad population.

Effects on gametes.

Live/Dead Sperm Assay. No general trend was observed when sperm exposed to Cu were assayed for compromised membranes (Table 1). Typically, sperm only showed compromised membranes when exposed to the highest concentration of Cu (470 µg Cu/L). The sperm exposed to Bayluscide were not distinguishable from controls except at the highest concentration of Bayluscide (1000 µg/L) in Penn Cove sperm whereas Carlsbad sperm showed that effects as low as 0.1 µg/ml maintained a similar level of effect out to 1000 µg/ml. Cl₂ treated sperm (Carlsbad animals) showed increasing effects beginning as low as 26 µg/L with 96 percent affected at 2400 µg Cl₂/L.

Table 1. *Mytilus galloprovincialis* EC50 values for cell assays and LC50 values for lethality tests determined for sperm exposed to CuSO₄, Cl₂ and Bayluscide.

Chemical	Assay	Population	EC50 (µg /L)	LC50 ¹ (µg /L)
Cu	Live/Dead	P ²	7.8 ³	25
	Mitochondria (JC-1)	P	2.0	
	Live/Dead	C	500	ND
	Mitochondria (JC-1)	C	ND	
Cl ₂	Live/Dead	C	625 ³	ND
	Mitochondria (JC-1)	C	39	
Bayluscide	Live/Dead	P	1000	12.1
	Mitochondria (JC-1)	P	0.1	
	Live/Dead	C	0.1	ND
	Mitochondria (JC-1)	C	0.1	

¹ See Millward et al. (2007) for LC99 values and 95 percent confidence intervals.

² P = Penn Cove population. C = Carlsbad Aquafarm population. ND = No data.

³ Peak effect was greater than controls, but <50 percent.

Sperm Mitochondrial Assay. The Cu results showed a peak in the percentage of affected mitochondria in 2.0 µg Cu/L but the peak level was not sustained at higher concentrations. For sperm treated in Bayluscide, distinctively greater percentages of sperm with depolarized mitochondria were observed in 0.1 – 15.6 µg/L and almost 100 percent of the sperm mitochondria were compromised at 1000 µg/L. The JC-1 Assay did demonstrate that mitochondrial respiration was substantially depressed beginning in 0.4-µg Cl₂/L and increased in higher Cl₂ concentrations. Mitochondrial depolarization resulting from Bayluscide exposures was first seen at 0.1 µg/L and was similar to all the higher concentrations.

Effects on early life stages (TUNEL assay).

4-hr sub acute post-fertilization treatment. For Cu, the apoptotic index (API) of both populations increased in 2.0 µg Cu/L with a peak at 7.8-µg Cu/L but were indistinguishable from controls in higher concentrations (Table 2). Cl₂ did not induce APIs different from the control in Carlsbad animals until the highest concentration of 2500 µg Cl₂/L. The Bayluscide treatments presented similar trends with cell damage sharply increasing in 0.1 µg/L and peaking in 0.2 µg/L for Carlsbad and 1.0 µg/L for Penn Cove, but followed by a decreasing API in 3.9 to 1000 µg/L.

24-hr acute post-fertilization treatment. Notable cell damage and a correspondingly high API were observed in the 7.8-µg Cu/L, analogous to the sub acute 4-hr exposure. In contrast, Carlsbad embryos were indistinguishable from controls in the Cu treatments. A low API was also observed in Cl₂ exposures compared to controls with notable shift in the API (to 80 percent) not occurring until 397-µg Cl₂/L. The Penn Cove API shifted only slightly from the 4-hr exposure API of Bayluscide to 0.2 µg/L while the Carlsbad presented a larger shift to a peak in 63 µg/L. In both populations, the Cu and Bayluscide exposures had no observed apoptotic cells in the higher concentrations.

4-hr sub acute treatments of 24-hr-old larvae. (Carlsbad only) API levels were consistently higher in 7.8- to 5-µg Cu/L compared to controls. When treated with Cl₂, results were parallel to the 24-hr acute fertilization treatment with the only API different from controls occurring in 2500-µg Cl₂/L. The Bayluscide API EC₅₀ increased to 3.9 – 63 µg/L and peaked in 250 µg/L.

24-hr acute treatments of 24-hr-old larvae. Relative to the control, the API peaked in the 7.8- to 31.3-µg Cu/L treatments, and decreased to levels of no apoptotic cells in 500-µg Cu/L. The Cl₂ (Carlsbad only) treatments did not show any increases in API distinguishable from controls until 625-µg Cl₂/L and the API was 100 percent by 2500-µg Cl₂/L. The Penn Cove and Carlsbad Bayluscide exposures both displayed API peaks in 0.2 µg/L and 16 µg/L, respectively, but less than an EC₅₀ value. At higher concentrations of Bayluscide, the API was not distinguishable from controls.

4-hr sub acute treatments of 48-hr-old larvae. APIs were not distinguishable from control levels in any Cu exposures. In the Bayluscide treatments, API's were greater than the control, indicating greater potential for cell death, with an EC₅₀ of 63 µg/L and increasing at higher concentrations.

24-hr acute treatments of 48-hr-old larvae. The API in Cu treatments increased in 125-µg Cu/L, peaking at 500-µg Cu/L compared to the 4-hr subacute treatments of 48-hr larvae. The Bayluscide APIs were variable in 0.1 – 63 µg/L, but increased in 250 µg/L with a peak in 1000 µg/L.

4-hr sub acute treatments of 72-hr-old larvae. (Penn Cove only.) The development of both exposed and unexposed Penn Cove embryos was slower than anticipated and the normal treatments that were to be performed at 48 hr were delayed until 72 hr after fertilization. The larvae at this time point did not show any apoptotic effects from Cu until the API jumped to 100 percent in 125-µg Cu/L. In Bayluscide treatments, a single peak in the API was observed at 250 µg/L. At all other concentrations of Bayluscide, the APIs were indistinguishable from the controls.

Table 2. *Mytilus galloprovincialis* apoptotic index values (EC50) and acute lethality values (LC50) determined for early life stages (i.e., embryos and larvae) exposed to CuSO₄, Cl₂ and Bayluscide.

Chemical	Life Stage	Population	Exposure	API (EC50)† (µg /L)	LC50 (µg /L)
Cu	Post-fertilization	P P	4 hr 24 hr	7.8 ^b 7.8	8.0
	24 hr	P P	4 hr 24 hr	ND 7.8^a	40.7
	48/72 hr	P P	4 hr 24 hr	125 ^c ND	47.3
	Post-fertilization	C C	4 hr 24 hr	7.8^a **	34.2
	24 hr	C C	4 hr 24 hr	7.8^a 31.3 ^b	28.8
	48 hr	C C	4 hr 24 hr	** 500 ^c	ND
Cl ₂	Post-fertilization	C C	4 hr 24 hr	2500 625^a	716.9
	24 hr	C C	4 hr 24 hr	2500 625	ND
Bayluscide	Post-fertilization	P P	4 hr 24 hr	1.0^a 0.2^a	16.8
	24 hr	P P	4 hr 24 hr	ND 0.2 ^b	12.4
	48 hr	P P	4 hr 24 hr	250 ^b ND	125.0
	Post-fertilization	C C	4 hr 24 hr	0.1^a 63.5 ^c	31.9
	24 hr	C C	4 hr 24 hr	63.5 16 ^b	31.8
	48 hr	C C	4 hr 24 hr	63 1000	ND

P = Penn Cove population. C = Carlsbad Aquafarm population. ND = No data.

† The apoptotic index (API), based on the presence of greater than five apoptotic cells present in the larvae, was used to calculate effects concentrations in 50 percent of the test population (EC50).

* See Millward et al. (2007) for LC99 values and 95-percent confidence intervals.

** No distinguishable differences from control.

^a Concentration with peak API less than concentration predicted by LC50, suggesting greater sensitivity.

^b Maximum API was greater than controls, but <50 percent.

^c API increases abruptly from <30 percent to >70 percent.

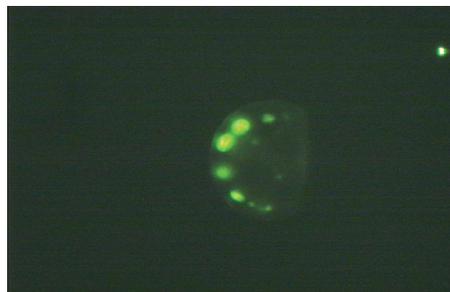
DISCUSSION: The cell bioassays for apoptosis provided a sensitive marker for degenerating cells in the early life stages of *Mytilus galloprovincialis* based on the number of cells with degenerating DNA. In the parallel study, Millward et al. (2007) demonstrated that early life stages of *M. galloprovincialis* were significantly more sensitive to chemical control than were the adult animals. The apoptotic assays, however, suggest that the post-fertilization life stage has even greater vulnerability to the toxicants, copper and Bayluscide, than reported by the more traditional toxicological lethality data after a 24-hr exposure. The current data using an apoptotic index indicate potential for impacting population recruitment using even lower toxicant levels. The ability to detect changes in the DNA as a marker of fitness rather than overt, premature death has great promise in long-term assessment of ecosystem integrity (Evenden and Depledge 1997).

Traditional embryological studies have established that loss of even a single blastomere, as predicted by our cell assays, at these early cleavage stages will adversely affect the continuation of development. The results may include death, deformities suppressing reproduction, or loss of developmental competence when embryos cannot metamorphose into later developmental stages (Wilson 1904, Render 1998, Mwatibo and Green 1997). At the cleavage stage, which predominates during the first 12 hr post-fertilization, the significance of the number of apoptotic cells may be understated since the presence of only 1 or 2 apoptotic nuclei during early cleavage may predict arrest of the life stage in invertebrates exhibiting determinative developmental patterns. In the early cleavage life history stage, any number of apoptotic cells will result in critical cell damage. In these studies, however, detrimental effects were conservatively estimated as requiring at least five apoptotic cells.

Embryos assayed using the TUNEL method appeared most sensitive during the earliest life stages when mollusc embryos are likely the most sensitive to loss of blastomeres. Past a given toxicant threshold, the API was not as great and probably reflects rapidly induced cell death that results in mortality at concentrations reported by Millward et al. (2007). The detection of apoptosis in the earliest stages (i.e., cleavage and swimming blastula) occurred at lower dosages than the reported LC50 in their cohorts (Table 2, Millward et al. 2007). The API shows its greatest utility at these early stages (to 24-hr) where it predicts toxic effects, particularly in Cu and Bayluscide treatments, at levels below the concentrations determined by traditional mortality data. A key factor in these observations is that death does not have to occur at the earliest stages (fertilization through formation of the trochophore larvae) to have significant effects on developmental competence.

In the trochophore and D-shell stages, larvae were about equally sensitive to Cu and Bayluscide, whether they were exposed for 4 hr or 24 hr within a given size class. Once the developmental stages passed cleavage and swimming blastula, three additional phenomena were noted. First, later life stages generally required higher levels of toxicant for cell death and were similar to or exceeded the acute lethality values reported by Millward et al. (2007). Secondly, the peak API required higher doses of the toxicant for the sub acute 4-hr exposures than for the acute 24-hr exposures. For example, compare the peak API for Bayluscide in the 24-hr class between the 4-hr exposure (63 µg/L) and the 24-hr exposure (16 µg/L). In the later-stage embryos, apoptotic cells showed some tendency to group near the periphery of the embryo, suggesting partial inhibition of toxicant penetration into the deeper cell masses of the embryo; this was particularly evident for the D-shell stage (Figure 1).

Figure 1. D-shell stage larvae showing six apoptotic cells (green fluorescence) following the arch of the vellum (i.e., curved) side of the embryo. Embryo was treated with Bayluscide for 4 hr at a concentration of 63 $\mu\text{g/L}$.



Lastly, the API is not always the best indicator for developmental competence in every type of toxicant. Surprisingly, Cl_2 did not induce apoptosis at levels beyond the control until the highest concentration of 2500 $\mu\text{g/L}$, indicating that an acute exposure to Cl_2 was not necessarily inducing DNA fragmentation in *M. galloprovincialis* larvae even at 24 hr post fertilization.

Although the focus of this technical note was to evaluate several cell integrity assays for comparing with traditional mortality parameters such as LC_{50} 's, the cell integrity assays chosen were not always amenable with the life stages tested. For example, the Live/Dead Assay was performed on both sperm and ova. While the ova and cleavage stage embryos may have had compromised cell membranes, the egg coat was of such integrity that the fluorescent dyes were not able to penetrate into the cell.

In addition, when ova were assayed with JC-1, the mitochondria were uniformly depolarized in all of the samples, indicating a reduced respiration level. Prefertilization ova are quiescent and cellular respiration is naturally low for this life stage. Mollusc embryos show only a modest increase in respiration following fertilization and during the cleavage stages (Needham 1963). Together, these observations would indicate the ability to discriminate the mitochondrial states may be beyond the detection limits of the assay used for this particular species. Nevertheless, both types of assays merit future investigation to further assess their usefulness as a cell bioassay in the earliest life stages and for other ANS.

CONCLUSION: This technical note describes cell bioassays chosen to measure the developmental competence of an embryo or larval stage. The data presented suggest that apoptotic cell assays, which predict cell death, are a sensitive methodology to detect early changes in embryos challenged with some, but not all, chemical treatments. This methodology could be very useful as short-term validation of control efficacy. Data collected from the same cohorts in a separate, but complementary, study (Millward et al. 2007) are summarized as follows:

- Apoptotic cell assays were capable of detecting damaged embryos at lower concentrations for Cu and Bayluscide treatments, but not Cl_2 .
- Pulse treatment (4-hr sub acute treatments) induced apoptosis in a pattern similar to longer (24-hr) acute treatments.
- Cleavage and swimming blastula stages were more sensitive than the later trochophore and D-shell stages.

In general, for the earliest life stages, the apoptotic index (API) was predictive of LC50 values in a much shorter time frame (4 hr vs 24 hr), further supporting this as a tool for quick screening of control efficacy when time is of the essence. Although the data seem to predict 168-day LC50 values from the 4-hr assays as well, confirmation will require additional pulsed treatments with tracking of the life stages to predict effects on actual recruitment.

Based on the above data, the authors recommend use of an apoptotic index (API) for very sensitive monitoring of early life stages of ANS with four specific implementations:

- 1) API monitoring during toxicant treatments to determine lethal effects in chemical control strategies.
- 2) Use of API to evaluate field specimens from contaminated environments.
- 3) Use of API to monitor developmental competence of early life stages of model systems to evaluate environmental contamination.
- 4) Use of API to rapidly determine control efficacy of new invasions that necessitate immediate management.

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