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Research Article

Use of a differential simple stain to confirm mortality of dreissenid mussel veligers in field and laboratory experiments

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Editor's note:
This study was first presented at the 19th International Conference on Aquatic Invasive Species held in Winnipeg, Canada, April 10–14, 2016 (http://www.icais.org/html/previous19.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Assessment of mortality in toxicological trials of dreissenid mussel veligers is often time consuming and can be erroneous. Recommended methods for scoring mortality or survival of veligers include observing each individual to detect external or internal movements or lack thereof. Post-mortem infestation of tissue with decomposers frequently mimics movements of internal organs and leads to inaccuracy in assessment. Conversely, studies determining the toxic effect of potassium have found that veligers may appear dead after exposure, with shells agape, vellum exposed and no movement. However, after a recovery period in potassium free water, the test subjects may recover and move. Differential stains have been used to aid in scoring mortality or survival and we evaluated fast green stain compared to direct observation without stain.
Fast green stain is used as a food coloring dye, and is a differential stain that penetrates dead tissues only, allowing for easy and accurate determination of live and dead tissues. The stain is used for assessment of saltwater mussel spat viability and more recently with freshwater dreissenid larval and juvenile life stages. Fast green allows for a differentiation between paralyzed and dead veligers following exposure to potassium without the need for a recovery period. In this study, we exposed dreissenid veligers to elevated levels of potassium chloride and evaluated the proportion of live and dead, and empty shells using two methods: one with the fast green stain and one without. We found the staining method resulted in more rapid and precise results without need for a long recovery step. We recommend this technique as beneficial to both novice and experienced microscopists. The fast green stain method is easy to use in the field or laboratory setting.

Key words: quagga and zebra mussels, invasive species, toxicity, health analysis, food coloring

Introduction

Toxicity studies with zebra mussel, *Dreissena polymorpha* (Pallas, 1771) and quagga mussel *Dreissena bugensis* (Andrusov, 1897) veligers rely exclusively on determining the status of the veligers post exposure. The most frequently used methods for observing and scoring condition of treated dreissenid mussel veligers are to observe each individual for up to 1 min and identify internal movement or other signs of life (Horvath and Lamberti 1999; Edwards et al. 2000; APHA 2005). Alternately, samples are retained for 3–24 hours post-exposure in recovery water and observed for internal movement (Fisher et al. 1991;

Waller et al. 1996; Sykes 2009; Pucherelli et al. 2014). Observation of veligers is complicated by post-mortem infestation of tissues with decomposers, such as bacteria. Observed movements due to these decomposers can be interpreted as movement of veliger organs or cilia. Determination of veliger status post exposure is further complicated when potassium is used as a toxicant in dreissenid mussel toxicity studies. Potassium causes muscle tissues in veliger and adult life stages to relax and cease movement (Fisher et al. 1991; Sykes 2009). When potassium is removed from the system, movement returns (O'Donnell et al. 1996; Sykes 2009; Pucherelli et al. 2014). When observing the veligers immediately after a potassium treatment, researchers found that veligers look dead with shells agape and vellum exposed with no movement; but after a recovery period in potassium free water, the test subjects were alive and swimming (Sykes 2009; Pucherelli et al. 2014).

Other methods for distinguishing live from dead individuals have been proposed for dreissenid species. Neutral red dye has been used to distinguish live from dead veligers, but researchers found high variation in the absorption among veligers and accuracy required a protracted staining time of up to 3 h (Horvath and Lamberti 1999; Gray 2005; Sykes 2009). Snider et al. (2014) investigated the use of neutral red and trypan blue to determine survival, independent of behavior, but found results were too ambiguous and variable to be used reliably.

Fast green FCF, a synthetic food dye (Food Green 3), has been used to determine viability of veligers in recent studies (Link et al. 2013; Whitledge et al. 2015). This dye has been used on mammalian cells as an exclusion dye for viability testing in cancer research (Weisenthal et al. 1983). In aquaculture, fast green is used to determine health of mussel spat prior to distribution from the nursery (Webb and Heasman 2006). Fast green stain has also been used to differentiate tissue damage in Chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792) (Elliott et al. 2009).

In this study, we compare the precision of results and time needed for analysis using fast green staining with a protocol using only direct examination of veligers without the aid of special stain after treatment with potassium chloride.

Methods

Quagga mussel veligers were collected at Willow Beach National Fish Hatchery (WBNFH), AZ (35º52′N; 114º39′W) from pumped Colorado River water (18.7 °C) in a raceway with a 50 µm plankton tow net. The collection was passed through a 300 µm filter onto a 63 µm filter and back flushed into a beaker with 30 mL of 35 µm filtered river water to obtain a concentrated sample of veligers. Veliger concentrate was assessed for density of live, (physically moving or tissue movement of veliger), dead, (cracked shells or degraded tissue), or empty shell (with no tissue and shell remaining). If veliger concentrate contained >1% dead to live veliger density, the plankton collection was not used. The veliger concentrate was divided into testing beakers to achieve approximately 100 to 200 live veligers per beaker, ranging in size from D-shaped (>63 µm) to pediveligers (< 300 µm) per replicate in 10 mL of filtered river water. This division was done within 1 h after density evaluation. Three replicate beakers were used to evaluate the staining or no staining methods after a treatment with potassium chloride (KCl) toxicant. We also evaluated two replicates that were held with no KCl treatment to serve as controls. We applied 90 mL of a stock solution (9.35 g/L KCl) of analytical grade KCl (CAS 7447-40-7; FW 74.55, Macron Chemical Company, Center Valley, PA, lot 66919) to obtain a final treatment concentration of 8.5 g/L KCl. For the controls, we added 90 mL of 35 µm filtered river water. All vessels were held for a 3 h duration before analysis at room temperature (20 ± 2 °C).

To end each replicate, the contents of each beaker were poured onto a 63 µm filter and the beaker was rinsed three times with filtered river water onto the filter for analysis. Veligers were back flushed from the filter into a recovery beaker with <5 mL filtered river water or placed into fast green stain.

Assessment of veliger condition and time for processing

The proportion live and dead in the samples of quagga mussels was analyzed using two methods: without and with stain. In the no stain method, veligers were examined post exposure with the aid of a compound microscope. A 2 mL sample of veliger concentrate was removed from each recovery beaker with a disposable pipet and individuals were recorded as live, dead, or empty using a gridded Sedgewick-Rafter counting cell and compound microscope (total magnification of 40 and 100×). Each veliger was observed individually for up to 30 sec to assess ciliary movement and/or organ activity (Figure 1). When movement was difficult to assess at 40×, the veliger was examined at 100× to determine if the veliger had tissue or cilia movement. If movement was not observed, the veliger was scored dead. Live veligers and empty shells with no tissue were also recorded.

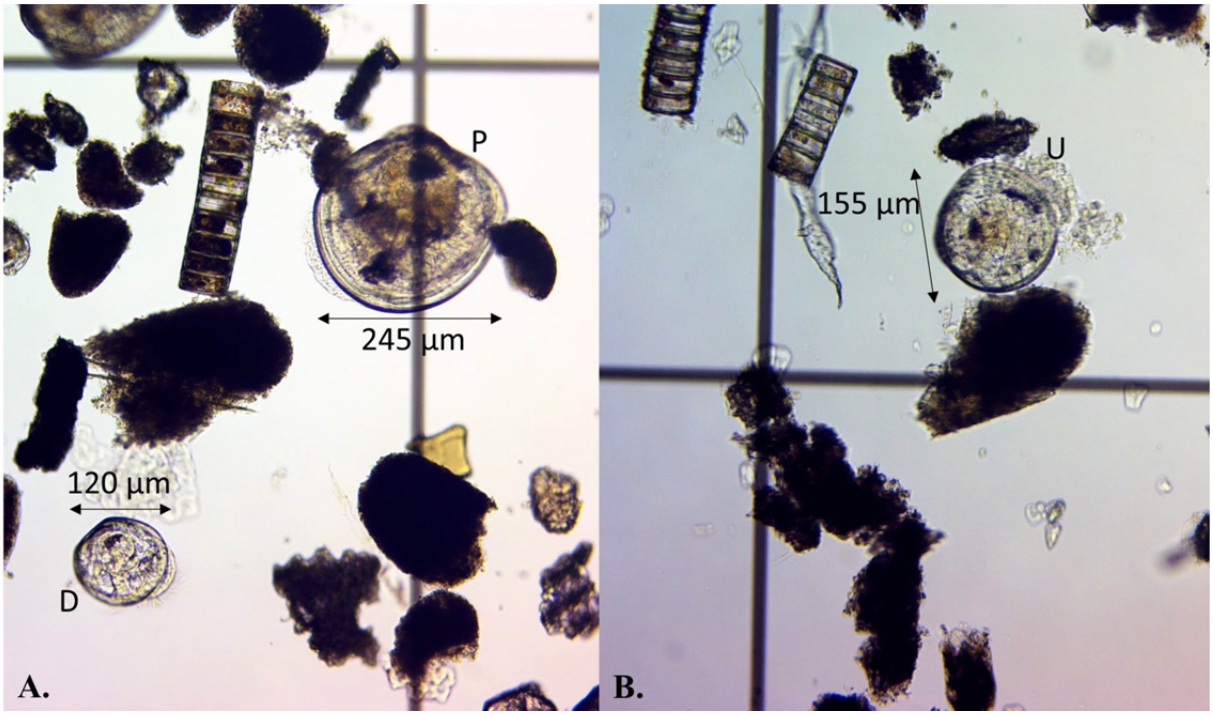

Figure 1. Veligers at 100× with a compound microscope using no stain of A. live and B. dead conditions. P = pediveliger, U = umbonal, and D = D-shaped life stage of veliger (Photos taken by Kelly Stockton-Fiti).

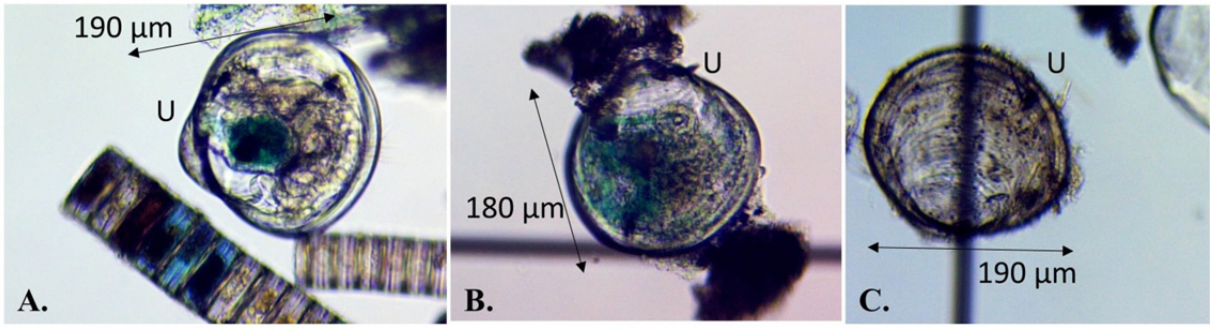

Figure 2. Veligers at 100× with a compound microscope using fast green stain of A. live, B. dead, and C. empty shells. P = pediveliger, U = umbonal, and D = D-shaped life stage of veliger (Photos taken by Kelly Stockton-Fiti).

In the stain method, fast green stain was used to stain the veliger sample prior to observation. An aqueous solution of 0.4% fast green FCF (Sigma-Aldrich, Co St. Louis, MO, lot MKBR8790V) was prepared with filtered river water. The sample concentrate on the 63 µm filter was placed into a dish with < 5 mL fast green stain and held for 20 min. The contents were then rinsed with filtered river water until dye was not visibly present and then back flushed from the filter into a recovery beaker with < 5 mL of filtered river water until the sample could be evaluated. A 2 mL sample of veliger concentrate was removed from each recovery beaker with a disposable pipet and visually evaluated as above. Live veligers were found minimally-stained with mantle intact, dead veligers had stained mantles, and open empty shells were not stained and had no mantle present (Figure 2). Some of the live veligers had a green spot in the viscera which we interpreted as dead food material staining green, while the veliger tissue remained stain free (Figure 2A).

The total time for microscopic assessment of replicate individual samples was recorded for each method. The content of one Sedgewick-Rafter counting cell

was used to obtain a minimum of 75 veligers per test beaker. Veligers were returned to their beakers and 90 mL of filtered river water was added to the beaker. Samples were retained for an additional 24 h recovery and reassessed using the same methods. Dissolved oxygen, conductivity, pH and temperature were measured with a Hach HQ40d with a LDO 101, CDC401, and PHC705 probes (Hach, Loveland, CO) and recorded.

Statistical analysis

The proportion of live veligers compared to the total of live and dead counted in each sample was used to determine percent survival. The time for completing assessment of each sample was recorded, and expressed as seconds per veliger, based on the total count of live, dead and empty shells. We compared the proportion of live and dead veligers in replicate treatments between immediate assessment and the 24 h assessment for each method of analysis with chi-square tests. Chi-square tests were also used to determine if differences in frequency of counts were observed between the two methods of analysis. Tests were conducted using frequency tests in SAS 9.4 (SAS Institute, Cary, NC). A general linear model was used to evaluate the relationships between the different analysis time for each method and treatment condition at each assessment period, and assessment time per veliger as a function of method or treatment with combined data for the variables. When there were significant differences in the variables, further analysis was conducted on the variables individually to determine if the relationship remained for the individual treatments or methods. Additional evaluations were conducted to determine the relationship of assessment time and time per veliger as a function of assessment period for each treatment. The GLM process was conducted in R 3.1.3 (R Development Core Team 2008) with package lme4 (Bates et al. 2014). An alpha of 0.1 was used to determine significance due to small sample sizes.

Results

In the immediate assessment, the proportion of veligers live and dead was similar regardless of assessment method. Control survival from the no stain method was not significantly different from that calculated with the staining method ($\chi^2 = 1.679$; $P = 0.432$; Table 1). Treated survival was not significantly different between the two assessment methods either ($\chi^2 = 1.648$; $P = 0.439$; Table 1). Survival estimates made with the fast green stain in the immediate assessment had smaller standard deviations (1%) in treated counts over the non-stained samples (6.8%). The standard deviation for the controls at the immediate assessment was very similar regardless of method used to assess the veligers. The immediate assessment standard deviations for survival were lower compared to the 24 h assessment, except for the fast green stain control (Table 1). The variation was due to the natural variation in the health of the population. Standard deviation of the treatment survival with the stain method were similar over the two assessment periods.

At the 24 h assessment, the proportion live and dead in the control samples without stain was significantly different from that observed with the use of stain ($\chi^2 = 7.018$; $P = 0.030$; Table 1). The assessment of live and dead in KCl treated samples did not differ between the two methods ($\chi^2 = 0.114$; $P = 0.945$; Table 1). The staining method had smaller standard deviations in treated and control samples compared to the samples analyzed without stain. Proportional survival in the controls at the 24 h assessment significantly decreased from the initial survival analysis with both the non-stained ($\chi^2 = 8.383$; $P = 0.004$) and stained method ($\chi^2 = 13.306$; $P = 0.000$; Table 1).

Assessment of samples immediately following KCl exposure was faster using stain for both the control (mean = 7 ± 0 min; Table 1) and treated samples (mean = 7.8 ± 2.02 min; Table 1), compared to the assessment time without stain for both the control (8 ± 0 min) and treated (12.3 ± 3.06 min) samples. When control and treated samples were combined, the difference in average time of assessment of non-stained compared to stained was significantly different ($F_{(1,8)} = 3.829$; $P = 0.086$). Assessment of the treated samples took more time than the control samples regardless of assessment method, though differences in total time of processing were not statistically different ($F_{(1,8)} = 2.201$; $P = 0.176$).

The control samples required significantly less assessment time per veliger ($F_{(1,8)} = 6.005$; $P = 0.040$; Table 1). Assessments made immediately without stain took longer than assessments made with stain to confirm that dead veligers were not moving, and often required adjusting the microscope to the higher power, $100^{\times}$ (Figure 3A; Table 1). Less time per veliger was needed with the stained assessment because it was easier to observe the dye-colored mussels at $40^{\times}$ (Figure 3B) especially at the initial assessment.

When the time per veliger was analyzed across for treated and control samples combined, we found no significance in time attributed to the use of stain ($F_{(1,8)} = 0.775$; $P = 0.404$). However, when samples of controls and treated veligers were analyzed separately, shorter analysis times for stained samples were

Table 1. Number of live, dead, and empty shells in samples of veligers counted by each replicate evaluated. The proportion survival (of live and dead), assessment time, and assessment time per veliger by method of analysis for treated and control. Data are reported for assessment made immediately and after 24 h recovery. Standard deviation is provided in parenthesis.

		Live	Dead	Empty	Total	Survival	Assessment Time (min)	Time/veliger (sec)
Immediate Assessment								
No Stain								
Control	1	34	2	137	173	0.944	8	2.77
	2	30	1	156	187	0.968	8	2.57
	Average				180	0.956 (0.012)	8 (0.0)	2.67 (0.10)
Treated	1	13	14	57	84	0.481	13	9.29
	2	16	22	72	110	0.421	9	4.91
	3	18	34	107	159	0.346	15	5.66
	Average				117.7	0.416 (0.068)	12.3 (3.1)	6.62 (2.34)
Fast Green Stain								
Control	1	33	4	185	222	0.892	7	1.89
	2	34	3	176	213	0.919	7	1.97
	Average				217.5	0.905 (0.014)	7 (0.0)	1.93 (0.04)
Treated	1	10	16	48	74	0.385	10	8.11
	2	23	34	60	117	0.404	6	3.08
	3	20	31	113	164	0.392	7.5	2.74
	Average				118.3	0.393 (0.010)	7.8 (2.0)	4.64 (3.01)
24 h Assessment								
No Stain								
Control	1	39	4	158	201	0.907	8	2.39
	2	26	13	67	106	0.667	8	4.53
	Average				153.5	0.787 (0.120)	8 (0.0)	3.46 (1.07)
Treated	1	20	11	53	84	0.645	10	7.14
	2	8	14	59	81	0.364	6	4.44
	3	16	29	83	128	0.356	7	3.28
	Average				97.7	0.455 (0.165)	7.7 (2.1)	4.96 (1.98)
Fast Green Stain								
Control	1	22	11	163	196	0.67	5	1.53
	2	62	29	81	172	0.68	6	2.09
	Average				184	0.67 (0.007)	5.5 (0.5)	1.81 (0.28)
Treated	1	9	19	48	76	0.32	6	4.74
	2	21	17	68	106	0.55	6	3.40
	3	15	24	99	138	0.38	6	2.61
	Average				106.7	0.42 (0.119)	6.0 (0.0)	3.58 (1.08)

apparent (($F_{(1,2)}$ = 44.080; P = 0.022) for control samples; and ($F_{(1,4)}$ = 4.528; P = 0.100) for treated samples; Table 1).

Assessment at 24 h was also faster with the stain method compared to the no stain method and mean assessment times were significantly different ($F_{(1,8)}$ = 8.333; P = 0.020; Table 1). The time for assessment of control and treated combined did not significantly between stained and non-stained samples ($F_{(1,8)}$ = 0.007; P = 0.936).

Assessment time of the controls at the 24 h recovery assessment was significantly different between the two assessment methods ($F_{(1,2)}$ = 25; P = 0.038; Table 1); 8 min for non-stained and 5.5 min for stained samples. For treated samples, the assessment time after the 24 h recovery did not differ significantly (7.7 min versus 6 min unstained and stained samples).

Time for assessment of treated samples after 24 h was significantly shorter than that required for samples evaluated immediately after treatment (Table 1): 7.7 min compared to 12.3 min ($F_{(1,4)}$ = 4.780; P = 0.094). After 24 h, the veliger tissues were more degraded and no movement was more apparent. More veligers were counted in the initial assessment than at the 24 h assessment for the treated non-stained samples; assessment time spent per veliger showed that there was not a statistical difference when comparing initial and 24 h assessment for the treated non-stained samples. Assessment time at 24 h for the stained treated samples was not significantly different from the initial assessment time.

It took significantly less time to analyze the control samples with the stain after the recovery period: 5.5 min compared to 7 min at initial assessment ($F_{(1,2)}$ = 9; P = 0.096), which was in part due to more

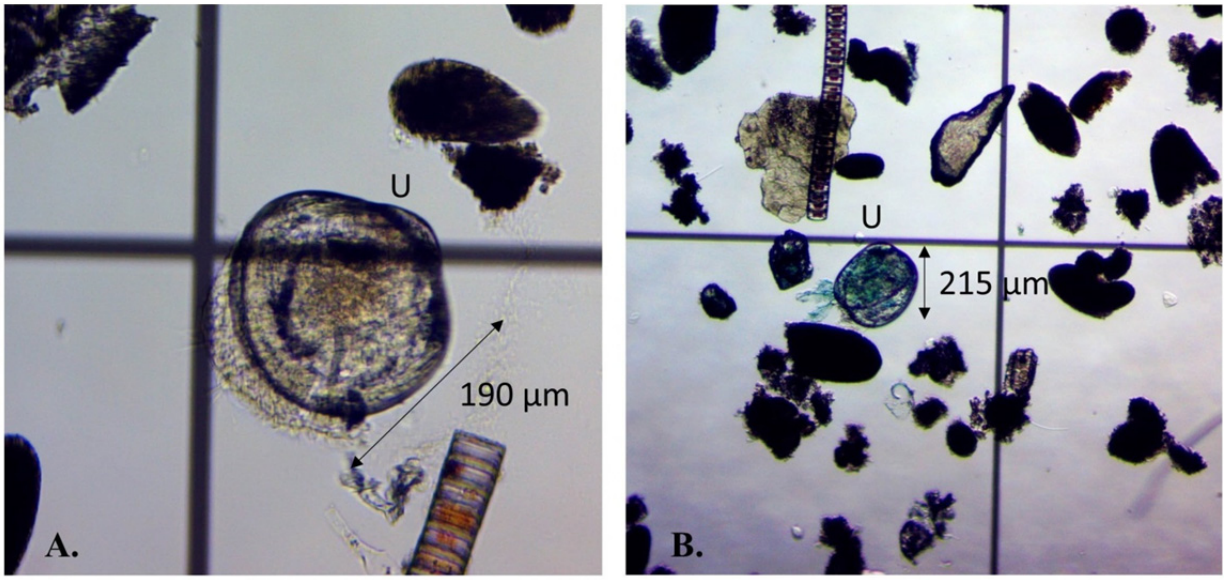

Figure 3. Dead veligers immediately after exposure with A. no stain at 100× and B. fast green stain at 40×. The increased magnification for the no stain was necessary to determine if the veliger was alive or dead, where it was not needed with the fast green stain as the dyed tissue indicating death was apparent at the lowest magnification. P= pediveliger, U= umbonal, and D=D-shaped life stage of veliger (Photos taken by Kelly Stockton-Fiti).

Table 2. Summary of mean water quality measurements of the treatment and recovery water.

Treatment	Temperature (°C)	Specific Conductance (mS/cm)	Salinity (ppt)	pH	Dissolved Oxygen (mg/L)
Control	22.0	0.96	0.49	7.98	8.25
Treated	22.0	1.48	8.63	8.08	8.59
24 h Control	20.6	1.01	0.50	7.95	8.05
24 h Treated	20.6	1.01	0.50	7.90	8.03

veligers examined per slide during the immediate assessment. When accounting for the differences in veligers counted by analyzing the assessment time per veliger, there were no significant difference between the times. There were no significant differences in the time to analyze control samples with the no stain method as both the initial assessment and 24 h assessment took 8 min. The analysis time per veliger was not significantly different for the control replicates for the two assessment periods and this was despite a drop in number of empty veligers counted in control replicate 2 between the two assessment periods (Table 1). Counts of empty shells did not add a significant amount of time to the assessment time.

Specific conductivity and salinity was increased from the control levels with additions of potassium chloride (Table 2) during treatment. Average testing temperature of this study was at 22.0 °C, mean pH was 8.03 and dissolved oxygen levels were above 8 mg/L (Table 2). After the 24 h recovery period at 20.6 °C, treated beakers had very similar specific conductivities and salinities to the controls, indicating that the potassium chloride toxicant was effectively removed before being placed into recovery (Table 2). The dissolved oxygen and pH did not decrease below suitable levels for veliger survival (Sprung 1993).

Discussion

We found that assessment of veliger status after exposure to potassium chloride can be accomplished with and without the use of fast green stain. However, the use of fast green allowed for a quick differentiation between paralyzed and dead veligers following exposure to potassium without the need for a recovery period. Further, using the stain provided replicates that had lower variation in the proportion of survival over evaluations made without stain. The fast green stain was applied to a wide range of veliger sizes and in all size classes stain had similar effect.

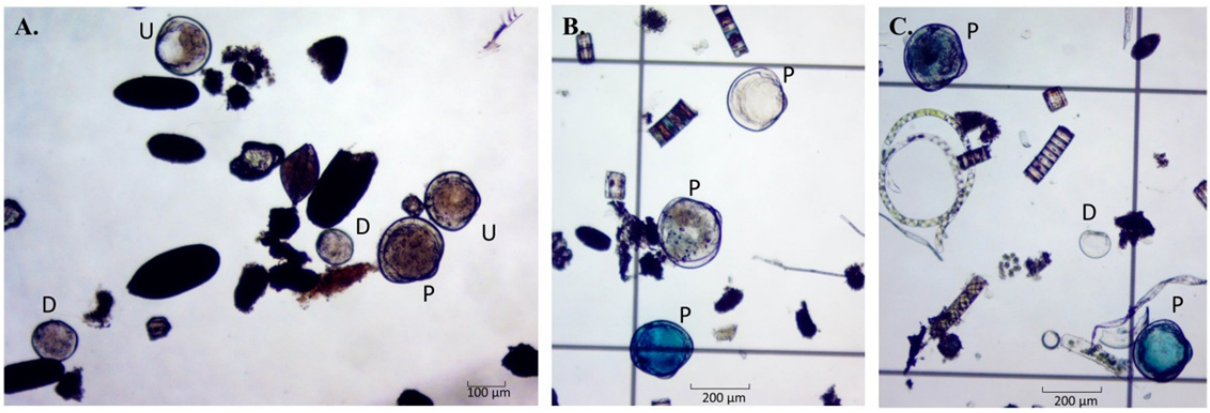

Figure 4. Dead veligers 24 h post exposure with A. no stain at 40× were brown and tissue was degraded and with the fast green stain at 40× the dead veligers stained B. degraded tissue partially and empty shells containing mantle or C. dead tissue fully and empty shells containing mantle. Completely empty shells are opaque and did not stain. P = pediveliger, U = umbonal, and D = D-shaped life stage of veliger (Photos taken by Kelly Stockton-Fiti).

The use of fast green has been used in aquaculture industry for marine mussel (*Perna canaliculus* (Gmelin, 1791)) to confirm the health of spat (Webb and Heasman 2006). They found staining was a good surrogate for activity as an indicator of group health. They reported fast green staining was highly advantageous over activity assessment for its ease and brevity. The specificity of this tool to recognize dead tissue can provide a reliable way to quantify live and dead veligers. Elliott et al. (2009) used the dye to quantify skin injury sites in Chinook salmon, and because it was rapid and specific for staining of integumental injuries, they suggested that this tool had widespread application in fish health and surface injury evaluations.

Use of stain reduced the time needed to analyze samples even after 24 h recovery period. The fast green stain enhanced the appearance of the veligers from the background plankton, which made it easier to find the live and dead veligers reducing time spent per slide. With the stain, the dead veligers were stained a dark green color, leaving the observer confident that the veligers were dead. The stain also colored recently empty shells, which were counted as dead since there was still dead mantle tissue present to be stained.

Assessments made after 24 h without the use of stain were easier, but studies that use an extended recovery period can introduce factors that may be confounding the true survival if released into the environment. In this study, the health of the veliger population was declining in the river environment as control survival after a 24 h recovery period decreased to 65% and there were also many empty shells in the plankton tow collections. The proportion of empty shells varied across each plankton tow collection due to time of day or river current passing by the pump. Death of the veligers was apparent with both methods at 24 h (Figure 4). With the non-stained samples at 24 h post exposure, the tissue turned a brown color and individual organs could not be discerned (Figure 4A). It was observed at the 24 h assessment highly degraded tissue did not stain as noticeably (Figure 4B and C).

One advantage that the no stain method had over the stain method was immediate analysis could be performed and there was no wait time for staining. With the stain method the 20 min stain period could be considered part of the analysis time, which would increase the time of analysis of one sample to 29 min. The stain method starts to have an advantage if 6 samples were stained at once, with a total time to analyze 6 samples with the fast green stain method would be 74 min. Using no stain, the time to analyze 6 samples at 12.3 min would also be 74 min. However, during the 20 minute wait time for stain other tasks could be accomplished, such as evaluating previously stained samples, setting up another treatment round, etc., which results in the fast green stain method being a better use of time.

The precision of the assessments was increased with the fast green stain method as this method consistently had smaller standard deviations. Studies that used this stain protocol were able to perform model analysis and have tight confidence intervals around those models showing significant differences and models of effect (Whitledge et al. 2015; Moffitt

et al. 2016). Moffitt et al. (2016) used the 0.4% concentration fast green stain to determine mortality of quagga mussel veligers exposed to potassium chloride with reliable results and allowed for high confidence in the assessment results with low processing time for high number of organisms observed. The authors have used the fast green stain method with other studies investigating other chemical and physical toxicants. Each study has resulted in low standard deviations around results and ability to confidently state results and effect of the toxicant.

Reducing the time needed for microscopic analysis is important to mitigate fatigue and eye strain of the analyzer (Howard and van Ginkel 1989; Helander et al. 1991; Wang and Huang 2004). Eye strain and fatigue levels are important to manage to ensure quality counts and accurate results (Wang and Huang 2004; Culverhouse 2007). If sample analysis is staggered, the sample analysis that uses a 20 min staining procedure can allow for a break in microscope time.

This study was conducted by an observer experienced with use of both methods; and was not conducted as a blind study. By using an experienced observer using both methods, the times reported and standard deviations reported should be considered conservative minimums. A novice observer would take more time, especially in determining live and dead based on ciliary or other gut movement of the veligers. The staining method offers a more definitive way of determining mortality by removing some of the subjective judgement inherent in this type of analysis as problems associated with movement by decomposers such as bacteria is ignored as the dead tissue is stained. While the fast green stain is of benefit to an experienced microscopist, it is particularly useful for those new to working with dreissenid veligers.

Assessments were made with precision shortly after exposure, eliminating the need for a recovery period. The advantages of no recovery time would allow for fast processing times for a study, which in turn could lead to more replicates, more treatment exposure times or treatment concentrations being performed, and direct further testing to obtain needed information for dose-response models. Potassium toxicity tests would be the most improved with the incorporation of the fast green stain protocol because if veligers were agape and not moving from the potassium toxicity (Sykes 2009; Pucherelli et al. 2014), the stain would still function and status would be assessed independent of the veligers physical condition. Other toxicity testing of dreissenid veligers would be greatly improved with the fast green stain protocol, and this method could be applied to monitoring activities, early detection and rapid response, and where other uses of determining the status of a dreissenid veliger was warranted. Further work to find a method to stabilize the fast green in the dreissenid veligers in the field prior to a preservation protocol would be very beneficial in monitoring activities.

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