

Research Article

Effectiveness of a neutral red viability protocol developed for two colonial tunicate species

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Abstract

In order to evaluate the potential risk posed by transporting invasive tunicates away from infested sites it is important to be able to measure the viability of these organisms. A Neutral Red (NR) viability assay was developed using a colonial invasive tunicate, *Botrylloides violaceus* (Oka, 1927) that could be applied to other species. Unlike some colonial tunicates *B. violaceus*' mortality can be determined under light microscopy by observing changes in the physiological parameters of filtration and reaction to tactile stimulus making them a good study organism. A total of 32 *B. violaceus* segments (3 cm²) were allocated to treatment and control groups. The treatment group was subjected to acetic acid which resulted in 100 % mortality. Viability was then assessed by comparing treatment and control group responses to NR uptake. There was no mortality in the control that demonstrated a response to tactile stimulus and a noticeable amount of stain uptake inside zooids. In contrast, the treatment groups did not respond to tactile stimulus and no stain was taken in by the zooids. In this study the viability of *Didemnum vexillum* (Kott, 2002) was assessed under controlled experimental conditions by simulating the air exposure encountered during the normal transportation and processing of tunicate fouled Pacific oysters, *Crassostrea gigas* (Thunberg, 1793). Clusters containing *C. gigas* and *D. vexillum* were collected from an aquaculture lease, in Lemmens Inlet, BC, for an air exposure trial comprised of 9 equally divided treatments (0, 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours). Three segments of *D. vexillum* on each cluster were removed and evaluated with the NR protocol developed for *B. violaceus*. Preserved tissues were analyzed and no sign of stain was noticeable, even with the control segments. The NR protocol developed worked efficiently with *B. violaceus*, but appeared to fail with *D. vexillum*. The findings are consistent with *D. vexillum* demonstrating a high tolerance in situations they are not accustomed to reflecting their ability to enter a dormant state during adverse environmental conditions (hence lack of response to clinical or NR markers via lack of filtering). Further studies are required to determine the viability of *D. vexillum* and potential risk movements pose.

Key words: neutral red, aquatic invasive species, mortality, viability, *Didemnum*, *Botrylloides*

Introduction

The colonial invasive tunicate, *Didemnum vexillum* Kott, 2002, is found at Pacific oyster (*Crassostrea gigas* (Thunberg, 1793)) aquaculture sites on the West Coast of Vancouver Island and the Sunshine Coast of British Columbia (BC) (Valentine 2003; Cohen 2005; Herborg et al. 2009). Although its detailed distribution is not known and keeps evolving, some areas on Vancouver Island and along the BC coast are considered to be free of *D. vexillum* and therefore vulnerable to future introduction or spread (Cohen 2005; Daniel and Therriault 2007). On Vancouver Island, transfers of oysters colonised with *D. vexillum* to processing

plants in non-infested areas occurs (pers. obs.). As standard processing practice employed by industry, shucked oyster shells, along with any attached biofouling, including tunicates, are discarded on the intertidal zone near processing plants in shell piles. The potential risk of introducing *D. vexillum* to non-infested areas is real and for this vector introduction success is a function of tunicate viability after transfer and processing. Thus, to characterize the potential risk it is critical to determine the viability of *D. vexillum* related to husbandry activities, particularly air exposure from transportation and processing activities.

The objective of this study was to assess the viability of *D. vexillum* under controlled experimental

conditions by simulating the air exposure encountered during the normal transportation and processing of oysters. The viability of colonial tunicates can be evaluated by several clinical markers including siphoning (filtration) activity, circulation (heartbeat) activity and morphological (discolouration) changes (Dijkstra et al. 2008; Rinkevich et al. 1992). Previous use of vital stains has proven valuable in assessing the health of marine species, including tunicates. For example, Neutral Red (NR) was used to detect the presence of acidic bladder cells in different species of the suborders *Stolidobranchia*, *Phlebobranchia* and *Aplousobranchia* (Hirose 2001). It also was suggested that NR stains tunicates in areas containing proteolytic enzymes (Bradway 1936). Although used in the past, NR has not been evaluated as a viability marker for tunicates. In order to conduct the treatment trials, a validation was deemed necessary. We conducted validation experiments using the violet tunicate (*Botrylloides violaceus* (Oka, 1927)) to determine the effectiveness of NR as a potential marker. *B. violaceus* was selected as a proxy for several reasons. First, *D. vexillum* was not available locally. Second, the violet tunicate has similar biological features to *D. vexillum* (colony formation and colour morphs) (Carver et al. 2006; Daniel and Therriault 2007) but is easier to work with given its larger zooid size that allows mortality to be determined under microscopy by observing its filtering action or its response to tactile stimulation (Dijkstra et al. 2008; Rinkevich et al. 1992). Given the small zooid size in *D. vexillum* visual observation techniques are unreliable for determining tunicate viability hence the need for a reliable method to ascertain *D. vexillum* viability. Information on viability can help assess the risk of spread associated with the current oyster harvesting and processing activities in BC or other invasion vectors of interest. Furthermore, it will assist with risk assessment, rapid response, and development of mitigating measures in the event *D. vexillum* is introduced to the Atlantic coast of Canada.

Material and methods

Viability markers for tunicates

Clinical viability markers

In this study, a positive result for filtration consisted of the detection of movement of zooids and/or movement of particles in the water by monitoring zooid activity using a dissection microscope. The

particles in the water came from the substrate attachment point of the tunicate segment. For tactile stimulation, positive results consisted of zooid contraction followed by a relaxation to the previous undisturbed state. Tactile stimulus was applied by the light contact of a dissecting probe. If no reaction occurred, this was repeated five times. A negative result (zooid mortality) occurred when both filtration and tactile response were absent.

Vital stains

To be used as an indicator of tunicate viability, the vital stain needs to be integrated to the organism by filtration or otherwise absorbed by cells while still viable. The requirements for a positive and negative stain result are explained below.

Procurement and preparation of *B. violaceus*

Thirty-two colonies of *B. violaceus* were obtained from different natural substrata; blue mussels (*Mytilus edulis* Linnaeus, 1758), eel grass (*Zostera marina* Linnaeus, 1753) and rockweed (*Fucus vesiculosus* Linnaeus, 1753) at various locations on Prince Edward Island. Colonies were removed from substrate, cleaned and a 3 cm² segment was dissected carefully to avoid unnecessary tissue stimulation. Segments were observed under a dissecting microscope to ensure that zooids were siphoning and responding normally to tactile stimulation prior to treatments. To ensure viability, segments were kept in fresh artificial seawater (ASW), 28 ‰ until experiments were conducted upon arrival at Atlantic Veterinary College (~1 hour).

Development and validation of a neutral red staining protocol for *B. violaceus*

Tunicate segments were allocated to either treatment or control groups. The treatment group was exposed to 5 % acetic acid (household strength) by submerging tunicate segments for 15 minutes followed by monitoring of the clinical markers to ensure 100 % mortality. Acetic acid has been shown to be an effective way of killing tunicates (DFO 2006; Forrest et al. 2007; Piola et al. 2010). A previous study reported that an exposure to a 15 second spray of 5 % acetic acid was sufficient to impact *B. violaceus* (Carver 2006). Therefore, the submersion time was chosen to ensure complete mortality. After exposure, the tissues were then soaked with light agitation in ASW for 10 minutes to remove residual acetic acid.

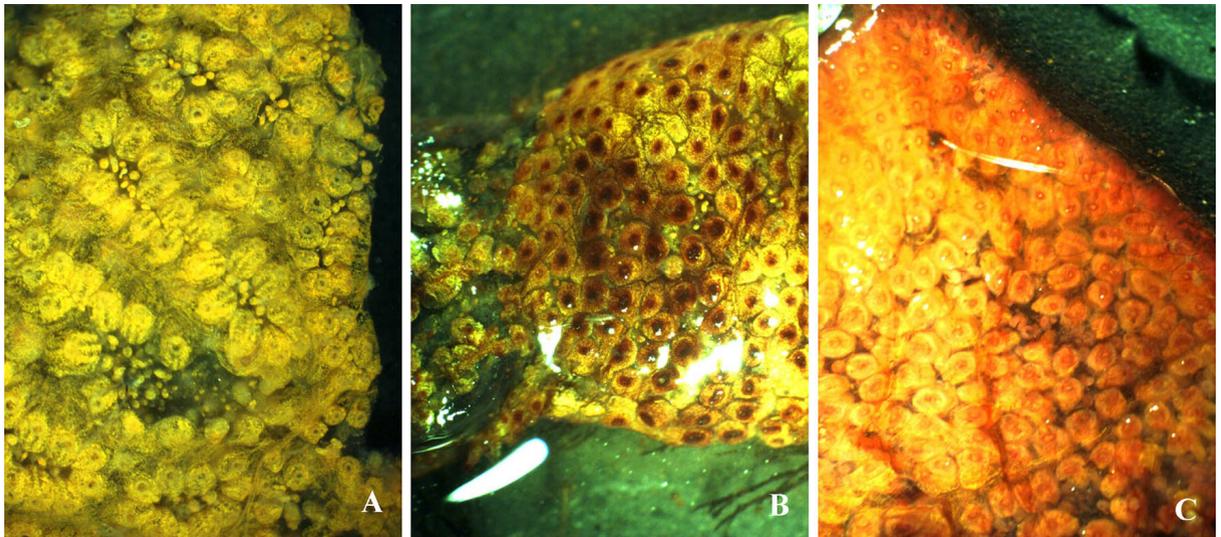


Figure 1. The three different zooid states used to compare a dead and a live colony of *B. violaceus*. (A) Intact tunicate showing a healthy zooid. (B) Control group - Healthy zooids after a 15 minute submersion in 1% Neutral Red and ASW solution. (C) Treatment Group - Dead zooids after a 15 minute submersion in 5% acetic acid followed by a 15 minute submersion in 0.01% NR. All images were at a 0.63x magnification. Photographs by Louis F. Ferguson.

The segments of *B. violaceus* were then subjected to NR stain trials. A 0.01 % concentration was prepared from 0.01 g of NR (Acros Organic, New Jersey, USA) per 100 mL of filtered ASW (63 μ m) (Cook 1974). Segments were then submerged in this staining solution for 15 minutes. All tissues were subsequently bathed in ASW for 5 minutes to remove excess stain. Viability of segments was then assessed under a dissecting microscope (Zeiss Stemi 2000C, Carl Zeiss Jena GmbH, Zeiss Group, Jena, Germany) at a 6.3 \times and a 12.6 \times magnification. A segment was considered positive when stain was present inside the zooid wall combined with siphoning and response to tactile stimulation. A lack of stain uptake combined with no indication of filtering or lack of response to stimulation was considered a negative result. Fresh, live zooids (Figure 1A) were used as baseline to compare and describe zooids from control and treatment groups (Figure 1B, C).

Viability trial on D. vexillum after air exposure

Trial protocol

Twenty-seven clusters of Pacific oysters (*C. gigas*) fouled with *D. vexillum* were collected in March 2009 from an aquaculture site in Lemmens Inlet, BC. The site cultures oysters on a long line system with clusters intertwined in two stranded

rope. Clusters are composed of a mother shell (clutch) with several live oysters attached and epibionts, including *D. vexillum*. Clusters were transported from the site to a holding area (wharf) near the laboratory in Tofino, BC in buckets containing seawater from the holding area. The duration of the transit was 20 minutes. The air exposure from transportation and processing activities was simulated by suspending the fouled clusters, with *D. vexillum*, in a field laboratory. This laboratory offered a controlled environment where the suspended specimens were sheltered from the variable environmental factors (wind, rain, sun exposure...), allowing a uniform air exposure at temperatures that ranged from 9 $^{\circ}$ C (mornings and nights) to 13 $^{\circ}$ C (mid-afternoons). The exposure durations were set at 0 (control), 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hours.

Three replicate clusters were allocated to each of the nine air exposures. Following treatment, clusters were returned to the holding area for an acclimation period (30–60 minutes) to allow surviving zooids to recover filtering activities. The holding area was located in the same water body that the *D. vexillum* was collected. Three segments (3 cm²) per cluster were then observed microscopically for clinical markers (as defined above) and exposed to the NR staining protocol. All segments were preserved in 10 % formalin and filtered seawater (63 μ m solution).

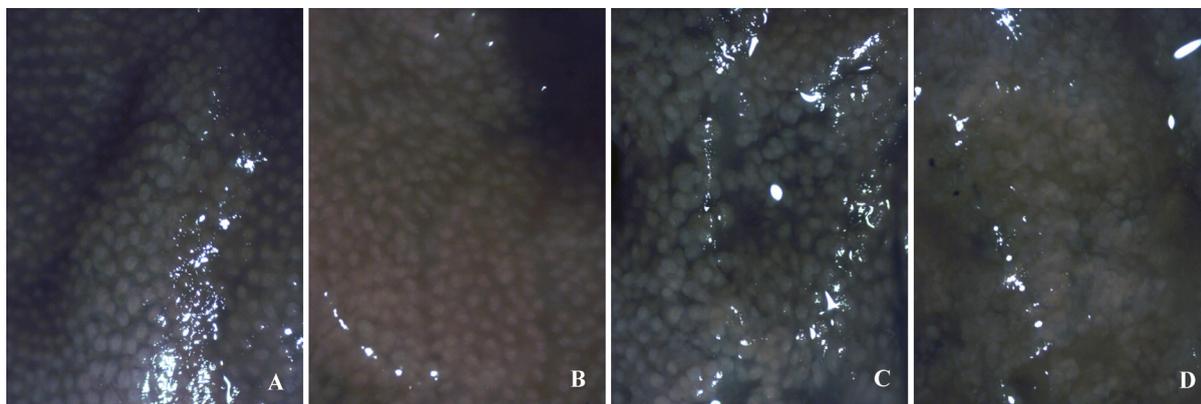


Figure 2. Grading scale for the deterioration of the morphology of *D. vexillum* zooids. From left to right, the colonies have a weaker grade on a scale of 0 to 3. (A) 0 - Represents a fresh colony (control) where all the zooids show no sign of deterioration. (B) 1 - Represents a colony that received a mild treatment effect, a few zooids started to lose their shape and/or degrade. (C) 2 - Represents a colony that received a moderate treatment effect, more than half of the zooids are irregular and degraded, (D) 3 - Represents a colony that received a severe treatment effect. Almost all the zooids are irregular to the point that they are unrecognizable. All are at a 0.63x magnification. Photographs by Louis F. Ferguson.

Table 1. 2×2 contingency table for the observed stained *B. violaceus* colonies.

	Treatment		Total
	NR	Acetic Acid + NR	
Alive	16	0	16
Dead	0	16	16
Total	16	16	32

The preserved tissues were transported to the Atlantic Veterinary College, Charlottetown, PEI, where all tissue samples were observed under a dissecting microscope at a 6.3 × and 12.6 × magnifications, for NR stain and any other morphological observations. Changes in morphology were graded by two observers using a double-blind protocol using the scale developed in Figure 2.

Results

Validation trials on B. violaceus

Clinical assessment of all 32 segments before the trials showed no evidence of compromised viability. The zooids were sensitive to tactile stimulation and were actively filtering.

The control stained colonies showed no difference from the baseline zooids. Figure 1b shows that the inside and the circumference of the siphon were stained. Conversely, no siphoning

action or movement was observed in the treatment group (Figure 1C). All of the control groups were confirmed to be alive, demonstrating physiological activity and a noticeable amount of stain inside the zooids. All the treatment groups were confirmed to be non-responsive to tactile stimulation and did not uptake stain.

The experimental results are summarized in a 2×2 contingency table (Table 1). The observations were consistent with a systematic staining of live *B. violaceus* while dead colony fragments did not respond to the stain and did not result in a false positive (i.e., stain uptake by dead or dying cells).

Air exposure on D. vexillum

No signs of staining were observed in any of the full and sectioned segments of *D. vexillum*, including the controls. The control showed no clinical signs of viability (siphoning and reaction to tactile stimuli). The only change noted over time of exposure was the general morphology of the colonies. A theoretical grading is given in Figure 2 to represent this morphological change.

Discussion

Although there are no published studies on the use of vital stains to assess the viability of colonial tunicates, various experiments have been conducted on these biochemical stains for other purposes. NR stain was used to detect acidic bladder cells in different species of the

Table 2. Contrast between *D. vexillum* and *B. violaceus* viability markers after staining of the control segments with neutral red.

<i>D. vexillum</i>	Viability Marker	<i>B. violaceus</i>
No particle movement	Filtration	Particle movement near siphons
No zooid movement	Tactile Stimulus	Zooid contraction and return to relaxed state
No Staining of tissue	Stain	Stain inside zooids

suborders *Stolidobranchia*, *Phlebobranchia* and *Aplousobranchia* (Hirose, 2001). It also was suggested that NR stains tunicates in areas containing proteolytic enzymes (digestive tract) (Bradway 1936). In this study, a viability protocol using NR stain was developed and validated on *B. violaceus*, in Prince Edward Island, with the goal to assess the viability of *D. vexillum* under controlled experimental conditions by simulating the air exposure encountered during the normal transportation and processing of Pacific oysters on Vancouver Island.

In order to manage the risk of introducing *D. vexillum* into new areas, it is essential to ensure the death of the entire colony since establishment can occur from small fragments (Bullard et al. 2007; Valentine et al. 2007). Although the results from this study are not conclusive for *D. vexillum*, a time efficient protocol would provide management options that could be implemented at processing sites before disposal of tunicate fouled shells. Denny (2008) noticed that *D. vexillum* controls had a 65 % mortality, which would indicate a negative impact coming from manipulations (dipping declumped mussels in mesh bags). Therefore, handling that occurs during the pre- and post-processing of oysters could increase the mortality of tunicates which could potentially lower the risk of spread. Katayama and Ikeda (1987) observed mortality of *Didemnum moseleyi* (later confirmed as *D. vexillum* by Lambert 2009) after 30 minutes or more of air drying in direct sunlight in June and July, while it took over 5 hours to detect mortality in March. Even though the method of shell disposal has not changed in the past few decades, the zones surrounding the processing plants in BC have not become infested with *D. vexillum* (Cohen 2005; Daniel and Therriault 2007), at least to our knowledge. It is still critical however to know that the *D. vexillum* is dead before it leaves the processing site as this would prevent the establishment of this invader in surrounding waters.

The degradation of the *D. vexillum* segments was scaled over time (Figure 2). Although we cannot set a threshold for mortality, the degradation follows a steady slope.

The trials on *B. violaceus* demonstrated that all of the control groups were alive and took up NR stain while all the treatment groups were dead and did not. Thus, NR appears to be a useful method for determining the viability of the violet tunicate and could be applicable to other tunicates. The stain enters the siphon opening during active filtration and stains the surrounding structures. Although the acetic acid treatment killed the violet tunicate, residual NR stain was still present within the siphon muscle of a few specimens. This staining deposition could have occurred due to acetic acid that was not flushed out following artificial seawater washing. This would be consistent with the chemical properties of NR stain since it also works as a pH indicator, changing to a darker red in pH lower than 6.8 and yellow above a pH of 8.0. To ensure identification of potential false positives careful examination of the stained tissue segments is needed. When NR is embedded in internal tissues, there is no doubt that the staining occurred while the organism was alive. In contrast, NR residue on the tunicate rather than within the tunic represents a false positive.

A challenge arose in determining viability of *D. vexillum* using the techniques such as filtering ability and reaction to tactile stimulus that were successful for evaluating *B. violaceus*. No filtration or response to stimuli was noticeable using microscopy even with fresh samples, potentially due to the small zooid sizes (*D. vexillum* (1–2 mm) are smaller in comparison with *B. violaceus* (2–4 mm) (Carver et al. 2006; Daniel and Therriault 2007)) and sensitive response to disturbance (i.e., tunicate closes siphons and enters resting state). The trials employing the NR vital stain also proved inconclusive (Table 2). Although not verified with *B. violaceus*, the elapsed time between the staining and the analysis of the segments (approximately one month) might explain the result. However, the absence of physiological markers of viability is still more plausible. Assessments of these clinical markers in *D. vexillum* indicated a poor applicability. An observational trial executed after the negative results of the prior trials used menthol crystals to relax the siphons to facilitate the uptake of NR but the same results were encountered. No stain was taken up even if the menthol crystals relaxed the siphon muscle via analgesia (Galeotti et al. 2002).

D. vexillum demonstrates a tolerance to uncommon situations in comparison with other colonial tunicates and is suspected to induce and maintain dormancy when exposed to adverse environmental conditions. This may be associated with the ability of this species to close their siphons for extended periods in order to overcome environmental changes. Ascidians close their siphons for various reasons, ranging from physical disturbance (involuntary movement), light variation or to avoid obstructions. For example, Forrest et al. (2007) showed that 4% acetic acid killed the colonial ascidians *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides leachii* (Savigny, 1816), which are morphologically and functionally similar to *D. vexillum*. However, Forrest et al. (2007) found that acetic acid was ineffective at killing 100% of the *D. vexillum*. Similarly, Denny (2008) did not observe any effects of a 10 minute freshwater immersion on the survival of *D. vexillum* while Coutts and Forrest (2005) found that the solitary tunicate, *Styela clava* Herdman, 1881, was able to withstand 1 hour of freshwater immersion presumably by closing their siphons for an extensive period. However, there is a trade-off when siphons are closed. At salinities lower than 20 ‰, ascidians can close their siphons for long periods of time but this can eventually lead to zooid death (Stoner 1992). Heavy turbidity (sedimentation) cause ascidians to close their oral siphons to prevent the siphon and brachial filtering wall from clogging and ceasing respiration to avoid suffocation (Monniot et al. 1991). Therefore, *D. vexillum* may employ a similar strategy and might not release the siphon muscles due to adverse or changes in its surrounding area with dormancy extending beyond the duration of the environmental hardship which introduces potential costs to the reproductive capacities of the species (Cáceres 1997). Although attributed to winter dormancy, Marks (1996) found that ascidian could remain dormant for up to five months.

This study found that *B. violaceus* was susceptible to acetic acid treatment and that an established protocol that included physiological (filtration and response to stimuli) and NR vital stains could be used to assess viability. However, these methods were not transferable to *D. vexillum* as tested during the trials. Due to this, further studies in the determination of *D. vexillum*'s viability are needed. Other researchers have encountered difficulties in keeping *D. vexillum* alive in a closed system and have had problems in accurately determining if it is dead or alive

(Forrest BM pers. comm., Valentine PC pers. comm., Therriault TW pers. comm.). Many suggestions could be brought forth on how to overcome these difficulties. Developing a successful method to keep *D. vexillum* in a recirculation system would certainly enable us to test the NR protocol. *In situ* trials could also be developed. This would entail using mesocosm surrounding the fouled cluster without removing it from the aquaculture site. The NR would then be introduced in the mesocosm. If the protocol works, our suspicions of dormancy due to disturbance or handling would remain. We still believe that the NR vital staining is possible on this colonial ascidian, and that this protocol can be transferred to other colonial or solitary tunicates.

Future studies to verify the viability of *D. vexillum*, without the use NR, could include molecular analysis to evaluate the degeneration of *D. vexillum*. Quantification of DNA/RNA after performing treatments similar to this trial, could give a measure of internal degradation of nucleic acids up to the time of death or a plateau for a dormant stage. Others studies could include the quantification of particle depletion or the uptake of O₂ marking metabolism rate. The results would provide a better understanding of the biology of *D. vexillum*. The ability to determine mortality in *D. vexillum* would certainly help the potential for spread via human-mediated dispersal vectors.

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