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**ARTICLE**

# Abalone withering syndrome disease dynamics: infectious dose & temporal stability in seawater

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Running head: Abalone Withering Syndrome Infectious Dose & Temporal Stability

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***Abstract***

**Withering syndrome (WS) is a chronic bacterial disease that affects numerous northeastern Pacific abalone species, *Haliotis* spp. The causative agent of WS is an obligate intracellular *Rickettsiales*-like bacterium (WS-RLO) that remains unculturable thereby limiting our understanding of WS disease dynamics. The objectives of our study were to: (1) determine the temporal stability of WS-RLO DNA outside of its abalone host in 14°C and 18°C seawater, (2) develop a standardized protocol for exposing abalones to known concentrations of WS-RLO DNA and (3) calculate the dose of WS-RLO DNA required to generate 50% infection prevalence (ID50) in the highly cultured red abalone *Haliotis rufescens*. WS-RLO stability trials were conducted in October 2016, February 2017, and June 2017 during which qPCR analysis was used to quantify bacterial DNA for 7 days in seawater collected at an abalone farm in southern California where the pathogen is now endemic. For all trials and temperature treatments, WS-RLO DNA was not stable in seawater longer than 2 days. To determine an ID50, groups of uninfected juvenile red abalone were subjected to 3 hour bath exposures of WS-RLO at four concentrations: 0, $10^3$, $10^4$, and $10^5$ DNA copies/mL. Abalone feces were monitored bi-weekly for the presence of WS-RLO DNA and abalone tissues were sampled 9 weeks after dosing for histology and qPCR examination. Results from the ID50 indicated that our protocol was successful in generating WS-RLO infections and a pathogen dose of 2.3 x $10^3$ DNA copies/mL was required to generate 50% infection prevalence in the tissue of red abalone as assessed by qPCR. These findings are critical components of disease dynamics that will help assess WS transmission risk within and among abalone populations and facilitate appropriate management and restoration strategies for both wild and cultured abalone species in WS-endemic areas.**



## Introduction

Withering syndrome (WS) is an infectious marine wasting disease that affects numerous northeastern Pacific abalone *Haliotis* species. The causative agent is the *Rickettsiales*-like

organism "*Candidatus* Xenohaliotis californiensis" (WS-RLO; Friedman et al. 2000), which is an obligate intracellular bacterium that is transmitted horizontally via a fecal-oral route (Friedman et al. 2000, 2002). The WS-RLO creates bacterial inclusions in abalone post-esophageal (PE) epithelial cells and the digestive gland (DG) epithelia undergoes metaplasia, the transformation of one differentiated cell type to another mature cell type, which allows the bacterium to proliferate (Friedman et al. 2002). As WS progresses, abalones lose the ability to properly digest and absorb nutrients from their food. In response, abalones catabolize their pedal musculature for energy and begin to exhibit clinical signs of the disease including lethargy, mantle retraction and anorexia (Gardner et al. 1995, Friedman et al. 2002, Braid et al. 2005).

WS was initially observed in wild black abalone *Haliotis cracherodii* populations on the south shore of Santa Cruz Island, California, USA in 1985 (Tissot 1995). Since then, WS has spread to numerous abalone species and is considered continuously distributed from Baja California, Mexico to Sonoma County, California, USA including the Channel and Farallon Islands (reviewed by Crosson et al. 2014). Research investigating potential environmental drivers of the WS epidemics revealed that as seawater temperatures increased, the prevalence and severity of the disease also increased (Steinbeck et al. 1992, Tissot 1995, Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). The onset of warmer than normal seawater conditions associated with the large El Niño Southern Oscillation (ENSO) events of 1982-83 and 1997-98, as well as the anthropogenic transport of WS-RLO infected abalone to previously uninfected areas (Friedman & Finley 2003) likely facilitated the spread of the disease (Tissot 1995, Crosson et al. 2014).

The threat of WS to abalones has substantial ecological implications. Abalones are considered ecosystem engineers that, via herbivory, maintain open space on rocky substrate for utilization by other benthic organisms and conspecifics (Miner et al. 2006). Currently, five out of seven northeastern Pacific abalone species are experiencing population declines and receive varying levels of federal protection, ranging from "Species of Concern" (pinto abalone *Haliotis kamtschatkana*, green abalone *Haliotis fulgens*, and pink abalone *Haliotis corrugata*) to "Endangered" (white abalone *Haliotis sorenseni*, and black abalone; reviewed by Crosson et al. 2014). Many of these abalone species overlap in geographic range and demonstrate differential susceptibility and resistance to WS (Crosson & Friedman 2018). Therefore, the need to understand potential WS transmission risk is critical to facilitate more informed management

decisions for the successful protection and restoration of threatened and endangered abalone species.

WS can also have profound economic effects on the commercial abalone industry. Abalones are one of the world's most expensive seafood products and current production has shifted from wild to farmed abalones with 95% of the global market supplied by aquaculture (FAO, 2017). Over the last 10 years, global abalone aquaculture production has increased a dramatic 500% (24,400 metric tons in 2006 to over 150,000 metric tons in 2015; FAO, 2017) and production in the United States (California and Hawaii) for 2015 was an estimated 362 metric tons (Cook 2016) valued at over $13.1 million dollars (Ray Fields, The Abalone Farm, Inc., pers. comm.). Given the economic importance and explosive growth of abalone aquaculture combined with declining wild abalone numbers, concern has risen regarding potential WS transmission between wild and cultured abalone populations, including both pathogen spillback (wild to cultured abalones) and spillover (cultured to wild abalones; Daszak et al. 2000). A previous study implicated shore-based abalone aquaculture in southern California as a potential threat to critical habitat for endangered wild abalones (Lafferty & Ben-Horin 2013). However, without fundamental information regarding WS transmission mechanisms and associated pathogen dynamics, the potential threat is largely unknown.

The paucity of baseline information on WS disease dynamics can be primarily attributed to the inability of the WS-RLO bacterium to grow *in vitro* and the lack of established marine invertebrate cell lines making disease transmission experiments a challenge (Rinkevich 2005). Many studies attempting to explore WS transmission mechanisms have relied on cohabitation of healthy abalone with WS-RLO infected abalone (e.g. Friedman et al. 1997, 2002, Braid et al. 2005). While cohabitation experiments more closely simulate natural WS infections, the amount of pathogen or dose needed to establish infection remains unknown. Development of tools and protocols that facilitate both *in vitro* and *in vivo* studies with the WS-RLO are needed. Friedman et al. (2014a) recently developed and validated a quantitative PCR (qPCR) assay for the detection of WS-RLO DNA in abalone tissue, feces and seawater. While the presence and amount of WS-RLO nucleic acid does not confirm the pathogen's viability or infectivity, it can serve as a proxy for pathogen detection and potential transmission risk. Other studies comparing bacterial quantification via PCR-based assays to traditional culture-based methods found that

PCR-based assays are a viable alternative, although relative quantities of DNA copies detected and limits of detection relative to traditional methods can vary by taxa being examined (Wright et al. 2007, Ricchi et al. 2017). These findings are especially valuable for the detection and quantification of unculturable bacteria such as the WS-RLO.

To better understand important components of WS disease dynamics, our study aimed to: (1) determine the temporal stability of WS-RLO DNA outside of its abalone host in 14°C and 18°C seawater, (2) develop a standardized protocol for exposing abalones to known concentrations of WS-RLO DNA and (3) calculate the dose of WS-RLO DNA required to generate 50% infection prevalence (ID50) in the highly cultured red abalone *Haliotis rufescens*. These findings will provide critical information for modeling potential WS transmission risk within and among abalone populations and will aid management decisions for the restoration and culture of abalone species in WS-endemic areas.

[A]Methods

[B]WS-RLO temporal stability

[C]*Experimental design*. - Approximately 19 L of effluent seawater from a commercial abalone farm located in Goleta, CA, US, where the WS-RLO pathogen is established in local waters, was shipped overnight on ice to the School of Aquatic and Fishery Sciences-University of Washington-Pathogen Quarantine Facility [SAFS-UW-PQF (Seattle, WA, US)]. Upon arrival, effluent seawater was thoroughly mixed, immediately aliquoted into glass beakers (500 mL each), covered with parafilm, and placed in dark incubators at their respective temperature treatments, 14°C and 18°C. Each temperature treatment consisted of 16 replicate effluent seawater beakers and 4 sterile seawater (SSW) beakers used as negative controls, all with independent aeration. Two effluent seawater beakers per treatment were randomly sampled daily for 8 days and two SSW control beakers were sampled per treatment on days 2 and 8 to ensure no pathogen cross contamination at the beginning or end of the experiment. Three experimental trials were conducted in October 2016, February 2017 and June 2017 to assess seasonal variability of WS-DNA levels in the farm effluent seawater.

[C]*Seawater sampling*. - Sampling consisted of aseptic filtration of seawater replicates through a 0.2 µm Supor® membrane disc filter (Pall Corp., Port Washington, NY, US) along with 5 mL of 1X TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] for nucleic acid preservation. All filters

were aseptically halved; one half was stored at -80°C for subsequent DNA isolation and the remaining half was stored in 1 mL of TRI Reagent® (Sigma-Aldrich Corp., St. Louis, MO, US) at -80°C for subsequent RNA isolation (methods below).

[B]WS-RLO infectious dose

[C]*Experimental design.* - Independent shipments of WS-RLO-free juvenile red abalone (n = 120), to serve as experimental animals, and WS-RLO infected adult red abalone (n = 26), to serve as a source of infectious material, were received from a commercial abalone farm located in Cayucos, CA, US and housed at the SAFS-UW-PQF. WS-RLO infected abalone were placed in a 270 L recirculating seawater system and healthy abalone were equally distributed among four 175 L recirculating seawater systems containing triplicate 11.4 L tanks with 10 abalone per tank and system-independent biological/mechanical filtration, UV-irradiation, and temperature control (Fig. 1). Abalone were acclimated for approximately two weeks prior to WS-RLO dosing (method below) and maintained at 18°C, a temperature known to augment pathogen proliferation (Moore et al. 2000). Feces were tested by qPCR to ensure abalone were free of detectable levels of WS-RLO DNA prior to experimentation. Abalone were fed 8-10 pellets of S-A diet (Cosmo Business Support, Japan) per tank daily for the duration of the experiment. Seawater pH, dissolved oxygen and ammonia were assessed on alternate days, while temperature and abalone mortality were assessed daily. Lethargic or dead abalone were immediately removed and sampled for histology and quantitative PCR (qPCR) analyses (methods below). Abalone tank feces were monitored bi-weekly for the presence of WS-RLO DNA via qPCR and abalone post-esophageal tissues were sampled at 9 weeks post WS-RLO dosing for histology and qPCR examination.

[C]*WS-RLO dosing.* - Post-esophageal (PE) tissue, the target tissue type for WS-RLO infections (Friedman et al. 2000), was excised from all infected adult red abalone. PE was pooled, suspended in 100 mL of SSW and homogenized on ice with a 7 mL Tenbroek homogenizer (Fisher Scientific, Pittsburg, PA, US). Approximately 19 L of effluent seawater was received from the same commercial abalone farm from where the infected adult red abalone originated and filtered on to 0.2 µm Supor® membrane disc filters (n = 5). All filter retentate (primarily WS-RLO infected abalone feces) was added to the infected tissue homogenate to ensure a maximum yield of WS-RLO source material. To generate an appropriate volume for dosing, 3.3 L of SSW was added to the WS-RLO homogenate and two 1:10 serial dilutions in SSW were

generated. The homogenate stock ($10^0$) and dilutions ($10^{-1}$, $10^{-2}$) were sampled via the seawater sampling method (described above) except the entire filter retentate was immediately processed for DNA isolation and quantified via qPCR (methods below) to determine WS-RLO dosing concentrations in DNA copies per mL. Abalone treatment tanks received 1 L of the appropriate WS-RLO dose, while control tanks received 1 L of SSW. All abalone remained submerged for 3 hours with aeration after which the dosing medium was decanted and replaced with flow-through SSW.

[C]*Abalone tissue & fecal sampling.* - For histological examination, a 2-3 mm tissue cross-section of PE was aseptically excised posterior to the right kidney-DG junction. Tissues were preserved in Davidson's fixative (Shaw & Battle 1957) for 24 h and stored in 70% ethanol until processing by routine paraffin histology. Deparaffinized 5µm sections were stained with hematoxylin and eosin (Luna 1968) and examined by light microscopy. WS-RLO infection intensities in PE tissues were scored on a (0) – (3) scale that estimated the number of bacterial inclusions per 20x field of view: (0) no infection, (1) 1-10, (2) 11-100, and (3) > 100 (Friedman et al. 2002). For each abalone examined, all tissues were screened and the mean score per field of view was reported. A 150-200 mg sample of PE was also excised directly adjacent to the histology cross-section and preserved in 100% molecular grade ethanol until subsequent DNA isolation and qPCR analysis described below. Abalone fecal samples (~ 200 mg) were collected bi-weekly from each tank (or pooled by dosing treatment when tank fecal quantities were low) and immediately processed for DNA isolation and qPCR analysis for continuous monitoring of WS-RLO DNA levels.

[B]DNA & RNA isolation

DNA was isolated from seawater filters using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's "Quick-Start" protocol with the following modifications: volume of lysing buffer was doubled to ensure complete filter coverage and DNA was eluted in 100 µL. DNA was isolated from abalone fecal and tissue samples using a QIAamp DNA Stool Mini Kit (Qiagen Inc.) according to the manufacturer's "Isolation of DNA from Stool for Pathogen Detection" protocol with the following modifications: DNA was eluted in 100 µL. All DNA samples were stored at -20°C until qPCR analysis. RNA was isolated from seawater filters using TRI Reagent® according to the manufacturer's protocol and suspended in 50 µL of 0.1% DEPC-treated water. To ensure complete removal of genomic DNA (gDNA), all

RNA was treated with DNase using the Turbo DNA-free Kit (Invitrogen Corp., Carlsbad, CA, US) according to the manufacturer's protocol and verified gDNA-free via qPCR. RNA was then reverse transcribed to complimentary DNA (cDNA) using M-MLV reverse transcriptase, 10 mM dNTPs and oligo Dt primer (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. All cDNA samples were stored at -20°C until qPCR analysis as described below.

[B]Quantitative PCR

Quantification of WS-RLO nucleic acids was conducted using the validated qPCR assay of Friedman et al. (2014a), which amplifies the single copy per bacterium of the16S ribosomal gene as determined by the annotation of the WS-RLO partial genome (Langevin et al. 2019). Thus qPCR copy numbers quantified may serve as a proxy for bacterial cell numbers present, but do not signify bacterial viability (Friedman et al. 2014a). Briefly, qPCR reactions were conducted using 0.5 µL 2x GoTaq, 12.5 µm qPCR Master Mix (Promega Corp., Madison, WI, US), 0.8 mg/µL BSA (New England Biolabs Inc., Ipswich, MA, US), 2uL of template, and sterile water for a total volume of 25 µL per reaction. Thermal cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was run in duplicate along with a negative template control and a plasmid-based standard curve of known WS-RLO copy numbers with a limit of detection of 3 gene copies for both genomic and cDNA (Friedman et al. 2014a). WS-RLO copy number was determined via regression analysis of the standard curve. Seawater filter quantities were calculated as copies per mL, while tissue and fecal quantities were calculated as copies per gram. All samples negative for WS-RLO via qPCR were evaluated using a Qubit™ 3.0 fluorometer (Invitrogen Corp., Carlsbad, CA, US) to ensure the presence of nucleic acid.

[B]Statistical analyses

All data were analyzed in JMP 12.0 (SAS). Histological data were analyzed using least squares regression (LSR) with dose and time as fixed factors and tank as a random factor. qPCR data were natural log transformed for normalization and analyzed by ANOVA with pairwise multiple comparisons using the Holm-Sidak method at a significance level = 0.05 to test for relationships between temperature, replicates, and time (JMP 12.0). Infection prevalence per tank (fecal shedding) and per individual abalone (PE tissue) were estimated using qPCR data (WS-RLO DNA copies). Differences in fecal shedding were tested using LSR with dose and day nested

within dose as factors. Differences among factors were post-hoc tested using the Tukey-Kramer multiple comparison method. Infectious dose values were calculated according to the methods of Reed & Muench (1938) with the percentage of infected abalone per replicate as the unit of measurement due to the fact that infection by cross-contamination during a relatively short incubation period was unlikely to occur as demonstrated by previously published cohabitation infection experiments with red abalone (Crosson & Friedman, 2018).

[A]Results

[B]WS-RLO temporal stability

Initial mean ± SE WS-RLO DNA levels were 18.09 ± 0.61 copies/mL, 2.99 ± 0.39 copies/mL, and 93.60 ± 27.20 copies/mL in water received from the commercial abalone farm for the October 2016, February 2017, and June 2017 trials, respectively (Fig. 2). The highest mean ± SE WS-RLO DNA levels were detected for the first 24 h post-arrival for all trials (October = 32.60 ± 5.20 copies/mL, February = 3.61 ± 0.73 copies/mL, June = 95.50 ± 1.50 copies/mL; Fig. 2). WS-RLO DNA copies declined rapidly between 24-72 h for the October trial, and between 24-48 h for the February and June trials ($P < 0.05$; Fig. 2). WS-RLO DNA levels declined below the qPCR assay limit of detection (3 copies/mL) between 72-168 h of the October trial (Fig. 2A), by 48 h of the February trial (Fig. 2B), and by 120-144 h of the June trial (Fig. 2C). No significant temperature or replicate effects on WS-RLO DNA stability were observed (Table 1). WS-RLO RNA was below qPCR detection at all time points for all trials (data not shown).

[B]WS-RLO infectious dose

Initial WS-RLO homogenate dosing concentrations were 4.08 x $10^5$ ("$10^0$ stock"), 6.49 x $10^4$ ("$10^{-1}$ dilution") and 5.07 x $10^3$ ("$10^{-2}$ dilution") WS-RLO DNA copies/mL, which were used to calculate infectious dose curves. The infectious dose required to generate 50% infection prevalence based on qPCR of abalone PE tissues and shed feces were similar: 2.26 x $10^3$ and 3.16 x $10^3$ WS-RLO DNA copies/mL, respectively. An ID50 could not be calculated using visual observation of bacterial inclusions within abalone PE tissues via histology. However, the infectious dose required to generate 25% infection prevalence (ID25) based on histology was 6.98 x $10^4$ WS-RLO DNA copies/mL (Table 2, Fig. 3).

Only abalone from the highest dose ($10^5$) shed significantly more WS-RLO DNA than did the other doses ($P < 0.05$; Fig. 4). The infection prevalence of abalone tanks positive for WS-RLO

DNA as determined by qPCR of fecal samples was 100% for the $10^5$ dose, 75% for the $10^4$ dose and 25% for the $10^3$ dose (Fig. 3). Abalone feces from SSW control tanks contained no WS-RLO DNA throughout the duration of the experiment. No WS-RLO DNA in abalone feces was detected in any treatment 6 days post-dosing (Fig. 4). The amount of WS-RLO DNA in abalone feces increased with time ($P = 0.0068$) and by dose ($P = 0.0042$).

Mean ± SE WS-RLO DNA copies/g of abalone PE tissue for the $10^5$, $10^4$, and $10^3$ doses upon termination of the experiment on day 61 were $8.87 \times 10^5 \pm 3.75 \times 10^5$, $4.16 \times 10^5 \pm 1.49 \times 10^5$, and $6.03 \times 10^4 \pm 2.45 \times 10^4$, respectively. Abalone PE tissues from SSW control tanks contained no WS-RLO DNA. The infection prevalence of abalone positive for WS-RLO DNA as determined by qPCR of PE tissues was 87% for the $10^5$ dose, 69% for the $10^4$ dose, and 40% for the $10^3$ dose (Fig. 3). Mean ± SE WS-RLO infection intensity and prevalence in PE tissues scored by histology were 0.10 ± 0.05 and 28% for the $10^5$ dose, 0.02 ± 0.02 and 10% for the $10^4$ dose, and 0.07 ± 0.04 and 5% for the $10^3$ dose (Fig. 3)

[A]Discussion

[B]WS-RLO temporal stability

This was the first study attempting to assess WS-RLO temporal stability outside of its abalone host at seawater temperatures spanning the seasonal range of southern CA waters (https://www.nodc.noaa.gov). Despite differences in initial pathogen levels during the three seasonal trials, WS-RLO DNA was not stable in seawater longer than 48 h. WS-RLO DNA levels fell below the qPCR assay's limit of detection (3 copies/mL) by 48-168 h, while WS-RLO RNA levels were below detection at all time points of all trials. Cellular and extracellular DNA is often very persistent in the environment (Taberlet at al. 2012). However, bacterial DNA persistence in seawater can vary considerably, as short as 6.5 h to an estimated 2 months (see review by Neilsen et al. 2007). Key environmental factors influencing DNA persistence in aquatic ecosystems are temperature and sunlight as prolonged exposure to high temperatures and/or UV radiation result in single stranded and fragmented DNA molecules (Paul et al. 1987, Huver et al. 2015). All of our stability trials were conducted in the dark to eliminate the risk of UV-induced DNA degradation and allow us to examine the influence of seawater temperature (14°C and 18°C) and time outside of an abalone host (up to 168 hours or 7 days) on WS-RLO DNA persistence. Time proved to be the only significant factor tested that influenced WS-RLO

DNA stability. Although we observed no influence of temperature on WS-RLO DNA stability, the strong correlation between pathogen abundance and seawater temperature remains an important consideration when conducting disease transmission experiments and managing both wild and cultured abalone populations. Seawater temperatures of 18°C are known to facilitate WS-RLO proliferation (Moore et al. 2000, Friedman et al. 2002, Braid et al. 2005, Crosson & Friedman 2018) and likely explains the higher observed levels of pathogen DNA detected in our June trial when mean ambient coastal seawater temperatures are typically highest in CA (https://www.nodc.noaa.gov). Although elevated seawater temperature is the key environmental factor driving WS expression, thermal variation has been shown to influence WS-RLO transmission in black abalone (Ben-Horin et al. 2013). Coastal seawater temperature records for southern CA consistently show high variance over time and often include intervals best described as chaotic; future studies should consider the influence of thermal variation on WS-RLO stability.

Although WS-RLO DNA proved to be relatively unstable *in vitro*, transmission of WS continues to occur in the natural environment (VanBlaricom et al. 1993, Altstatt et al. 1996, Miner et al. 2006). Host behavior and pathogen reservoirs or vectors may influence the potential for WS transmission. The WS-RLO is transmitted horizontally via a fecal-oral route (Friedman et al. 2002) and abalones are gregarious by nature with some species, such as the intertidal black abalone, even displaying a stacking behavior (Douros 1987, VanBlaricom et al. 1993). This behavioral dynamic can readily facilitate the spread of disease within a population. Unknown reservoirs or vectors of the WS-RLO bacterium can also serve as potential disease transmission mechanisms. A marine bacterium in the family *Rickettsiaceae* was discovered living in the cytoplasm of the protozoan ciliate, *Diophrys appendiculata* (Vannini et al. 2005). While the novel bacterium does not appear to cause disease in the ciliates, this symbiotic relationship reveals that members of the *Rickettsiaceae* are more diverse and widespread than once thought. More recently, an intertidal snail belonging to the *Turbinidae* family was discovered harboring WS-RLO inclusions in Japan (Iku Kiryu, pers. comm.), demonstrating that the WS-RLO bacterium has the ability to infect other marine vetigastropods. Expansion of the WS-RLO host range coupled with episodic ENSO or strong seasonal warm water events could readily facilitate spread of the pathogen among abalone populations. The potential geographic spread of

pathogenic RLOs in the natural environment by non-target organisms and variable oceanic conditions has significant ecological implications and warrants further research.

The role of virus-induced bacterial mortality in seawater should also be considered when assessing WS transmission risk. Bacteriophages constitute a significant controlling factor for bacterial abundance and activity with estimates of bacteriophage infection being responsible for ~10-50% of bacterial mortality in both seawater and sediment (Fuhrman & Noble 1995, Glud & Middelboe 2004). Friedman & Crosson (2012) recently discovered a bacteriophage infecting the WS-RLO and, while it remains unclear as to whether viral replication occurs via a lytic or lysogenic cycle, co-infection was shown to attenuate WS disease progression in black abalone (Friedman et al. 2014b). The potential for the bacteriophage to modulate WS disease dynamics has significant ecological and economic impacts with regards to abalone survival and disease management. Future studies further characterizing the role of the bacteriophage on WS-RLO survival are needed.

Our study confirms that southern CA abalone farm effluent seawater contains WS-RLO DNA and that RLOs could spillover to nearby wild abalone populations as suggested by Lafferty & Ben-Horin (2013). However, no established method exists to determine the source of the pathogen and results from our WS-RLO stability trials indicate pathogen DNA persistence is limited to ~2 days making transmission over broad geographic ranges unlikely. These observations combined with the low concentration of WS-RLO DNA in the farm effluent (a maximum of 94 copies/mL) and the large dilution factor once the RLO reaches coastal waters bring question to the assertion of Lafferty & Ben-Horin (2013) that an abalone farm was a likely source of WS-RLO DNA over 20 km from the farm outfall. The potential for species other than abalone, such as kelps or other macroalgae to which the WS-RLO may adhere, could serve as vectors or a link from sources of the WS-RLO in the natural environment to off-shore abalone populations (Fuller, 2017). It is important to note: (1) the WS-RLO was first detected in wild abalones and has since spread to farmed populations, (2) the WS-RLO is now considered established in southern and central CA waters and thus farm influent seawater may contain the pathogen creating spillback concerns, and (3) due to the rapid decline (degradation) of detectable WS-RLO nucleic acid in seawater, the relative risk of an abalone farm acting as a significant source of pathogen spillover depends on its proximity to wild abalone populations and local

seawater circulation patterns. Future research should examine oceanographic patterns surrounding CA abalone farms and determine their proximity to wild abalone populations in order to accurately assess WS-RLO transmission risk. A recent sentinel study conducted in southern CA estimated WS-RLO transmission risk *in situ* by deploying modules containing uninfected abalone at two sites, one near an onshore commercial abalone farm and one in proximity to wild aggregations of abalone (Fuller, 2017). WS-RLO DNA was detected in seawater via qPCR at the site where modules were deployed near wild abalones but not at those near farm site modules even though WS-RLO DNA was detected in the farm's effluent seawater (Fuller, 2017). Infection prevalence and intensity of the sentinel abalone were also very low and similar at both wild and farm site modules. These *in situ* WS-RLO transmission data support our findings of minimal temporal stability of the WS-RLO and highlight the importance of thoroughly assessing all aspects of WS disease dynamics for the proper management of both wild and cultured abalones.

[B]WS-RLO infectious dose

This was the first study to successfully infect abalone with known concentrations of WS-RLO and calculate an infectious dose. We demonstrated that $2.26 \times 10^3$ and $6.98 \times 10^4$ WS-RLO DNA copies/mL of seawater are needed to infect 50% (using qPCR as a proxy for infection) or 25% (using histology to determine infection), respectively, of juvenile red abalone with a 3 h exposure period. The difference between quantification methods is likely due to the increased sensitivity of qPCR relative to histology. In both white (Friedman et al. 2007) and black abalones (Friedman et al. 2014b) exposed to WS-RLO, 23-30% more individuals were positive for WS-RLO DNA using qPCR as a proxy relative to histology. In fact, visual observation of bacterial inclusions correlated with qPCR copy numbers of $10^6$ copies/mg of tissue (Friedman et al. 2007, 2014b). The WS-RLO DNA levels from our infectious dose experiment were 100-1,000 times higher than those quantified from abalone farm effluent seawater during our stability trials (~3 to 94 WS-RLO DNA copies/mL). The ID50 as assessed by qPCR was similar when calculated using PE tissues from individual abalone ($2.26 \times 10^3$ copies/mL) or feces shed per tank of abalone ($3.16 \times 10^3$ copies/mL). These data suggest that determining WS-RLO DNA levels via abalone feces is a reliable alternative to lethal PE tissue sampling. Quantification of WS-RLO DNA from PE tissues was performed on individual abalone upon experimental termination, while fecal sampling was pooled by treatment tank allowing us to track WS-RLO DNA levels

over time. Future dosing experiments should include replicates of individual abalone to avoid concern of possible WS-RLO cross-transmission.

Efficient disease transmission relies primarily on initial infectious dose (including pathogen concentration and host exposure time), pathogen virulence, host immunity and important environmental drivers such as temperature and salinity (Grassly & Fraser 2008). Dose effect must be considered as a function of the bacterial strain and the life stage and species of the host. Our study demonstrated a relatively high WS-RLO dose was needed for a 3 h exposure period at an optimal pathogen temperature (18°C) for the moderately susceptible red abalone (Crosson & Friedman 2018). The composition of at risk abalone populations will be an important factor given the substantial differences in susceptibility and resistance to WS that have been observed among abalone species (Crosson & Friedman, 2018). Fuller's (2017) sentinel study illustrated that red abalone developed low level infections after 6 weeks of exposure in CA waters near an abalone farm where no WS-RLO DNA was detected in the seawater and near wild abalone populations where WS-RLO DNA was detected in the seawater. These data suggest a disease vector, such as macroalgae to which the WS-RLO could adhere and concentrate, may be responsible for transmission and warrants further examination. Collectively, these studies highlight the need to characterize the relationship among pathogen dose, exposure time, host species and seawater temperature in order to better understand WS-RLO transmission dynamics. Our novel dosing method can be used to facilitate these types of studies and will help address remaining questions associated with WS disease dynamics such as bacterial reproduction, shedding rates (pathogen spread potential) and critical thresholds (host density needed for pathogen invasion).

In order to generate predictive models and accurately assess transmission risk associated with WS, more studies addressing the fundamentals of disease dynamics need to be conducted. Host, pathogen, and potential vector diversity/species composition should be explored in relation to ecological processes at community, ecosystem and landscape levels. Collectively, our findings are essential components of understanding WS disease dynamics and will help to assess transmission risk within and among abalone populations for better informed management decisions to successfully protect and restore abalone species in WS-endemic areas.

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561 Table 1. ANOVA output examining the relationship among temperature, replicates, and time for
562 the WS-RLO stability trials. Significant factors are bolded.

| Trial | Factor | df | SS | MS | F Ratio | *P* Value |
|---|---|---|---|---|---|---|
| October 2016 | **Whole model** | 4 | 29.35 | 7.34 | 26.31 | **0.0113** |
| | Replicate | 1 | 0.29 | - | 1.04 | 0.3837 |
| | **Time** | 3 | 29.07 | - | 34.74 | **0.0079** |
| February 2017 | **Whole model** | 10 | 22.15 | 2.22 | 25.67 | **< 0.0001** |
| | Temperature | 1 | 0.22 | - | 2.54 | 0.1247 |
| | Replicate | 1 | 0.03 | - | 0.31 | 0.5851 |
| | **Time** | 7 | 19.99 | - | 33.09 | **< 0.0001** |
| June 2017 | **Whole model** | 10 | 384.51 | 38.45 | 63.62 | **< 0.0001** |
| | Temperature | 1 | 0.11 | - | 0.18 | 0.6714 |
| | Replicate | 1 | 0.001 | - | 0.002 | 0.9650 |
| | **Time** | 7 | 327.02 | - | 77.30 | **< 0.0001** |

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564 Table 2. WS-RLO dose (DNA copies/mL) required to generate 50% or 25% infection prevalence
565 in red abalone (*H. rufescens*). na = not available.

| Infectious Dose | Feces qPCR | Tissue qPCR | Histology |
|---|---|---|---|
| 50% | $3.16 \times 10^3$ | $2.26 \times 10^3$ | na |
| 25% | na | na | $6.98 \times 10^4$ |

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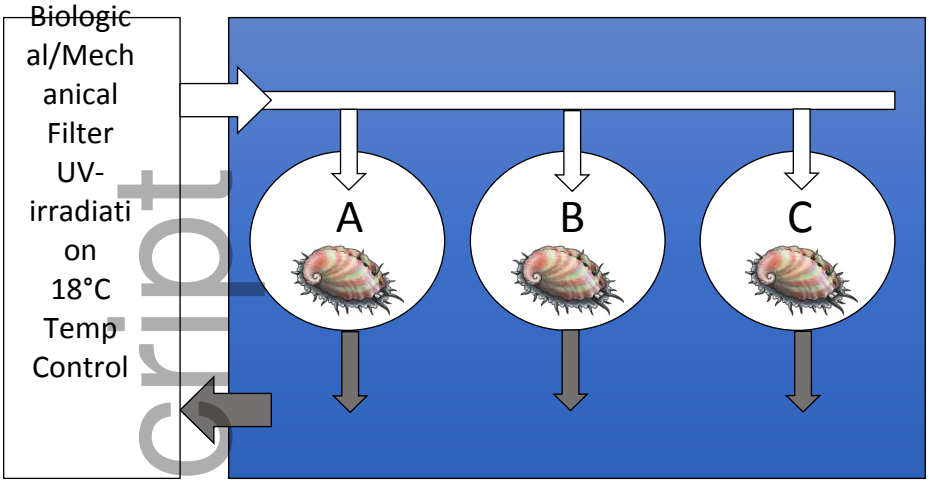
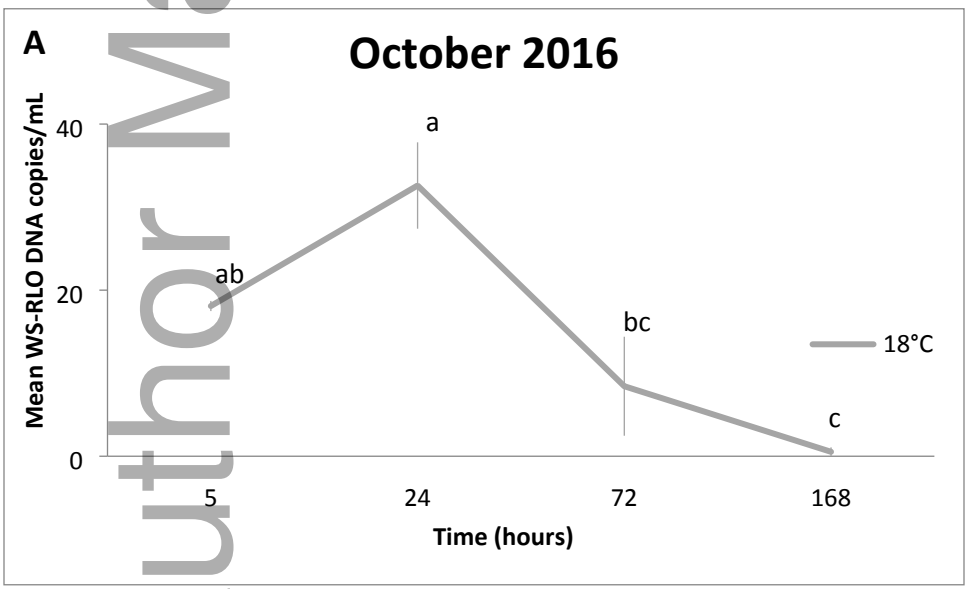



Figure 1. Schematic of recirculating seawater systems used for the WS-RLO infectious dose experiment at four concentrations: 0 (sterile seawater control), $10^3$, $10^4$, and $10^5$ WS-RLO DNA copies/mL. Each 175 L system contained triplicate 11.4 L tanks (A, B, C) of 10 juvenile red abalone (*Haliotis rufescens*). Post-dosing, effluent seawater (gray arrows) was pumped through a series of biological/mechanical filtration, UV-irradiation, and an 18°C temperature controller prior to reentry to individual abalone tanks (white arrows).



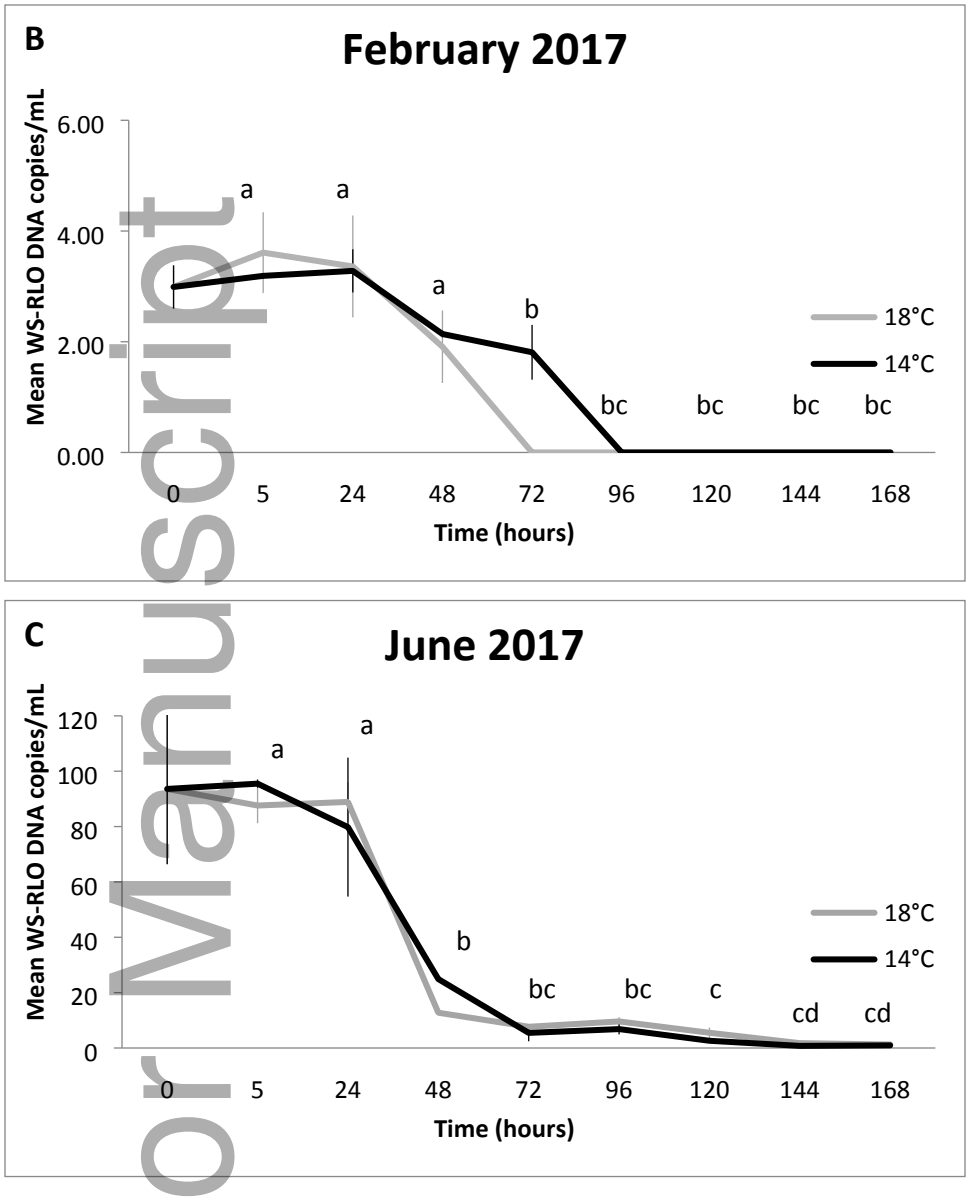



Figure 2. Mean WS-RLO DNA copies/mL in seawater effluent from a commercial abalone farm located in Goleta, CA, US and collected in October 2016 (A), February 2017 (B) and June 2017 (C). Bars represent standard error and letters indicate statistical differences ($P < 0.05$).

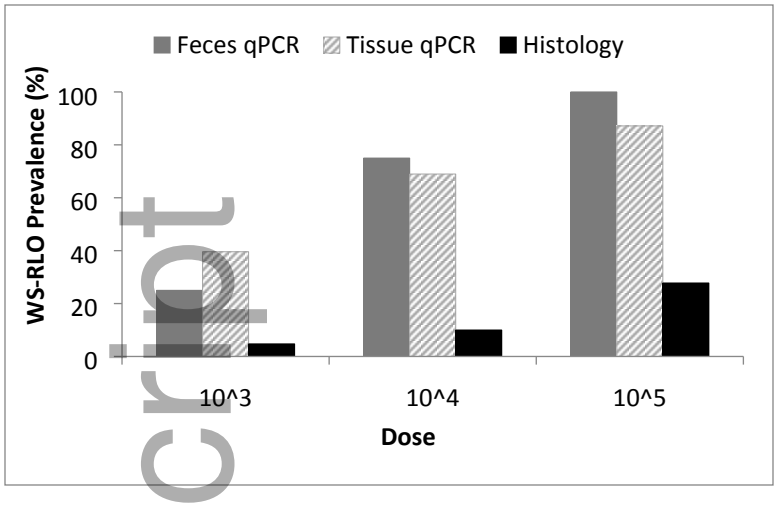



Figure 3. WS-RLO prevalence in red abalone (*Haliotis rufescens*) assessed by quantitative PCR (qPCR) of feces and post-esophageal (PE) tissues and by histological examination of PE tissues upon termination of the experiment on day 61.

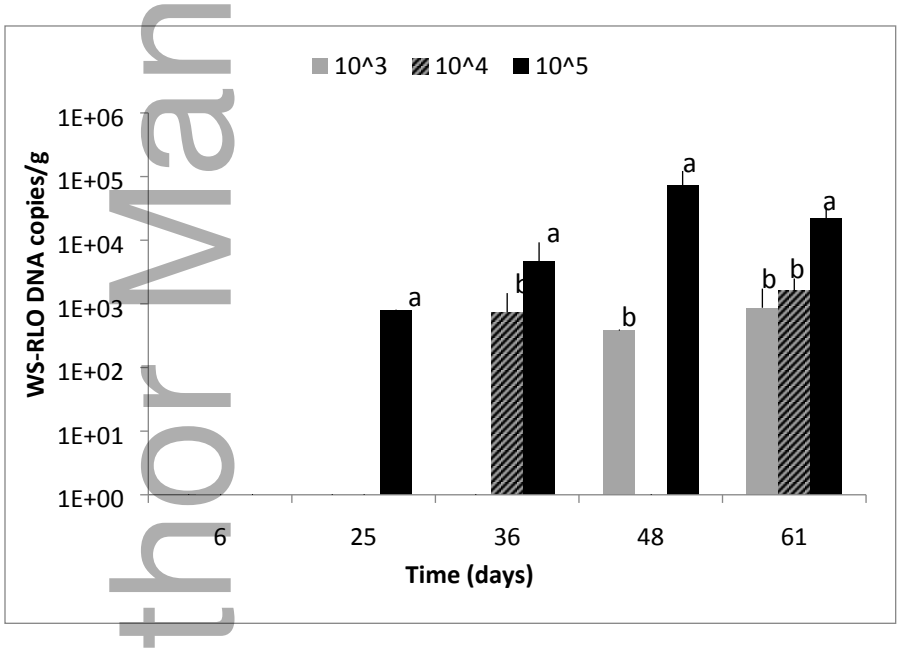



Figure 4. Mean WS-RLO DNA copies/g of red abalone (*Haliotis rufescens*) feces for the duration of the WS-RLO infectious dose experiment. Bars represent standard error and letters indicate statistical differences ($P < 0.05$).