Title

Tools for Management of Withering Syndrome in Abalone, Haliotis spp: PCR Detection and Feed-Based Therapeutic Treatment

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"Tools for Management of Withering Syndrome in Abalone, *Haliotis* spp: PCR Detection and Feed-Based Therapeutic Treatment."
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Withering syndrome is a chronic, often fatal disease of multiple species of California abalone. It was first observed in the mid-1980s at the Channel Islands off Southern California and has since spread along the main coastline as far north as the San Francisco bay area. Recent research has demonstrated that the causative agent is a Rickettsiales-like prokaryote (WS-RLP, Friedman et al. 2000), and that WS expression is much more rapid and severe at elevated water temperatures (Moore et al. 2000). Aside from impacts on wild populations, WS has resulted in substantial losses in red abalone farms. As a result of these losses and our observation that the WS-RLP is sensitive to tetracyclines, we developed and oral administration of oxytetracycline in conjunction with researchers at The Abalone Farm, Inc.

There is intense interest on the potential spread of the agent and the disease to the robust red abalone populations in Northern California. Extensive sampling over the past several years involving hundreds of red abalone from more than a dozen locations has demonstrated that most North Coast abalone populations remain free of the WS-RLP, although 5/29 abalone from Crescent City and 2/330 from Van Damme were positive. Continued monitoring is necessary, particularly in the vicinity of the two locations at which WS-RLP positive animals were found. A non-lethal detection method is highly desirable to obviate the need for sacrificing large numbers of these highly prized and slow-growing animals. A highly sensitive polymerase chain reaction (PCR) diagnostic method has been developed for detection of the WS-RLP in abalone tissues. The PCR technique consists of billion-fold replication of a gene fragment specific to the pathogen, which is visualized as a distinct band on an agarose gel. The aim of these studies was to optimize the PCR protocol and expand its applications, including an attempt to develop a PCR-based sampling method capable of detecting the WS-RLP in environmental samples. Collectively, the tools developed will significantly aid management and control of this devastating disease.

Experiment 1: Long term abalone oxytetracycline injection study.
A dilution of Liquamycin LA-200, *P*fizer to 0.20 ug/mL oxytetracycline was assayed using our protocol as 0.21 ug/mL. Survival of treated red and black abalone was significantly higher than that of sham treated controls ($p<0.001$ and $p=0.036$, respectively). In fact, only a single treated red abalone died in this study, while 35% of the untreated animals died from WS. The moribund animals sampled and those that died from WS contained heavy RLP infections. Visual and microscopic condition of the animals was correlated with RLP infection intensity ($p<0.05$). All sham treated (control) black abalone died from WS and had heavy RLP infections (average of 2-3 intensity rating), regardless of initial condition. Several treated black abalone died from WS prior to completion of all 12 IM injections. Microscopic examination of stained tissue sections revealed that these animals died with clinical WS. Only black abalone that were asymptomatic (0 rating) or had very early clinical signs (1 rating) survived the disease with oxytetracycline treatments. Feeding rates of treated abalone were also significantly higher than for sham treated control animals ($p<0.001$ for black abalone and $p<0.0001$ for red abalone), even after only administration of 3 of the 12 doses needed to the RLP infections. Microscopic examination revealed that our treatment regime successfully cured the WS-RLP infection in abalone and that all control abalone that died had advanced WS.

These data indicate that the RLP is the causative agent of WS and that abalone with visible signs are in the very end stages of the disease.

Results illustrated that multiple intramuscular injections of oxytetracycline (Liquamycin-LA) effectively eliminated the RLP from abalone tissues. As only those abalone that were either asymptomatic or some of those with very early signs (stage1), abalone with visible clinical signs are in the end stages of WS and that once clinical signs are observed, the animal cannot recover as tissue damage was beyond the ability of the animals to repair.

Experiment II: Development of an oral administration of oxytetracycline.

Water temperature averaged 15.3°C during the 30 day pre-treatment period with a 14.0°C minimum and a 19.2°C maximum. During the subsequent 14 day treatment period, the mean was 15.9°C, with a 14.5°C minimum and a 19.8°C maximum. Consumption of the medicated diet ranged from 0.06% to 0.33% per day with an average of consumption of 0.20% of tank biomass per day which resulted in a consumption of 3.89g OTC /100 lb of tank biomass versus the projected consumption of 10 g OTC/100 lb of biomass. Despite this, significant reductions in abalone mortality (6.45% in treated and 36.38% in control groups; Figure 1), intensity of RLP infection and degree of morphological changes in the digestive gland were observed in the medicated treatments relative to the controls 5 d after medication ($p<0.001$, $p<0.001$, $p<0.05$, respectively). Nearly 11 months after the average weights of the abalone that survived both treatments (medicated and unmedicated) were similar (129.97 g and 127.78 g, respectively; $p>0.05$). Yet, a significantly higher yield or biomass ($p<0.001$) was achieved per aquarium in the medicated treatments (15.55 kg) relative to the control aquaria (11.15 kg), an overall loss

of biomass relative to that stocked at the beginning of the experiment (13.35 kg; Figure 2). Treated abalone ate twice as much kelp as the non-medicated control animals (7.00% versus 3.14% biomass per day, respectively, $p<0.0001$).

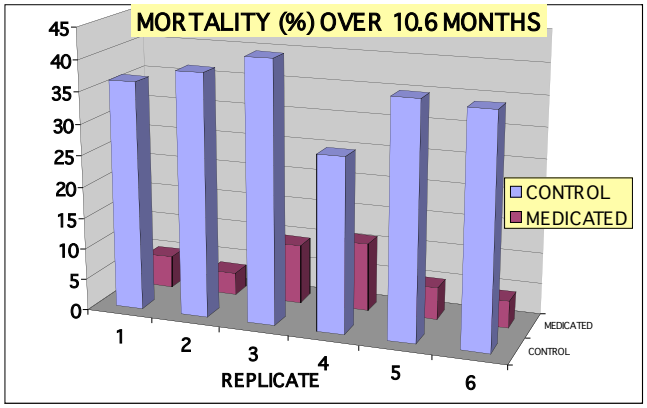


Figure 1: Mortality of medicated and control (unmedicated) abalone at a commercial abalone farm nearly one year after a single 2 week treatment.

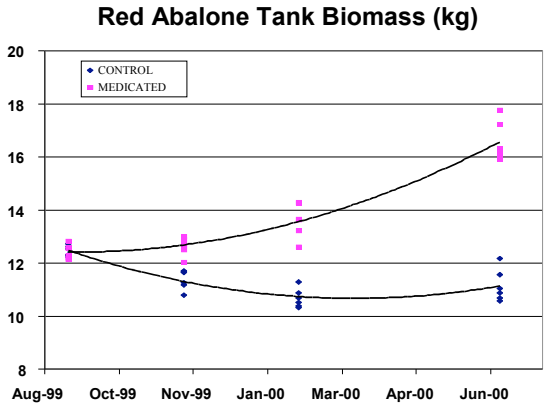


Figure 2: Yield (kg) of abalone in the medicated and control tanks. Note that the biomass in the treated tanks increased, while that in the control tanks actually declined over the duration of the study.

The possibility exists that secondary infections in control abalone may have contributed to elevated mortality in the control treatments (e.g. to opportunistic bacteria). However, we only observed one such infection as evidenced by microscopic examination of stained tissue sections; all other animals died with signs of WS and high rickettsial burdens.

Results from this trial illustrated that an oral medication is effective in reducing the prevalence and intensity of infection of RLPs in abalone. Although, some RLPs did survive treatment, RLP burdens were substantially reduced for at least one year after a single treatment regime. In fact, in laboratory studies, abalone that received OTC oral

medication were difficult to infect for several months after treatment (Braid, Moore, Friedman, pers. obs.)

Experiment III: Depletion of oxytetracycline residues in medicated red abalone, Haliotis rufescens.

A decline in oxytetracycline levels was observed over time, with a peak level of 27.42 ppm one day post withdrawal. All six individuals sampled on day 15 had OTC concentrations below the desired FDA tolerance level of 2 ppm (Figure 3). Concentrations of OTC in individual abalone were variable. No correlation between position of an individual abalone in the tank and oxytetracycline concentration in the abalone foot muscle ($p>0.05$). No detectable OTC was observed in any control foot muscle tissue. Additional studies examining the retention of OTC in the digestive gland and the influence of treatments to WS susceptibility are planned.

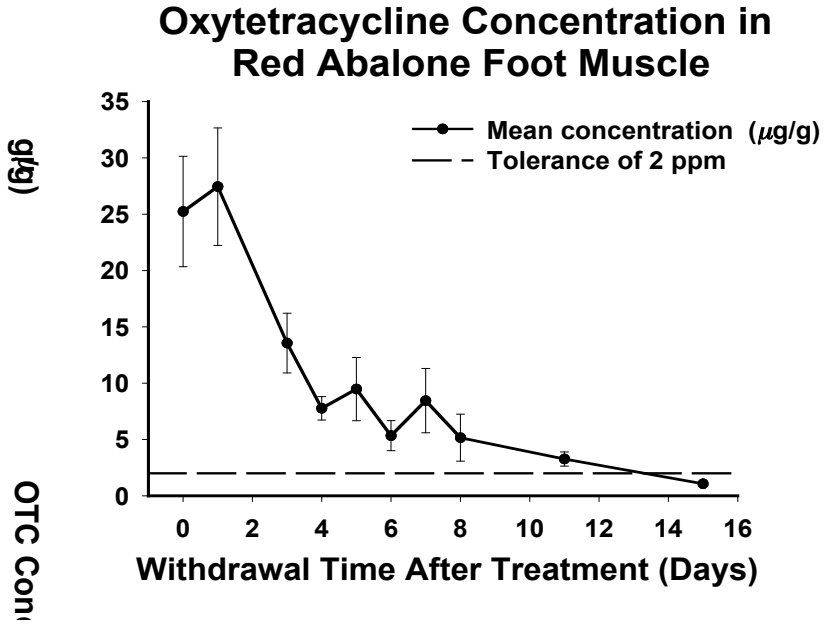


Figure 3. Depletion of oxytetracycline in the foot muscle of red abalone after an oral administration of the therapeutant for 2 weeks (15% Terramycin 100 w/w).

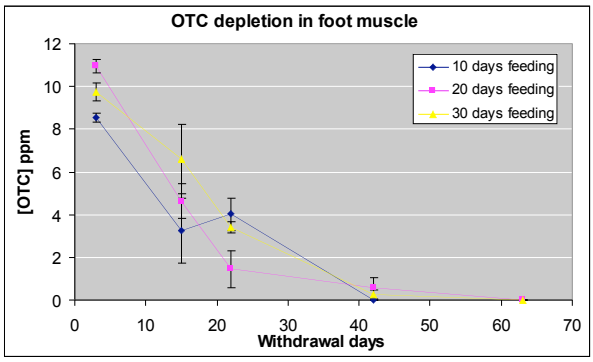
Data from Experiments I – III were published in the journal *Aquaculture*:

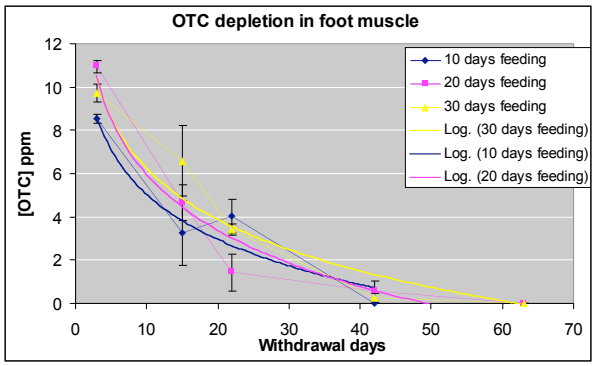
Friedman, C.S., Trevelyan, G., Mulder, E.P., and Fields, R. 2003. Development of an oral administration of oxytetracycline to control losses due to withering syndrome in cultured red abalone *Haliotis rufescens*. Aquaculture 224(1-4):1-23.

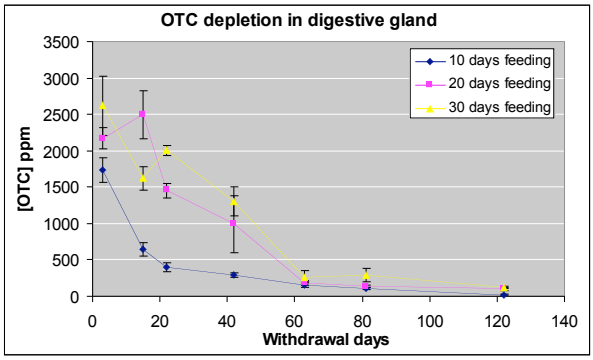
Experiment IV: Optimization of and Oral Oxytetracycline Administration

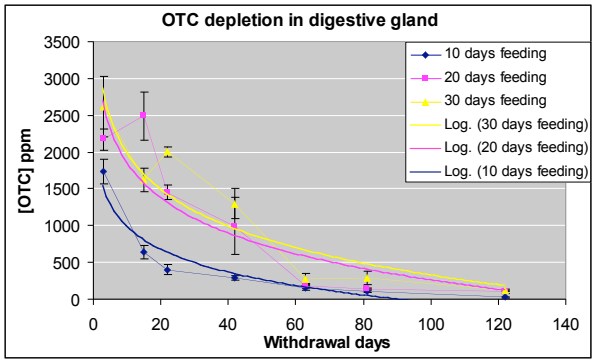
In order to assess the optimal *per os* (oral) administration of oxytetracycline, we fed triplicate groups of red abalone a medicated diet containing12% TM-100 (1.8% active OTC) at a rate of 0.45% body weight per day for 10, 20 or 30d. Control abalone were fed kelp *ad libitum*. The first day after cessation of feeding was considered withdrawal day 0. At withdrawal days 3, 15, 22, 42, 63, 81, and 122 d, samples were removed for 1) assessment of WS-RLP burden and disease by histology and 2) quantification of oxytetracycline in the foot muscle and digestive gland using a bacterial inhibition assay.

Abalones that received medicated feed for 10 d had significantly lower OTC levels in the digestive gland (DG) than those medicated for 30 d ($p<0.05$); those medicated for 20 days also contained more OTC than the 10 d group but the difference was not significant ($p=0.059$). No differences in OTC levels were observed in the foot muscle of abalone fed 10, 20 or 30 d of medicated feed ($p>0.05$). The foot muscle of those fed for 10 d was devoid of detectable OTC by 42 d, while those medicated for 20 and 30 d required 63 d for complete depletion. In contrast, the DG retained OTC in all groups until 122 d. At this time, the animals medicated for 30 d had significantly more OTC (126 ppm) in the DG than did those medicated for 10d (23 ppm). While abalone medicated for 20 d also had more OTC (102 ppm) than those fed for 10d, the difference was not significant ($p=0.099$). No differences in OTC levels at 122 d was observed in those medicated for 20 versus 30 d ($p>0.05$). Thus, we observed that feeding medicated feed for 30 days gave no added benefit to the abalones regarding OTC levels in the DG or foot muscle relative to providing 20 days of medication ($p>0.05$). In addition, the DG appeared to concentrate the therapeutant in the DG as evidenced by the up to 270-5000 fold more OTC in the DG than foot muscle (withdrawal days 3 and 63, 30 d medication, respectively). No added protection was observed against the RLP by the additional medicated feed as well. To date, only a single bacterial inclusion has been observed in any medicated abalone (up to 63 d of withdrawal) in the 3 d withdrawal sample from the 30 d medicated group. This inclusion is *not* the WS-RLP but a genetically distinct intracellular bacterium (Moore, Baxa and Friedman, pers. obs.) based on *in situ* hybridization and preliminary sequence data (see sections below). Control abalone were light to moderately infected with the WS-RLP and to a lesser extent, this other intracellular bacterium. The depletion dynamics of the DG and foot muscle are illustrated in the graphs below. Foot muscle depletion curves illustrated below had over 90% correlation with one another, while those of the DG varied between 75% to 89% correlations for the 10 and 20 d medication and 20 to 30 day medications, respectively. Based on these data feeding of 10-20 d of medicated feed appears to be effective. However, given the variation in medicated food consumed between individuals in a tank, 20 d may be the optimal.


OTC depletion in foot muscle
[OTC] ppm
Withdrawal days
10 days feeding
20 days feeding
30 days feeding


OTC depletion in foot muscle
[OTC] ppm
Withdrawal days
10 days feeding
20 days feeding
30 days feeding
Log. (30 days feeding)
Log. (10 days feeding)
Log. (20 days feeding)


OTC depletion in digestive gland
[OTC] ppm
Withdrawal days
10 days feeding
20 days feeding
30 days feeding


OTC depletion in digestive gland
[OTC] ppm
Withdrawal days
10 days feeding
20 days feeding
30 days feeding
Log. (30 days feeding)
Log. (20 days feeding)
Log. (10 days feeding)

PCR protocol optimization

Prior to this study, a PCR method to detect the WS agent was developed in our lab (Andree et al. 2000). Primers RA5-1 and RA3-6 amplify a 160 base-pair portion of the 16sRNA gene. The method was demonstrated to work well with DNA purified from abalone tissue, but had not been completely optimized. Optimal conditions for WS-RLP PCR with primers RA5-1 and RA3-6 were established using a 'PCR optimization kit' (Sigma). This is necessary because optimal conditions vary widely depending on the nature of the primers. The basic chemical conditions were optimized by varying each component or parameter in a series of reactions:

Component/Parameter	*Range studied*	*Optimal*
KCl concentration	25mM-75mM	25mM
$MgCl_2$ concentration	1.5-4.5mM	3.0mM
pH	8.3, 8.8, 9.2	8.8
Annealing temperature	50-70°C	60°C
Primer concentration	0.1-1μM	0.5μM

After identifying optimal basic conditions, the effects of specific adjuncts or additives were investigated. Single-strand binding protein, formamide, ammonium sulfate, bovine serum albumin (BSA), glycerol, and dimethyl sulfoxide (DMSO) are known to modify the sensitivity and specificity of PCR reactions. The effects of these chemicals were investigated by adding each separately to a known positive reaction at a recommended concentration. All except BSA inhibited the reaction (decreased the sensitivity) and BSA had no effect. The adjuncts other than BSA will be further investigated if non-specific reactions (decreased specificity) ever become a problem. We began adding BSA at a concentration of 400ng/μl to all reactions based on reports of its beneficial effect on diminishing the effects of PCR inhibitors (e.g., C. Kreador, 1996). We also began using a 'Hot-start' DNA polymerase, Sigma HotStarTaq, which has significantly reduced the presence of primer-dimers and likely increased sensitivity.

Our optimized PCR protocol for 50ul reaction volumes:

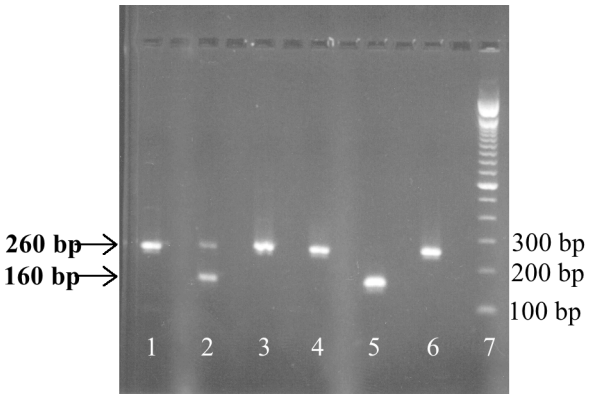
100ng of template DNA, 2U Sigma JumpStarTAq, 1x JumpStarTAq buffer, 3mM MgCl2, 400ng/ul BSA, 200uM dNTPs, and 0.5uM of each primer, RA5-1 and RA3-6. The PCR schedule is: 95C for 3 minutes, 40 cycles of 95C for 1min, 62C for 30 seconds, 72C for 30 seconds, and finally 72C for 10min. PCR products are visualized by electrophoresis in 2% agarose gels in TBE buffer

Controls to demonstrate amplifiable DNA

A negative PCR reaction can be due to the absence of a sufficient quantity of high-quality target DNA or to the presence of substances that inhibit PCR reactions. DNA purification protocols and kits are designed to remove inhibitors but the wide range of inhibiting substances and sample types makes this a challenging aspect of PCR diagnostics. In early studies we used a rapid and efficient method of DNA preparation, the Pierce Lyse N Go kit, but problems with inhibitors were indicated in some cases. We then found that the Qiagen DNeasy kit produces high quality DNA without inhibitors in most instances. We recently began using the Qiagen QIAmp Stool Kit, which follows a

protocol very similar to that of DNeasy but includes additional steps to remove inhibitors of the types present in human stool, such as plant-derived humic acids. Since compounds such these will also be present in abalone digestive gland, feces and concentrated seawater samples, this DNA purification method will help minimize presence of PCR inhibitors.

To validate that a negative WS-RLP PCR result for a sample of DNA purified from abalone is not due to inhibitors, we perform an additional PCR to attempt amplification of a portion of the gene encoding the cytoskeletal protein actin, using primers developed in Dr. Jane Burns' laboratory at the UC San Diego School of Medicine. The actin gene should be present in any sample of abalone tissue DNA. Verifying DNA presence and quality by amplification of the actin gene works well and in some cases we have shown that negative samples for WS-RLP were also negative for actin amplification, indicating presence of inhibitors or insufficient quantity of quality DNA. Actin is not expected to be present in environmental samples such as water or feces and therefore cannot be used as a control for these types of samples. We developed an internal control plasmid, designated WC9, that can be added to PCR reactions. This plasmid contains the 160bp WS-RLP amplicon with a 100bp insertion. Therefore, when added to a PCR reaction with the WS-RLP primers, a 260bp product will amplify regardless of whether the target DNA is present. If no 260bp band is present, the presence of inhibitors is strongly implicated. By adding an exceedingly small amount of the plasmid (a 10-billion fold dilution of the stock), competition with amplification of the target DNA can be avoided. The figure below shows an example of the use of this tool.



Ethidium bromide-stained 2% agarose gel after electrophoresis of DNA amplified with primers for the WS-RLP 16sRNA gene. The WC9 positive control plasmid was added to all six samples at a 10-9 dilution. In the absence of WS-RLP DNA, the 260bp control plasmid is amplified (lanes 1, 3, 4, 6) indicating that the lack of WS-RLP DNA amplication was not due to the presence of inhibitors. In the presence of WS-RLP DNA, the 160bp product is amplified (lanes 2,5). Lane 2 is an example of a weak positive reaction in which both the WC9 and the WS-RLP were amplified. Lane 7 is a 100bp DNA ladder for determination of molecular weight. bp = base pairs.
Lane 1: Tangential flow filtration sample of abalone farm intake water. Lane 2: Tangential flow filtration sample of abalone farm outflow water. Lane 3: Tangential flow filtration sample of Carmel Pt. tide pool water. Lane 4: Postesophagus tissue of WS-RLP free black abalone (negative control). Lane 5: Postesophagus tissue of WS-RLP positive black abalone (positive control).
Lane 6: Empty tube subjected to complete processing (negative control)

PCR amplification of WS-RLP in whole body samples from small animals

This study was conducted as part of a larger study to examine the potential utility of PCR to detect the WS-RLP in small seed abalone. WS-RLP-free red abalone were raised at BML and ranged in size from 5.7-7.2mm and weight (with shell) from 0.032-0.073gm. Tissues from three animals were spiked with 10-15ul of WS-RLP infected abalone postesophagus homogenate while three others were untreated. Positive PCR reactions were obtained from the three spiked samples and the spike material alone, while all untreated samples were negative. This study indicated that our DNA purification protocol is sufficient to remove potential inhibitors which may be present in whole body samples.

PCR investigation of the susceptibility of small animals to the WS-RLP

Is there a size below which the gastrointestinal tissue is refractory to infection by the WS-RLP? RLP-free red abalone 2.6-6.0mm were held in six containers receiving 18C water. Seven larger RLP-positive donor red abalone were placed in each of three of the containers. After 14 days the small abalone in the tanks with donors were placed in new tanks without donors. After an additional 35-day incubation period, all animals were sacrificed and whole body tissues were processed for PCR. All 21 animals in the exposed tanks and nine of ten sampled donors were PCR-positive, while all 16 animals in the unexposed tanks were negative. Successful amplification of a portion of the actin gene in samples from two randomly chosen animals from each of the negative tanks demonstrated the presence of amplifiable DNA. Histological analysis of these samples would have proven difficult due to the very small amount of RLP-susceptible tissue present in animals of this size. A second experiment addressed the susceptibility of even smaller seed abalone to the WS-RLP. Red abalone six weeks post-settlement were exposed (or unexposed) in a similar manner for two weeks and then all were maintained unexposed for four additional weeks. Size of animals at sacrifice was 1.1-2.2mm. All exposed groups were strong positive while each of the two unexposed groups were negative. It therefore appears that abalone are susceptible to WS-RLP infection within several weeks of settling.

Verification of single-species status of the WS-RLP in different hosts and analysis of strain variation between isolates

The WS-RLP has been detected by histology in red, black, pink, flat, white and green abalone species and over a geographic range from Mexico to near the Oregon border. The WS-RLP from diverse species and geographic locations appear identical by histology, but could possibly represent different species. If there is only one species, it is also very possible that significant differences between isolates have developed or will in the future, and the characterization of such isolates may be important in management of this disease. Therefore, we have amplified, using generic eubacterial primers, the 16sRNA gene from RLP-infected postesophagus tissue from a farmed red abalone from Southern California and a pool of black abalone from central California, and from digestive gland tissue from two wild red abalone from Crescent City and Van Damme in northern California. The PCR fragments obtained were cloned using TOPO TA cloning and *E. coli* transformants were screened for presence of the inserted gene. Numerous positive clones were obtained for all but the Van Damme sample. We have obtained six or more successful transformants for the other samples, and one clone of each has been partially sequenced (approximately 1000 bases; gene length is approximately 1500 bases).

Useful data has been obtained from the postesophagus samples from the Southern California farmed red abalone and the pool of black abalone from central California. Comparison of these data with the sequence obtained from a central California black abalone in our original description of the species (Friedman et al 2000) showed a very small amount of variation, clearly demonstrating that the WS-RLP isolates from red and black abalone are identical species.

Amplification from necrotic tissue

All tissues, but especially the digestive gland, undergo autolysis within a few minutes after death. Tissue samples for histology must be taken within minutes of sacrifice of the animal, and freezing tissue renders it worthless for this diagnostic technique. The failure of histology as a diagnostic technique has prevented the examination of important archival material and samples obtained by recreational sport fishermen. We evaluated the performance of our PCR protocol on necrotic tissue samples. In a preliminary study, tissue taken from severely necrotic tissue from two black abalone, that died after showing advanced stages of withering syndrome, was run with our PCR protocol. Both samples had strong amplification. In a follow-up study, withered animals were shipped overnight from a farm in southern California and held overnight in flowing seawater. The next day 11 dead and dying animals were placed in plastic bags and held at room temperature. Two animals were sampled at day zero and three each at days 3, 5 and 7. Every sample was positive for WS-RLP. Thus, PCR now provides an extraordinarily useful tool for monitoring the geographic distribution of this pathogen using material submitted by cooperating recreational fishers and resource agency personnel.

Amplification from formalin-fixed, paraffin-embedded tissue

The ability to examine the DNA from archived tissue blocks can be a powerful epidemiological tool. Formalin fixation and paraffin embedding significantly degrades DNA, but successful PCR amplification can be achieved, particularly if the amplicon is less than approximately 600bp. The amplicon for our standard WS-RLP diagnostic protocol is 160bp. We attempted amplification using frozen and paraffin-embedded digestive gland and postesophagus tissue from three adult red abalone unexposed to the RLP and four cohorts which were experimentally exposed to the RLP and had severe infections. As expected, all PCR results on frozen tissues completely agreed with histological results. All negative samples by histology and frozen sample PCR were also negative by paraffin PCR. For 3 of 4 animals positive by histology and frozen sample PCR, paraffin-embedded samples of PE and DG were also positive by PCR. For one animal, paraffin PCR for both were negative while frozen tissues were positive. Paraffin PCR samples generally had weak, although clearly positive, bands. This tool allows us to examine archived material and to determine whether our protocol will amplify related RLPs in blocks of tissue from other marine molluscs obtained from colleagues worldwide.

Distinction between the WS-RLP and a potential co-occurring RLP

For several years we have noticed, in some samples, the presence of what may be a second type of RLP in the same tissues infected with the WS-RLP. These inclusions are usually present at low intensities and has not been linked to a disease state. The relationship between these inclusions and the WS-RLP appears complex, in that substantial presence of the inclusions has been observed only in animals with moderate to severe WS-RLP infections. We amplified and produced 20+ clones expressing the 16sRNA gene from samples from three abalone with high densities of inclusions that may represent a second RLP. We have partially sequenced one clone from each of the three samples and BLAST searches indicate affinities with non-Rickettsial groups.

In addition, we examined the pathogenicity of this bacterium for red abalone in a pilot study in which red abalone that were lightly infected with only this newly observed bacterium were held at ambient and 18C for over 1 year. Periodic samplings of abalone revealed that prevalences remained low and infection intensities never increased over time. In addition, no abalone died from these infections. Abalone infected with the WS-RLP held at 18C typically succomb to the infections within 4-6 months. These data suggest that this bacterium in not pathogenic under our experimental conditions.

Sensitivity of PCR vs. histology

Optimized PCR protocols often provide significantly elevated sensitivity for detection of microscopic pathogens in host tissue relative to methods based on microscopic examination. To compare tissue-based PCR to our standard technique, examination of hematoxylin- and eosin-stained 5um paraffin tissue sections (histology), we obtained approximately 100 red abalone from a farm in Cayucos CA. These were processed using our standard histological and PCR methods (PCR sample of 100mg sample consisting of approximately 80% digestive gland and 20% postesophagus by weight). Tools and surfaces were soaked with 10% bleach to prevent cross-contamination between animals. Each DNA purification run included one positive control and internal negative controls for every six true samples. The negative control samples were digestive gland tissue from abalone collected at Elephant Rock, Point Reyes National Seashore, a location known to be WS-RLP-free.

The results are shown below:

	Histology	
PCR	Neg	Pos
Neg	31	1
Pos	33	15

Of the 80 samples examined by both methods, 16 were positive by histology and 48 were positive by PCR. 32 were negative by PCR and only one of these was positive by histology. As routinely found in other host-pathogen systems, PCR appears to provide greatly elevated sensitivity compared to histology.

PCR detection of WS-RLP in abalone feces samples.

The WS-RLP infects gastrointestinal epithelium. With histology we can readily observe large numbers of the bacterium being shed into the lumen of the gastrointestinal tract from infected cells. The spread of this bacterium is very likely to be fecal-oral, i.e. it is shed from one animal in feces, which then settle on food of adjacent animals and are ingested. Feces from infected animals, therefore, provide a naturally concentrated sample of the WS-RLP.

In a preliminary study, approximately 15ml of dilute feces and other sediment was collected from a tank holding approximately 100, 80mm red abalone at 14°C, and another holding seven, 60mm red abalone at 18°C. Tubes containing the samples were held at 4°C for 1hr, during which time most of the particulate material settled. Subsamples of the solid material and supernatant from each tube were collected and PCR was performed using Lyse n go DNA extraction. For each tank, the supernatants were PCR positive and the solid materials PCR negative for the WS-RLP. This pattern

suggests that the solid matter included components that inhibited the PCR reaction and the Lyse n go DNA extraction method was no longer used for these studies. Nevertheless, this study demonstrated the presence of amplifiable WS-RLP DNA in abalone feces. Since switching to the use of the QIAmp Stool Kit for DNA isolation, our results for PCR analysis of PCR samples show excellent correlation with those obtained using PCR on postesophagus tissue from the animals which produced the feces. During the course of this grant we have come to rely upon feces sampling as a key component of diverse WS research and management in our lab. These include monitoring the WS-RLP status of a marine laboratory after elimination of WS-RLP positive animals, verification of WS-RLP-free status of abalone held outside our quarantine facility, and monitoring of WS-RLP status of cultured individuals of the federally ESA listed white abalone held at the Channel Islands Marine Research Institute.

As an example, we processed the feces of abalone in various stages of growout at an abalone farm, with and without treatment with oxytetracycline, which as the other section of this report demonstrates, strongly reduces RLP presence. Feces from tanks holding older animals that had not been treated showed the strongest signal.

TANK	DATE STOCKED	TREATED WITH OTC?	Feces RLP PCR Result
D9-5	August-01	No	+
D11-5	August-01	No	-
C8-5	February-01	No	++
C13-2	February-01	No	+
E3-2	August-00	No	+
C15-5	August-00	Yes-high dose	-
C7-2	February-00	No	+++
C8-2	February-00	Yes-high dose	+
E25-5	August-99	No	+++
E26-5	August-99	Yes-high dose	-
E21-2	March-99	No	++
E19-5	March-99	Yes-low dose	+
Positive control			+++
Empty tube (neg. control)			-

PCR detection of WS-RLP in water samples

The WS-RLP must be present in sufficient numbers in a sample to be detected by PCR. Every PCR protocol has a lower limit of sensitivity, typically requiring from several dozen to several hundred copies of the targeted gene sequence. We are attempting to amplify a portion of the 16sRNA gene of a Rickettsiales bacterium; there are typically one to several identical copies of this gene present in members of this taxon. The minimum number of bacteria that must be present in a sample to detect it with our PCR protocol is likely in the tens to hundreds. The WS-RLP infects gastrointestinal epithelia and is shed from the abalone with feces. Feces are negatively buoyant and settle in the vicinity of the abalone; their dispersion is dependent on water flow.

For the above reasons, detection of the WS-RLP in water samples not immediately adjacent to infected abalone is an ambitious goal. The following set of experiments was conducted to develop the most sensitive water sampling method possible.

Concentration from water by centrifugation

We initially attempted to process water samples by centrifugation. A tank was set up containing approximately 20 suspected RLP-positive red abalone 25-35mm in shell length in 18°C water. The tank was cleaned and 48 hours later a 100ml water sample was taken by pipette, drawing it near each abalone. The water was turned off and a similar sample was taken five hours later. The samples were centrifuged at 10,000g for 20min

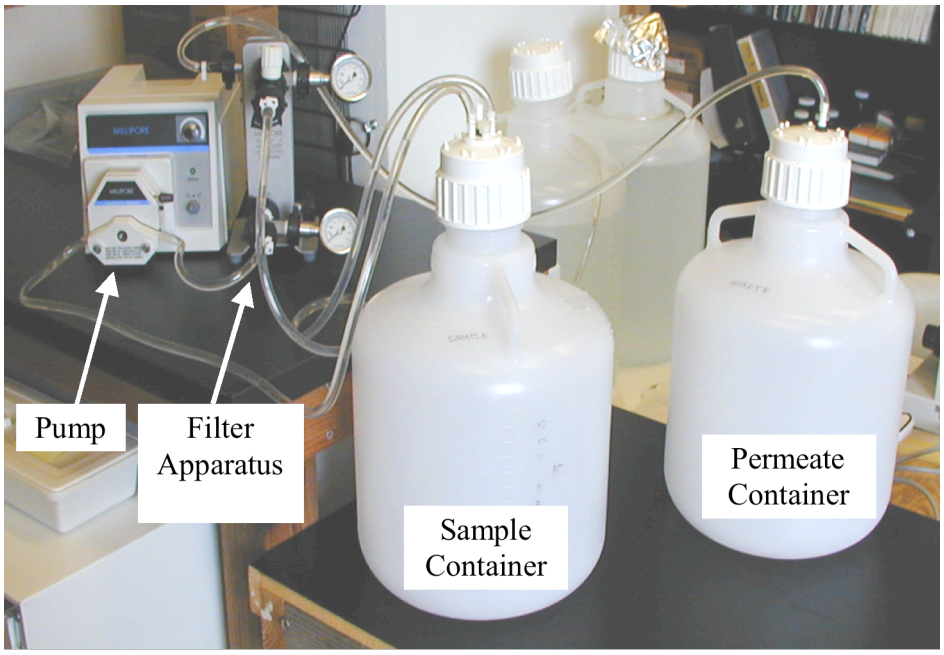
(which will pellet bacteria-sized particles) and the pellets frozen. A 100ml sample of seawater from Horseshoe Cove, Bodega Marine Laboratory was processed in an identical manner for a negative control. PCR was performed on all samples after Lyse 'n go DNA extraction and all samples were negative. The RLP burden in these small animals was likely low, but nevertheless these results suggested that more sophisticated techniques would be required for detection in water samples.

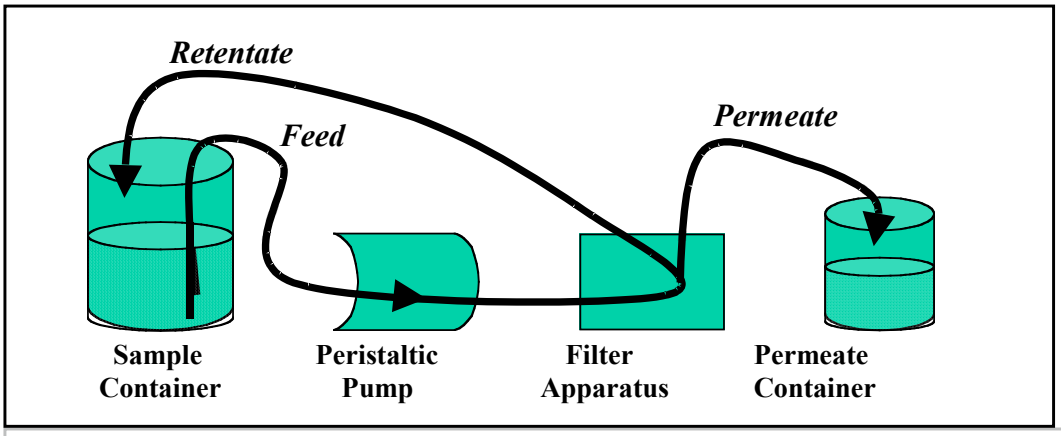
Concentration from water by classic filtration

Our initial approach was to capture bacteria-sized particles onto a paper filter and extract DNA from the filter. Water was shut off for 2h to four, 50-liter tanks containing 0, 3, 4 and 8 WS-RLP-positive adult red abalone respectively, while aeration was maintained. From each tank a 400ml water sample was collected and filtered through a 0.1µm filter, which is small enough to retain WS-RLPs. The filters were rinsed and scraped to harvest retained material and the harvested suspensions were centrifuged sufficiently to pellet bacteria (3200g, 30min, 4°C). PCR was performed with DNA extracted from the resulting pellet. The sample from the tank holding eight adult RLP-positive red abalone was PCR-positive and the others were negative. This indicated that detection in water was possible but suggested that sensitivity was not high. A sample of only 400ml proved difficult to process yet seemed far too small to provide sufficient material for detection in an open ocean sample.

Tangential flow filtration of spiked water sample

At this time we began searching for methods to process larger volumes of water and identified tangential flow filtration. This method, widely used in the pharmaceutical industry to concentrate proteins in solution, drives water out of a sample while particles remain in suspension. It has been used to concentrate marine bacterioplankton from open ocean water (Giovannoni et al. 1990). We purchased a tangential flow filtration system (Millipore) and developed a protocol with extensive assistance from Millipore Technical Services. A schematic of the system and a photograph is presented in the figures below.





Tangential flow filtration. Drives off water and particles less than 0.1μm (= permeate) while retaining larger particles, which become concentrated in solution but do not adhere to membrane.
Filter: 0.1μm Durapore TFF filter, loose screen, 0.1m^2, $500 each.
Sample: 14 liters seawater filtered through 1000, 100, 23μm sieves.
Procedure:
1. Flush filter (25min, requires 20 liters Millipore water).
2a. Run sample (20min). Final volume 50-100ml (continued as 2b-2e below).
3. Flush filter (25min, requires 20 liters Millipore water).
4. Clean filter (45min, 600ppm bleach, 50°C).
5. Flush filter (25min, requires 20 liters Millipore water).
6. Measure normalized water permeability (5min).
7. Perform module integrity test
8. Apply storage solution (30min, 0.1N phosphoric acid, 37°C).
9. Store at 4°C.
2b. Centrifuge 50-100ml sample 3200g, 30min.
2c. Re-suspend pellet to 1ml.
2d. Centrifuge 10,000g, 10min.
2e. Purify DNA with QIAmp DNA stool kit, perform PCR.

An outline of the filtration process and filter handling is provided in Appendix 1. Processing one sample requires approximately 60 liters of molecular biology grade distilled water and four hours of labor. The result is a highly concentrated sample of bacteria-sized particles, typically 0.1ml solid material from 14 liters of seawater. Using the Qiagen QIAmp Stool Kit, the DNA is extracted from this material and used for PCR detection of the WS-RLP. The 0.1ml pellet provides much more than enough DNA needed for PCR. Extracting the DNA, running the PCR reaction and determining the results by gel electrophoresis takes several more hours.

To establish the ability of the system to concentrate the WS-RLP, we first processed a 15-liter sample of BML seawater as a negative control. Then a withered 62mm red abalone was dissected and the digestive gland and postesophagus (target tissues for the WS-RLP) were homogenized in 7ml of BML seawater. A 300μl sample of the homogenate was processed directly for PCR to verify presence of the WS-RLP. The remainder was mixed well with 13 liters of BML seawater and processed via tangential flow filtration. The sample consisting only of particles concentrated from raw seawater was negative while the homogenate and homogenate-spiked samples were positive for the WS-RLP by PCR. This demonstrated that the principle of the method was sound.

Tangential flow filtration of water from a tank holding RLP-positive abalone

We next attempted detection in water in which infected abalone were being held. This differed from the previous experiment in that the source of the WS-RLP would be feces rather than homogenated gut tissue, and the WS-RLP would be present at a lower, more realistic, although undefined concentration. A 10-liter capacity tub containing approximately 20 50-70mm, RLP-infected farmed red abalone at 18°C was rinsed and the kelp removed. After 4.5 hours the water was shut off for 15min and the entire tank contents were processed by tangential flow filtration and used for WS-RLP PCR. The sample was positive. Flow was resumed and on a subsequent day a sample consisting of 20 liters of effluent from this tank was collected from outflow without disturbing the animals. This sample was also positive, indicating the ability to detect the WS-RLP in water exposed to undisturbed, infected abalone.

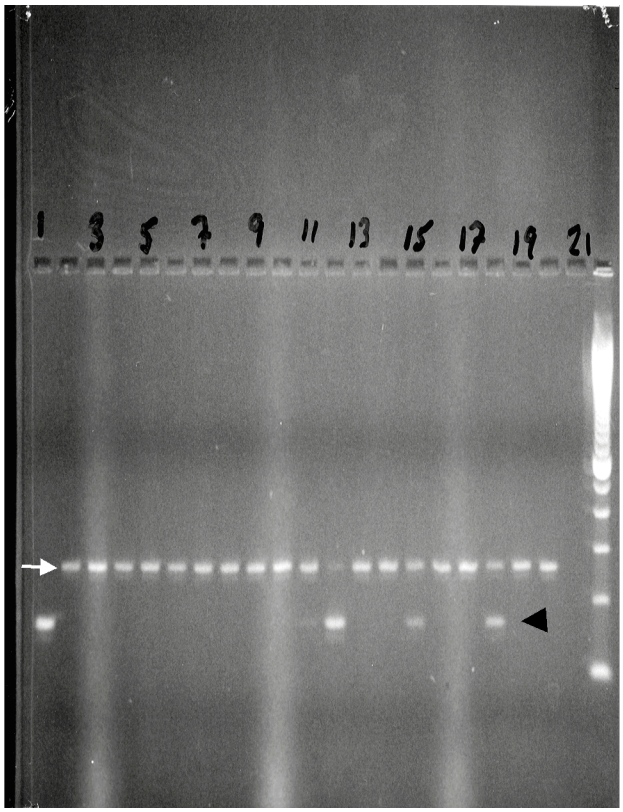
Tangential flow filtration of influent & effluent from abalone farms

We next attempted detection in the outflow of a large abalone farm in Santa Barbara. Two, 6.5 liter samples were taken from each of two main outfalls from the farm and combined. The sample was held overnight during transit and processed the following day. The sample was PCR positive for the WS-RLP.

Next we collected 14 liters of both influent and effluent of a large abalone farm in central California. The samples were held at room temperature for approximately 44 hours during transit. The intake water sample was PCR negative and the outflow water sample was PCR positive for the WS-RLP. The intake water is pumped from the ocean several hundred yards from the outfall, to the north. It would be impossible to predict the current flow here and the amount of exposure to outflow water by intake water is unknown. Nevertheless, the failure to detect the WS-RLP so close to a large input suggested that sensitivity may not be sufficient to detect the WS-RLP in open seawater samples, even if near a high density of infected animals in relatively warm water.

Tangential flow filtration of tide pool water from a location with RLP-positive animals

Carmel Point, near the town of Carmel, is a location that harbors at least several hundred black abalone in a small section of the intertidal zone. This population has an WS-RLP prevalence of approximately 5-10% (unpublished observations). We sampled 15 liters of water from crevices and pools at low tide from an area where black abalone were observed in the direct vicinity. The sample was processed for tangential flow filtration and PCR for detection of WS-RLP DNA. The sample was negative, further suggesting that this sampling method does not provide the sensitivity necessary to monitor WS-RLP presence in north coast abalone by processing seawater.



PCR products separated and visualized on an agarose gel. Note that the positive control (white arrow) is larger than the WS-RLP amlipcon (black arrowhead).

Legend

1	**Positive Control PE homogenate**
2-10	**Nursery abalone, seawater, or feces, all negative**
11-15	**Grow-out abalone**
16-17	**Juvenile system, feces and seawater**
18	**Grow out abalone feces**
19	**Blank**
20	**White abalone negative control dg**
21	**Negative control, water**

Combined study of histology, tangential flow filtration and feces sampling in various stages of production at an abalone farm

In a final study including water sampling, we attempted to amplify WS-RLP DNA from feces, water and animals taken in various stages of production at a central California abalone farm. Samples were collected from the nursery, juvenile rearing and growout production units. Nursery tanks hold animals approximately 3-7 months old. The animals come from the hatchery that receives ultraviolet light-treated seawater, and this is their first exposure to potentially WS-RLP positive seawater. The animals sampled were approximately 3mm in shell length, the total body weight (without shells) for all ten together was 0.07gm and they were combined into one pooled sample. A basket system is used for growing juveniles. The animals sampled were approximately 40-50mm in shell length and appeared healthy. The final stage of production is growout troughs. The animals sampled were 70-80mm in shell length and some were slightly shrunken. Juvenile and growout animals were pooled into groups of two animals per sample. The WS-RLP PCR results are shown below; - = negative, +, ++, and +++ = weak, moderate and strong signals respectively.

Sample Source	Sample Type	WS-RLP PCR
Black ab tissue (positive control)		+++
Nursery tanks	Water (15 L)	-
Nursery tanks	Feces	-
Nursery tanks	Animals (n = 10)	-
Juvenile baskets	Water (15 L)	-

Juvenile baskets	Feces	-
Juvenile baskets	Animals 1 & 2	-
Juvenile baskets	Animals 3 & 4	-
Juvenile baskets	Animals 5 & 6	-
Juvenile baskets	Animals 7 & 8	-
Juvenile baskets	Animals 9 and 10	-
Growout trough	Water (15 L)	not done
Growout trough	Feces	++
Growout trough	Animals 1 & 2	+
Growout trough	Animals 3 & 4	+++
Growout trough	Animals 5 & 6	-
Growout trough	Animals 7 & 8	+
Growout trough	Animals 9 and 10	++
Abalone tissue sample (negative control)		-
PCR grade water (negative control)		-

To summarize this experiment, inflow, nursery and juvenile samples were all negative for water, feces and tissue samples. Feces were positive in the growout tank as were three of the five pools of two animals each. There is no data for the growout tank water due mishandling of the sample at a critical stage. The lack of amplification of WS-RLP DNA in the two of the pools of growout animals is in agreement with our earlier studies indicating that some of these animals are truly uninfected. The failure to detect the WS-RLP in the nursery or juvenile tank water, which is unprocessed intake water, provides further suggestion that use of seawater samples for detection of the WS-RLP is reliable only for water samples that are closely associated with infected animals. We concluded that a more direct method is required, particularly for detection in north coast red abalone, in which low-level infections would predominate due to the cold temperatures. We therefore initiated the studies described below.

This table summarizes the results of experiments using tangential flow filtration to concentrate water samples:

	TFF PCR result
Clean seawater	-
Spiked seawater	++
Tank holding positive abalone: static sample	++
Tank holding positive abalone: outflow	++
Intake water, farm #1	-
Outflow water, farm #1	++
Outflow water, farm #2	++
Tidepool in area with positive abalone	-

SUMMARY

Our previous studies demonstrated that a Rickettsiales-like prokaryote (WS-RLP) is responsible for withering syndrome and that expression of clinical signs is often associated with elevated temperature. The bacterium (WS-RLP) replicates within vacuoles in host epithelial cells, forming large inclusions that burst into the lumen of the gastrointestinal tract. The inclusions are readily observed in formalin-fixed, hematoxylin and eosin-stained tissue sections, yet this diagnostic method is relatively expensive and time-consuming. We expanded the utility and applications of a recently developed polymerase chain reaction (PCR)-based detection method for the WS-RLP. PCR offers the potential to be vastly more sensitive than histology and can be used on frozen samples, which are useless for histology. With PCR methods we have detected the WS-RLP in red, white, flat, pink, green and black abalone postesophagus and digestive gland tissues and in whole body samples of red abalone as small as 2.0mm. We have amplified WS-RLP DNA from necrotic tissue held at room temperature for up to 7 days, and from samples fixed and embedded in paraffin. A non-destructive WS-RLP sampling method is desired since both wild and farmed abalone are highly valued. We developed PCR-based non-lethal sampling methods for the WS-RLP, including detection in feces and water samples. We developed a method involving tangential flow filtration that concentrates particles > 0.1μm from large volumes of seawater, allowing purification of DNA for PCR from 15+ liter samples. Although sample processing is laborious, using this method we have detected pathogen DNA in effluent from abalone holding units in both experimental and culture facilities. Field application of this sampling method indicated that, despite its high sensitivity, it would unlikely be able to reliably detect the WS-RLP in water samples taken adjacent to infected north coast red abalone. The feasibility of using feces as samples for PCR detection of the WS-RLP was demonstrated. Transient holding of abalone followed by PCR analysis of DNA isolated from pooled feces and/or TFF-concentrated holding water is recommended as the most sensitive and reliable sampling technique for non-lethal detect the WS-RLP in north coast abalone populations. PCR using fecal material appears to be useful for efficiently detecting the WS-RLP in various stages of farm production.

Appendix 1: Presentations of work supported by this grant.
Appendix 2: Tangential Flow Filtration procedure outline.

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APPENDIX 2: TANGENTIAL FLOW FILTRATION PROCEDURE OUTLINE

General notes:

See Millipore's publication, "Maintenance procedures for Pellicon and Pellicon-2 cassette filters" (Order # P17512) and the guides that come with filters, pump, pumphead, filter holder.

Prevent tubes from kinking at all times.

Filter: Durapore PVDF VVPP, screen type V (loose), 0.1μm (pH 2-11, temperature range 4-50°C.

Procedure:

Collect sample (typically 15 liters), pour through 1000μm, 100μm and 23μm mesh filters to remove large material. Note: all sample containers and mesh screens are treated with 10% bleach to decontaminate before each use.

Flush filter (see below)

Run sample.

Stop filtration when volume is reduced to less than 200ml.

Centrifuge sample in 50ml tubes (3200g, 30min, 4C).

Pool material, extract DNA with Qiagen Stool Kit, proceed with PCR.

Flush filter (see below).

Clean filter (see below).

Flush filter (see below).

Measure Normalized Water Permeability (see below)

Do module integrity test (see below).

Run storage solution (see below).

Store at 4°C in storage solution.

The details of each step are outlined below.

Flush filter:

Fill 20 liter carboy with Millipore water.

Attach to Feed tube.

Open retentate valve completely.

Flush retentate side of filter by adjusting pump speed to setting ~7-9 so that feed pressure is 1-2 psi. Filter 1.2 liters of retentate.

Flush permeate side of filter by adjusting pump speed to setting 10 and retentate valve so that retentate pressure is ~18psi. Filter 7 liters of permeate.

Run sample:

Direct feed and retentate tubes into sample container and permeate tube to waste. Adjust pump to setting 10 and close retentate valve so that feed pressure = 18psi. Run until volume is less than 100ml, then keep running several seconds until all is driven out of retentate line. Toward the end of the run, decrease pump speed to prevent splashing and spaying.

Clean filter:

This cleaning process is designed to remove particles and destroy DNA.
Mix 50ml bleach with 3.5 liters of water. Add 1.5 liters of boiling water. Solution should be 600ppm bleach, 50°C.
Direct permeate, feed and retentate tubes into bleach solution.
Filter with pump at setting 8.6 and retentate valve adjusted so that retentate pressure is 5psi. Run for 45min.

Storage:
Make 0.1N phosphoric acid solution
Warm to 37°C (cap loose).
Direct permeate, feed and retentate tubes into acid solution.
Adjust pump to setting 10 and retentate valve so that feed inlet pressure is 6-9 (adjust pump so that feed inlet is 6-9psi and differential with respect to retentate pressure is 1-4psi). Run for 30min. This step also acts as a cleaning process to remove/destroy nucleic acids.

Measure Normalized Water Permeability (NWP):
NWP value provides a measure of filter aging and some types of damage. After first use, NWP value should not vary more than about 10% between runs.
After cleaning and flushing, direct permeate, feed and retentate tubes to dH2O. Adjust pump to ~7.2 and retentate valve so that feed and retentate pressures are exactly 10 and 9 respectively. Run for 5min. Measure rate of permeate flow, e.g. by directly flow into 250ml cylinder for exactly 20sec.
NWP = (Permeate flow rate in liters/hour x temp correction factor)/0.95
The temperature correction factor is found in a chart in the "Maintenance procedures" guidebook.
Compare NWP to previous runs; should be within 10%. Higher values indicate improper cleaning.

Module integrity test:
This test verifies that the filter is not leaking.
Run dH2O with retentate pressure at 10psi and any pump setting. Simultaneously shut off pump and squeeze closed all tubes. Pressure should remain constant at 10psi (or similar) for numerous seconds.