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Control of horizontal transmission of white spot syndrome virus in *L. vanammei* by ultraviolet light

Control de la transmisión horizontal del virus del síndrome de la mancha blanca en L. vanammei mediante luz ultravioleta

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Resumen
Bacterial and viral diseases are common problems in aquaculture. The use of flow-through systems frequently represents a risk of contamination by introducing pathogenic microorganisms, resulting in diseases that economically damage the industry. White spot syndrome virus (WSSV) causes a shrimp disease responsible for substantial economic losses in many countries. The present study was conducted to determine if the specific pathogen-free (SPF) indicator shrimp might take WSSV from infected water flow after treatment with UV light: specific pathogen-free L. vannamei shrimp, 2.5-g avg Wt, were acclimated to 25 UPS artificial seawater. Once acclimated, they were distributed in four pairs of tanks (20/tank). Each pair of tanks had one tank infected by per os and one exposed to water from the infected tank. Water flow from the per os infected tank was pumped to the non-infected tank and returned to the per os infected tank by gravity. Two pairs of tanks had UV light (G15T8) to irradiate the flow of water before passing it to the non-infected tank. The other two pairs of tanks (control group) had no UV light irradiation to the recirculating water. Moribund and dead shrimp were collected and frozen to determine if they were infected with WSSV by using PCR. After 12 days of shrimp in, the tanks exposed to water contaminated with WSSV and treated with UV did not have any mortality. The water contaminated with WSSV and not treated with UV resulted in significant numbers of WSSV-infected and dead shrimp. This study demonstrated that it is possible to eliminate the transmission of WSSV by treating the water with UV.

Palabras claves: WSSV, biosecurity, aquaculture, pathogens, Pacific shrim.

Abstract
Las enfermedades bacterianas y virales son problemas comunes en la acuicultura. El uso de sistemas de flujo continuo suele suponer un riesgo de contaminación por la introducción de microorganismos patógenos, lo que da lugar a enfermedades que causan daños económicos al sector. El virus del síndrome de la mancha blanca (WSSV) provoca una enfermedad en los camarones responsable de pérdidas económicas considerables en muchos países. El presente estudio se llevó a cabo para determinar si el camarón indicador libre de patógenos específicos (SPF) podía contraer el WSSV del flujo de agua infectada tras el tratamiento con luz ultravioleta: camarones *L. vannamei* libres de patógenos específicos, con un peso medio de 2.5 g, se aclimataron a agua de mar artificial 25 UPS. Una vez aclimatados, se distribuyeron en cuatro pares de tanques (20 por tanque). Cada par de tanques tenía un tanque infectado por *per os* y otro expuesto al agua del tanque infectado. El flujo de agua del tanque infectado por *per os* se bombeaba al tanque no infectado y regresaba al tanque infectado por gravedad. Dos pares de tanques tenían luz ultravioleta (G15T8) para irradiar el flujo de agua antes de pasar al tanque no infectado. Los otros dos pares de tanques (grupo de control) no tenían irradiación de luz ultravioleta en el agua recirculada. Los camarones moribundos y muertos se recogieron y se congelaron para determinar si estaban infectados con WSSV mediante PCR. Después de 12 días con los camarones, los tanques expuestos al agua contaminada con WSSV y tratados con UV no presentaron ninguna mortalidad. El agua contaminada con WSSV y no tratada con UV dio lugar a un número significativo de camarones infectados con WSSV y muertos. Este estudio demostró que es posible eliminar la transmisión del WSSV tratando el agua con UV.

Keywords: WSSV, bioseguridad, acuicultura, patógenos, camarón del Pacífico

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Introduction

The white spot syndrome virus (WSSV) is a lethal and highly contagious virus that affects penaeid shrimp aquaculture worldwide (Walker and Mohan, 2009). This enveloped virus belongs to the Nimaviridae family; it has a circular, supercoiled double-stranded DNA that comprises 305 kb and is the largest penaeid shrimp virus in size (130 x 350 nm) (OIE 2007; Lightner *et al.,* 2012). WSSV causes up to 100% cumulative mortalities within 3-10 days under farming conditions. It has been estimated that from 1992 to 2005, economic losses of US$8 billion to several fresh and marine water species in Asia and America could be attributed to WSSV (Lightner, 1996; Walker and Mohan, 2009).

WSSV is a pathogen that is directly transmitted by cohabitation with living infected shrimp and from ingestion of an infected cadaver. It has been suggested by Soto and Lotz (2001) that WSSV is transmitted more effectively through ingestion of infected cadavers (*per os*) than through contact with infected hosts that are actively shedding viruses. Other authors confirmed that WSSV could also be effectively transmitted via contaminated water and infect the shrimp and many other crustaceans (Chou *et al.*, 1995; Kanchanaphum *et al.*, 1998; Supamattaya *et al.*, 1998).

Differences in susceptibility between species have been observed (Aguirre-Guzmán *et al.*, 2001; Tuyen *et al.,* 2014). Other authors demonstrated differences in susceptibility, depending on the size and physiological state of the organism (Pérez et al., 2005; Walker and Mohan, 2009).

Some external factors can influence the susceptibility of shrimp to diseases; management practices like disinfection, the use of therapeutics, feed additives, algaecides, pesticides, and fertilizers can be potential stressors and cause problems during shrimp production (Bainy 2000). However, physicochemical factors produced by the environment are more commonly involved in WSSV outbreaks (Nga *et al.*, 2005; Walker and Mohan, 2009). Water temperature is considered to be the most important of these factors since it has been demonstrated to have direct effects on all biological processes (Spanopoulos-Hernández et al., 2005; Moser *et al.,* 2012). By increasing the water temperature (hyperthermia), it is possible to reduce the expression of WSSV and result in a decrease of viral replication (Jiravanichpaisal *et al.*, 2004; Jiravanichpaisal *et al.*, 2006; Rahman *et al.*, 2006; Reyes *et al.*, 2007; Esparza-Leal *et al.,* 2010). Previous studies demonstrated that increasing the temperature from 26 to 33 ºC completely inhibited mortality of shrimp infected with WSSV (Vidal *et al.*, 2001) and that the WSSV replication was affected (Jiravanichpaisal *et al.*, 2006). Subsequent studies found that increasing temperature improves the immune response by producing an increased number of apoptotic cells and has a direct effect on WSSV by reducing the viral load (Granja *et al.*, 2003; Granja *et al.*, 2006). Reyes *et al.* (2007) demonstrated that the inhibited mortality effects of hyperthermia are due to decreased viral replication, rather than improving the immune response. Additional studies to elucidate the mechanisms of how temperature interferes with WSSV infection are needed to understand this host-virus relationship better.

Lightner *et al.* (1998) wrote that disease is the result of a complex interaction between the shrimp, its environment, and the pathogen itself (Lightner *et al.*, 1998). Salinity is a fundamental environmental factor in marine shrimp culture and is frequently found to be related to WSSV outbreaks (Tuyen *et al.,* 2014). Abrupt fluctuations in salinity are often provoked by rain and seem correlated with the increase of WSSV load within the shrimp populations (Peinado-Guevara and López-Meyer, 2006). Moreover, Bray *et al.* (1994) reported a relationship between salinity and infectious hypothermal and hematopoietic necrosis virus (IHHNV) in *L. vanammei*, in that changes in salinity can trigger disease outbreaks, as also described by Peinado-Guevara (2006) and Spanopoulos-Hernández et al. (2005). Bacterial infections are also correlated with mortality of shrimp because of WSSV infection (Mohan *et al.*, 2002; Selvin *et al.*, 2004), as primary infections or opportunistic infections in diseased shrimp (Lightner, 1996). The water quality affects the virulence of WSSV as described by Jiang et al. (2004), reporting that ammonia-N decreases the virulence of WSSV by reducing the immunocompetence in *Farfantepenaeus japonicus* without affecting the shrimp's appetite, contrasting the patterns

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of the adverse effects of most of the other environmental parameters when they fluctuate in abnormal ways. The influence of environmental parameters, typically associated with disease outbreaks, needs to be investigated to understand their impact on the host's health and defense mechanisms, as well as on the virulence of WSSV.

The success of WSSV as a pathogen can be attributed to several factors: transmission mechanisms, including infected or mechanical carriers (Peng et al., 1998; Walker and Mohan, 2009), and the different routes to infect the host that WSSV uses (Supamattaya et al., 1998; Tuyen *et al.,* 2014). Experimental studies have demonstrated that WSSV can easily develop in shrimp culture conditions and successfully infect shrimp for long periods. However, the optimal conditions of the experimental challenges can be distant from what can really happen under farming conditions due to the complex biodiversity of organisms and organic matter that pond water has, and consequently, the influence on the mechanisms of infection. Moreover, the concentration of viral particles in a volume of water is usually greater; this raises more questions regarding how much the infection is altered by all these factors (Esparza-Leal *et al.,* 2014).

As mentioned before, there are two ways that WSSV can infect shrimp: by oral infection and waterborne routes. In experimental infections, both methods are employed, either by feeding on minced infected muscle tissue (sometimes introduced into the oral cavity using a catheter) or by inoculating the water with viral preparation (Esparza-Leal *et al.,* 2014). All the experimental shrimp must be exposed equally, at the same time, and with a uniform dosage (Gitterle *et al.*, 2006). In the present work, oral and waterborne exposure were used in a raceway-type system.

Ultraviolet irradiation is a standard disinfection method used to prevent diseases in aquaculture production systems (Bazyar-Lakeh *et al.,* 2013). There are numerous studies on pathogen inactivation by UV irradiation. Most of these studies are focused on wastewater treatment, drinking water, and human health concerns (Silva *et al.,* 2013; Calgua *et al.,* 2014; Poepping *et al.,* 2014). One limitation of the application of UV to aquaculture disinfection is that UV irradiation cannot be applied to the cultured organisms directly. However, there are several methods widely used to inactivate fish pathogens from water and equipment successfully (Liltved *et al.*, 1995; Liltved and Landfald, 2000; Liltved *et al.*, 2006). Recent studies have demonstrated that WSSV is sensitive to UV light irradiation, making the UV disinfection method a feasible alternative to physical and chemical treatments that sometimes are time-consuming, ineffective, and develop by-products in water that result in toxic compounds (Chang *et al.*, 1998; Balasubramanian *et al.*, 2006; Oseko *et al.*, 2006).

Although WSSV can be inactivated in water by UV irradiation, some studies have shown tolerance when compared with other shrimp diseases (Momoyama, 1989; LeBlanc and Overstreet, 1991). The sensitivity of WSSV to UV light irradiation is dependent on the UV irradiation time and the dosage (μW s cm^2) (Balasubramanian *et al.*, 2006; Oseko *et al.*, 2006). Most of the experimental work with UV light and shrimp diseases has been conducted in vitro (petri dish) with direct UV irradiation using the collimated beam apparatus in a dynamic running water system type raceway. The objective of the present work was to determine if the Specific Pathogen Free indicator (SPF) shrimp may become infected with WSSV from infected water flow after treatment with UV light.

Material and methods

Description of the system

The research design for this experiment is a joint effort of the University of Tamaulipas and the University of Arizona. The study was conducted at the Environmental Research Laboratory of the University of Arizona, Tucson, AZ. The goal of this particular trial is to determine the efficacy of UV radiation to reduce the incidence of WSSV transmission in a recirculating aquaculture system. An Integrated Water Filter System (IWFS) was used in this experiment. This system consists of a UV light unit and a biofilter in a PVC pipe frame. For this experiment, only the UV light was used. The substrate and the activated carbon shown in figures

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1 to 4 were omitted for this test. Figures 5 and 6 show the distribution of the experimental tanks, including the IWFS, heaters, pumps, and biofilters to keep the water quality within predetermined conditions. The test shrimp were distributed in the tanks (20 shrimp in each tank). Each IWFS (4 in total) was set to treat waterborne and *per os* infected shrimp tanks, (three tanks in total in a line as a raceway system) a pump, heater and biofilter (Fig. 5). The water flow was pumped from the lower tanks (Row 3) to the IWFS unit situated above the upper tanks (Row 1) for each treatment system. Row 3 was exclusively for the pumps, heaters, and biofilters and contained no shrimp (Fig. 6). The system was designed to pump water to the IWFS unit, which would then flow by gravity to Row 1 and then Row 2 and back to Row 3 to recirculate.

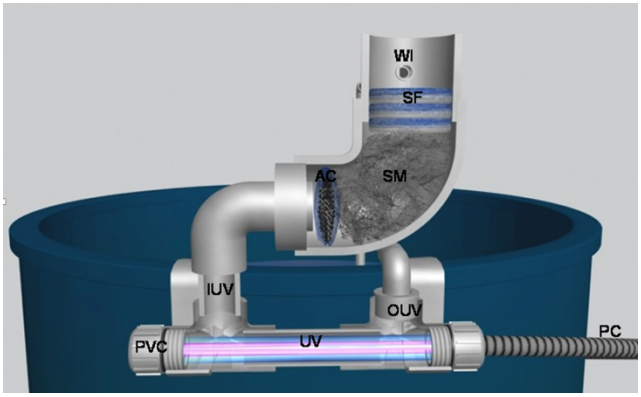

Figure 1. Frontal view of the ultraviolet light system with biofilter for aquaculture tanks. Unit water inlet (WI), Synthetic fiber substrate (SF), Synthetic Mesh substrate (SM), Activated carbon (AC), Water inlet to the ultraviolet bulb (IUV), Ultraviolet bulb enclosed by a PVC pipe (UV), Water outlet from the ultraviolet bulb (OUV), Power cord protected by aluminum conduit (PC).

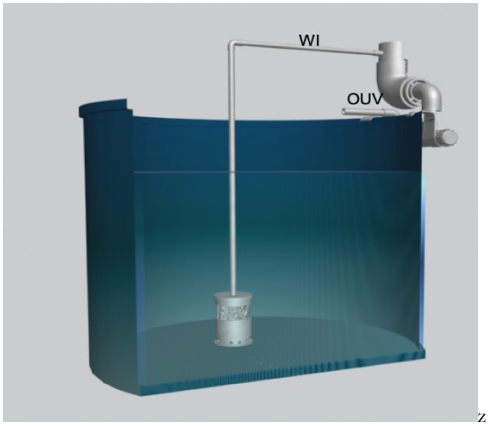

Figure 2. Horizontal view of the ultraviolet light system with biofilter for aquaculture. In this view, the unit water inlet (WI) and the water outlet from the ultraviolet bulb (OUV) are observed. The water after exposure to the ultraviolet irradiation is returned to the tank.

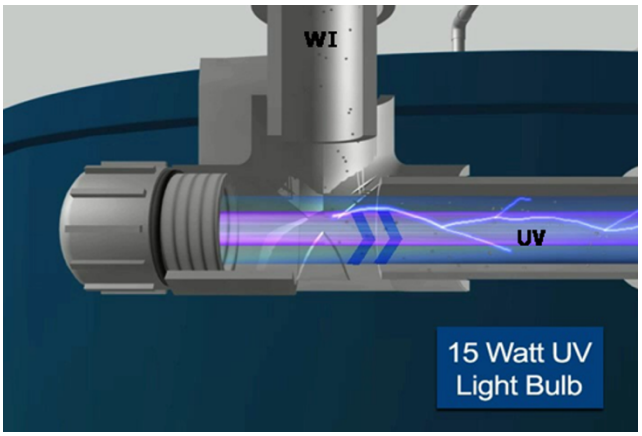

Figure 3. Frontal view of the 15-watt ultraviolet light bulb. Double arrowheads indicate the route of the water exposed to ultraviolet irradiation from the unit water inlet (WI) along the ultraviolet bulb (UV).

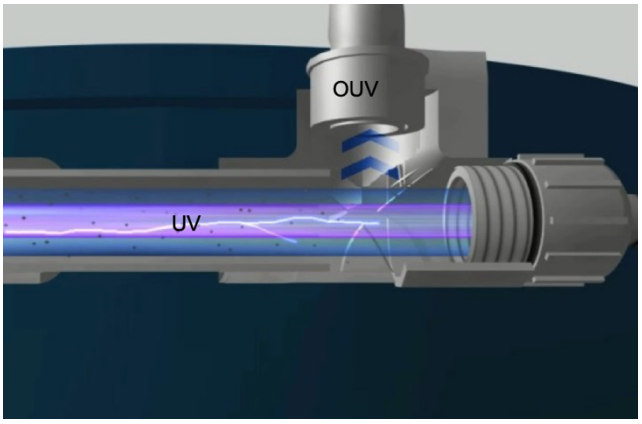

Figure 4. Frontal view of the 15-watt ultraviolet light bulb. Double arrowheads indicate the route of the water after exposure to the ultraviolet irradiation. The water, after exposure to ultraviolet irradiation, is returned to the tank through the OUV.

As shown in Figure 5, systems A and B were irradiated with UV light, and systems C and D were not. Shrimp in Row 2 were infected with WSSV by oral route (*per os*), feeding minced infected tissue at a rate of 10% of the biomass (White *et al.*, 2002). Moribund and dead shrimp were collected and frozen for analysis to determine if they were infected with WSSV.

Test shrimp

A total of 160 SPF juvenile shrimp *L. vannamei* (2.5 g mean body weight) were used in this trial. Commercial hatcheries provided shrimp, and they were randomly distributed among the tanks. Shrimp were acclimated to

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a salinity of 25 ppt and 27 ºC and fed twice a day with one pellet/shrimp. Each tank had individual nets and hoses for handling the moribund and dead shrimp. Each tank was stocked with 20 shrimp. Dead and moribund animals were collected and analyzed for WSSV by PCR and fixed for histopathology (Chou *et al.*, 1998).

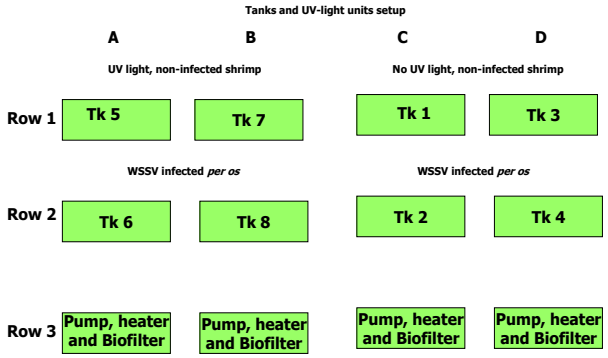

Figure 5. Water is pumped from the lower tanks (Row 3) to the upper tanks (Row 1). The water flow in Row 1 is irradiated with UV light (columns A and B) and non-irradiated with UV light (columns C and D). Water from Row 1 (tanks 5, 7, 1, and 3) overflows to tanks in Row 2 (tanks 6, 8, 2, and 4) and then to Row 3. Row 3 was not stocked with shrimp. The shrimp in Row 2 were infected with WSSV *via oral administration*. Tanks in Rows 1 and 2 were stocked with 20 shrimp each.

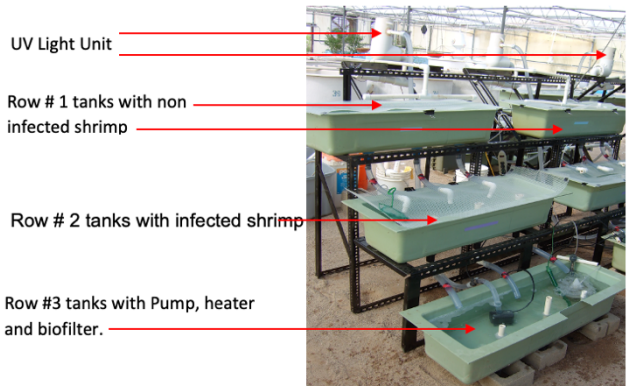

Figure 6. The water is pumped from the lower tanks (Row 3) to the UV light unit after treatment. Water overflows from the first tanks (Row 1), where the non-infected shrimp are located, and flows to Row 2, which contains the WSSV-infected shrimp.

Preparation of the tanks and UV light system

Each tank contained 60 L of 25 UPS artificial seawater. The water from the infected group of shrimp tanks (Row 2) was pumped and treated with UV light in the non-infected tanks (Row 1). A fine mesh (60 µm) screen was used to ensure that only water was pumped to the non-infected tanks and infected tissue was excluded. For the irradiated tanks, two UV lamps (two units) were set in the recirculation line. Each lamp, 15 W, 254 nm wavelength, was previously measured with a research radiometer (International Light IL 1700, Newburyport, MA 01950) to ensure that all the lamps were in the same radiation condition. To maintain good water quality, a separate biofilter with crushed oyster shell and activated charcoal was set in each system in Row 3.

Preparation of infected tissue and induced infection

Shrimp were starved before being fed infected tissue to ensure consumption of the infected tissue and a proper infection in the consuming shrimp. Frozen WSSV-infected shrimp were prepared by removing and discarding eyestalks, carapace, and telson. Minced tissue at 2-5 mm in size was homogenized, weighed, and stored in whirl-pack bags (1, 5, and 10 grams/bag) and frozen at -70 ºC. Infected tissue with WSSV was fed once to shrimp (10 % of each tank biomass). After exposure, the shrimp were kept under observation. Mortalities were recorded daily. All tanks, nets, and materials used to handle the shrimp were disinfected with chlorine, rinsed with tap water, and left to air dry.

System treatments and tanks distribution

As mentioned in table 1;

Treatment 1 (WBIUV), water borne infected and treated UV (tanks #5 and #7)

Treatment 2 (WBInotUV), water borne infected and no treated with UV (tanks #1 and #3)

Treatment 3 (WBI+POUV), water borne infected and per os infected and treated with UV (tanks #6 and #8)

Treatment 4 (WBI+POnotUV), water borne infected and per os infected and no treated with UV (tanks #2 and #4)

Data analysis

To evaluate the effects of UV irradiation on recirculation water and the virulence of WSSV for each treatment, a Survival Analysis Test was used (Kaplan and Meier, 1958). An event was defined as "time to individual mortality, with time measured in Days". Censored

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observations were survivors after all 16 days, the time that the trial ended. The number of individuals at risk remained constant at the beginning of the trials (40) and decreased as mortality occurred.

The Kaplan-Meier estimator was used to calculate and plot the survivor function of each treatment. The survival time was denoted as d (days) and defined as the day of each mortality or 16 in the case of survivors (censored observations). The Hazard function plot was used to depict each survival curve and compare the survival time among the groups.

The Gehan-Wilcoxon Test was used to compare the survivor curves. Differences were considered significant if the resultant *P* value was ≤ 0.05. All survival analyses were conducted with the analytical software package STATISTIXS® 8 (Statistix 8, Analytical Software, Tallahassee, FL)

Results

Overall effects

Significant differences between all the treatments were observed when recirculation water was irradiated with UV light compared to shrimp in systems without UV irradiation. Treatment 1 (WBIUV) (resulted in 100% survival at the end of the 16-day trial (Table 1). However, for the other three treatments (2 (WBInotUV), 3 (WBI+POUV), and 4 (WBI+POnotUV)), over 70% of the experimental shrimp developed characteristic symptoms of infection by WSSV, and survival was less than 30% (Table 1).

The different survival values for each of the treatments are shown in Tables 3 through 6. Survival values were: Treatment 2 = 25%, Treatment 3 = 30% and Treatment 4 = 2.4%. Although similar survival values were observed between treatments 2 and 3, they were, nevertheless, statistically significantly different (Table 2).

Differences between treatments were analyzed using Multi-Sample Survival Tests; Treatment 1 was excluded from these statistical tests as there were no mortalities. Table 2 shows the compared survival distributions for Treatments 2, 3, and 4. The calculated p-values were less than 0.05 for each of the three tests (Gehan-Wilcoxon, Log-rank, and Peto-Wilcoxon), indicating significant differences between each of the three treatments.

Table 1. Distribution of treatments (Tx) and tanks (Tk) included in each treatment. The two methods of infection are indicated: waterborne (WB) and *per os*. Percent of survival (St) at 16 days after infection. Treatment (Tx), Tank (Tk).

Tx	Tk	Methods of infection	UV Irradiated	St (%)
1	5, 7	WB	Yes	100
2	1, 3	WB	No	25
3	6, 8	WB + per os	Yes	30
4	2, 4	WB + per os	No	2.4

Table 2. Multi-Sample Survival Tests. Summary of the compared survival distributions. The p-values for all three tests are smaller than 0.05 and indicate highly significant differences among the three treatments. The Tx 1 was excluded from this statistical comparison as there were no mortalities.

		Gehan-Wilcoxon		Logrank		Peto-Wilcoxon	
Tx	N	Sum	Mean	Sum	Mean	Sum	Mean
2	40	1010.00	25.250	-9.4503	-0.2363	-8.1488	-0.2037
3	40	412.00	10.300	-7.2240	-0.1806	-3.7355	-0.0934
4	41	-1422.00	-34.683	16.6740	0.4067	11.8840	0.2899
Chi-Square			16.26		14.55		16.34
DF			2		2		2
p			0.0003		0.0007		0.0003

The onset of mortalities for treatments 2, 3, and 4 was days 5, 4, and 4, respectively, and the total number of dead shrimps on those days was 2, 1, and 6 (Table 8). This is a similar pattern when compared with the survival values, where the treatments resulted in the same numeric order (Tables 6 and 5). After the onset, mortalities were recorded daily until the end of the trial at day 16 and were statistically compared using the Kaplan-Meier Survivorship Percentiles. The survivorship was compared when each treatment reached 50% mortality (50% median survival time). We found that treatment 2 (0% of its population) survived longer compared with the 50% of tanks 6, 8, 2, and 4 (Table 7). The 50% median survival time indicates the

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survivorship pattern at the midpoint between the onset and the total mortality at the end of the trial at day 16.

Table 3. Treatment 1. Kaplan-Meier Product-Limit Survival Distribution. Summary of the survivorship presented for the UV-irradiated and waterborne-infected treatment. The values in the last four columns of the table were marked as NA and excluded from the statistical comparison because there were no mortalities. Percent survival St(%), Standard Error (SE), Hazard Function (H(t)).

Day	Died	Censored	At Risk	Lower 95% C.I.	St (%)	Upper 95% C.I.	SE St	H (t)
16	0	40	40	NA	100	NA	NA	NA

Table 4. Treatment 2. Kaplan-Meier Product-Limit Survival Distribution. Summary of the survivorship presented for the waterborne-exposed treatment without UV. Percent survival St(%), Standard Error (SE), Hazard Function (H(t)).

Day	Died	Censored	At Risk	Lower 95% C.I.	St(%)	Upper 95% C.I.	SE St	H (t)
5	2	0	40	83.9	95	98.5	0.0345	0.0513
7	3	0	38	74.5	87.5	94.3	0.0523	0.1335
8	2	0	35	68.5	82.5	91.0	0.0601	0.1924
9	5	0	33	55.8	70.0	81.1	0.0725	0.3567
10	5	0	28	43.5	57.5	70.3	0.0782	0.5534
11	1	0	23	40.1	55.0	69.0	0.0787	0.5978
12	4	0	22	31.9	45.0	58.8	0.0787	0.7985
13	3	0	18	25.2	37.5	51.6	0.0765	0.9808
14	4	0	15	17.4	27.5	40.5	0.0706	1.2910
15	1	0	11	14.5	25.0	39.4	0.0685	1.3863
16	0	10	10	-	-	-	-	-

Table 5. Treatment 3. Kaplan-Meier Product-Limit Survival Distribution. Summary of the survivorship presented for the UV-treated, waterborne, and per os exposure treatment. Percent survival St(%), Standard Error (SE), Hazard Function (H(t)).

Day	Died	Censored	At Risk	Lower 95% C.I.	St %	Upper 95% C.I.	SE St	H (t)
7	1	0	40	87.3	97.0	99.5	0.0247	0.0253
5	2	0	39	80.5	92.5	97.3	0.0416	0.0780
6	2	0	37	74.3	87.5	94.4	0.0523	0.1335
7	5	0	35	61.0	75.0	85.1	0.0685	0.2877
8	5	0	30	48.3	62.5	74.7	0.0765	0.4700
9	4	0	25	38.6	52.5	65.9	0.0790	0.6444
10	2	0	21	33.5	47.5	61.8	0.0790	0.7444
11	2	0	19	29.1	42.5	57.0	0.0782	0.8557
12	2	0	17	24.9	37.5	52.0	0.0765	0.9808
15	3	0	15	19.1	30.0	43.7	0.0725	1.2040
16	0	12	12	-	-	-	-	-

Survivor curves

The Kaplan-Meier survivor curves are designed for censored data (Cox, 1984; Hine *et al.*, 2002) and were plotted for the four treatments (Figure 8), where the highest percentage survival value was tanks 5 and 7 = 100% followed by tanks 6, 8, 1, 3, 2, and 4 (Table 1). The risk of death at a point in time is depicted using the Hazard Function Plot, which indicates that the highest risk was at day 15 in tanks 2, 4, 1, 3, 6, and 8. These values correspond with the values from the Product-Limit Survival distribution, with the maximum value four and the minimum zero.

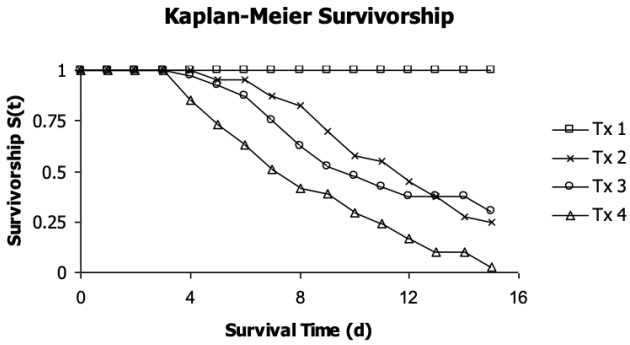

Figure 7. Kaplan-Meier survivor curves for *Litopenaeus vannamei* exposed to WSSV. Tx 1 = UV exposed and infected via WB, Tx 2 = non-exposed to UV and infected via WB, Tx 3 = UV exposed and infected via WB and per os, Tx 4 = non-exposed to UV and infected via WB and *per os*. Tx (treatment), WB (waterborne), *per os* (via oral by infected tissue), d (days).

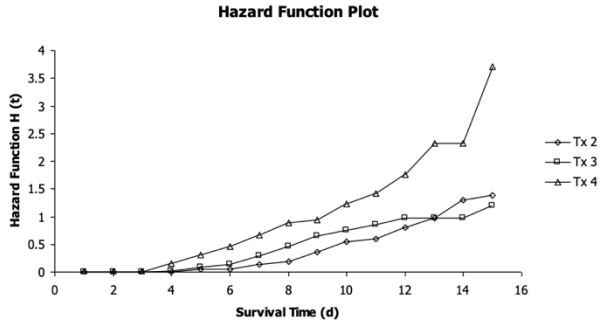

Figure 8. Kaplan-Meier hazard function curves for *Litopenaeus vannamei* exposed to WSSV. Tx 2 = no exposed to UV and infected via WB, Tx 3 = UV exposed and infected via WB and *per os,* Tx 4 = not exposed to UV and infected via WB and *per os*. Tx (treatment), WB (waterborne), *per os* (via oral by infected tissue), d (days). Tx 1 is omitted from this plot because no hazard function could be calculated.

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Methods of exposure

Waterborne. In tanks 5 and 7, the shrimp were exposed to waterborne and treated with ultraviolet irradiation (UV irradiated), resulting in 100% survival (Tables 1, 3, and 8). Tanks 5 and 7 were distinct from tanks 1, 3, 6, 8, 2, and 4 and excluded from the statistical comparison as there were no mortalities. Tanks 5 and 7 were considered the most successful treatments regarding survivorship. Tanks 1 and 3 were also WB-infected but were not UV irradiated. The lack of UV irradiation adversely affected this treatment and resulted in mortalities. The percent survival for tanks 1 and 3 was 25%, and the Hazard function was 1.38. Tanks 6 and 8, with waterborne and per os exposure to WSSV and UV irradiation, resulted in higher survival (30%) and lower hazard probability (1.20) at the end of the trial. This indicates that tanks 6 and 8 had a more successful treatment outcome than tanks 1 and 3 in terms of survival and hazard over time (Table 8). Tanks 1 and 3 onset started one day after tanks 6, 8, 2, and 4, suggesting that the infection was manifest earlier in tanks 6, 8, 2, and 4. The 50% median survivor time was also better for tanks 1 and 3 compared with tanks 6, 8, 2, and 4 at 12 days, contrasted with 10 days and 7.5 for tanks 6 and 8, 2 and 4, respectively. It can be inferred that the effect of the UV irradiation on the waterborne infection method was the factor that caused the difference between these treatments.

Waterborne + Per os. Tanks 6 and 8, 2 and 4 were fed WSSV-infected tissue and at the same time received the WSSV-contaminated flow, so they were exposed to both methods of infection at the same time (Table 1). The onset observed for tanks 6 and 8, 2 and 4, was the same at day 4. This suggests that the contact and cohabitation with the infection started at the same time as those tanks. Tanks 6 and 8, as well as 2 and 4, had the same infection methods but different water sources. Water received from the UV-irradiated treatment (tanks 5 and 7) had different effects than the water received from the non-UV-irradiated treatment (tanks 1 and 3). Tanks 6 and 8 received flow from tanks 5 and 7, resulting in higher survival (St) compared to tanks 2 and 4, which received flow from tanks 1 and 3 (30% and 2.4%, respectively). Moreover, the onset mortalities were 1 for tanks 6 and 8, and 6 for tanks 2 and 4. The Median Survivor Time was also higher at day 10 for tanks 6 and 8. And the Hazard Function at day 7 for tanks 2 and 4. The method of infection, WB + Per os, had a negative impact on tanks 6 and 8, as well as tanks 2 and 4, in a shorter period than WB alone. However, the UV-irradiated water flow increased survivorship in tanks 6 and 8 compared with the non-irradiated water flow coming into tanks 2 and 4.

Table 6. Treatment 4. Kaplan-Meier Product-Limit Survival Distribution. Summary of the survivorship presented for the WB and *per os* exposed treatment without UV. Percent survival St(%), Standard Error (SE), Hazard Function (H(t)).

Day	Died	Censored	At Risk	Lower 95% C.I.	St(%)	Upper 95% C.I.	SE St	H (t)
4	6	0	40	72.7	85.3	92.7	0.0552	0.1582
5	5	0	35	59.2	73.1	83.6	0.0692	0.3124
6	4	0	30	49.1	63.4	75.6	0.0752	0.4555
7	5	0	26	37.8	51.2	64.3	0.0781	0.6690
8	4	0	21	28.9	41.4	55.1	0.0769	0.8804
9	1	0	17	26.0	39.0	53.8	0.0762	0.9410
10	4	0	16	18.8	29.2	42.3	0.0711	1.2287
11	2	0	12	14.5	24.3	37.9	0.0671	1.4110
12	3	0	10	09.5	17.0	28.6	0.0588	1.7677
13	3	0	7	04.8	09.7	18.8	0.0463	2.3273
15	3	0	4	00.9	02.4	06.1	0.0241	3.7136
16	0	1	1	-	-	-	-	-

Discusion

WSSV causes a highly contagious, lethal disease characterized by massive mortalities during the first 10 days post-infection (Lightner, 1996; Lightner *et al.*, 1998). Throughout the present study, the characteristics observed for the disease were similar to those previously described, as massive mortalities were observed from day 3 to day 15 post-infection, confirming that the protocol followed for infection is well standardized and proper for this study. Given the suggestion that both temperature and salinity have direct effects on WSSV infection, we sought a uniform infection with constant mortalities across all tanks, which were monitored and controlled to ensure a proper infection for this experimental transmission.

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Table 7. Kaplan-Meier Survivorship Percentiles. Summary of the survival time for treatments 2, 3, and 4. The Tx 1 was excluded from this statistical comparison as there were no mortalities. The highest 50% Median Survival time is Tx 2 (12 days), then Tx 3 (8 days), and the lowest is Tx 4 (7.5 days).

Treatment	Percentile	Lower 95% C.I.	Time	Upper 95% C.I.
2	90	5.000	7.000	9.000
2	75	7.000	9.000	10.000
2	50	10.000	12.000	14.000
2	25	13.000	15.000	NA
2	10	NA	NA	NA
3	90	4.000	6.000	7.000
3	75	6.000	7.000	9.000
3	50	8.000	10.000	15.000
3	25	NA	NA	NA
3	10	NA	NA	NA
4	90	4.000	4.000	5.000
4	75	4.000	5.000	7.000
4	50	6.000	7.500	10.000
4	25	10.000	11.000	13.000
4	10	12.000	13.000	NA

Table 8. Summary of the survival results for treatments 1, 2, 3, and 4. The four treatment results are compared using the Product-Limit Survival distribution for Percent survival, Onset, and Hazard function. To calculate the Median survival time, the test Survivorship Percentiles were utilized. Note that Tx 1 had St = 100% and was not applicable (NA) for the other measures. The mortality for Tx 2 started later and reached the 50% Median survival time later than Tx 3 and Tx 4. In contrast, Tx 3 resulted in higher St and lower H (t) compared with Tx 2 and Tx 4. In all measures, Tx 4 had the weakest results. Treatment (Tx), Percent survival St (%), Hazard Function (H(t)).

Treatment	Percent survival	Onset		50%Median survival time	Hazard function	
			Mortalities			
Tx	St (%)	Day	(#)	Day	Day	H(t)
1	100	NA	NA	NA	NA	NA
2	25	5	2	12	15	1.38
3	30	4	1	10	15	1.2
4	2.4	4	6	7.5	15	3.71

The mortality of WSSV-infected shrimp can be reduced by increasing the water temperature to approximately 32 °C (Vidal *et al.*, 2001). This is described as a result of the reduction of the virus expression and replication (Jiravanichpaisal *et al.*, 2004/9; Rahman *et al.*, 2006; Jiravanichpaisal *et al.*, 2006; Reyes *et al.*, 2007) or the enhanced immune response as a consequence of an increased number of apoptotic cells that reduces the viral load (Granja *et al.*, 2003; Granja *et al.*, 2006). To avoid the reduction of infection from increased temperature, the experiment was conducted during a temperate climate season in March, when the ambient temperatures are low and the water temperature can be easily controlled indoors with immersed heaters.

Salinity was monitored using a refractometer and controlled by replenishing evaporated water, avoiding the salinity fluctuations that have been suggested to increase WSSV load in a shrimp population (Peinado-Guevara and López-Meyer, 2006). In contrast to salinity, the ammonia-N decreases the virulence of WSSV (Jiang *et al.*, 2004). Ammonia-N was controlled by means of a biofilter system using crushed oyster shell as substrate. In the present study, the potential effects of environmental parameters, such as temperature, salinity, and ammonia, were mechanically controlled to safe levels, and none of these parameters interfered with the susceptibility of the tested shrimp or the pathogenesis of the WSSV disease.

Cohabitation with living infected shrimp and ingestion of infected cadaver tissue (*per os*) are the successful means of transmission of WSSV (Supamattaya *et al.*, 1998; Soto and Lotz, 2001). Transmission of WSSV via water (waterborne) has been confirmed with shrimp and many other crustaceans as well (Kanchanaphum *et al.*, 1998).

Differences between these two methods of infection were observed in the present study. It was evident in this experiment that the dosage can be better controlled using *the per os method than the waterborne infection method,* as other authors previously described (Gitterle *et al.*, 2006). By using *per os,* it can be guaranteed that all the treatments (tanks) are fed with the same amount of infected minced tissue. However, this does not guarantee that all individuals in each tank will consume the same amount of tissue, as it may depend on differences in size and feeding patterns between individual shrimp.

In the present study, the *per os* treatment replicates were observed to have a similar onset of white spot disease between them. The onset for both treatments started at day 4 and was constant throughout day 6, when the non-UV-treated tanks increased the shrimp mortality. This suggests that the UV light was the factor that caused the

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difference between them and not the method of infection.

Using the waterborne infection method, it is unclear whether all shrimp will receive the same amount of virus at the same time in the tank. These gaps in the method leave room to question whether the method could establish a WSSV infection homogeneously distributed between the experimental populations (Gitterle *et al.*, 2006). A proportion of waterborne infected shrimp (non-UV light irradiated) resulted in cumulative mortalities of 70%. This suggests that waterborne infection is possible and represents a potential hazard for shrimp culture, as previously suggested by several authors (Kanchanaphum *et al.*, 1998; Supamattaya *et al.*, 1998).

Disregarding the method used in this experiment, we conclude that a complete WSSV infection was achieved as needed to test the effect of UV light irradiation on WSSV-contaminated water. The results were confirmed by a routine diagnosis using polymerase chain reaction (PCR) for white spot syndrome virus. The system was designed to maintain water recirculation and a constant flow from Row 1 to Row 2 and then to Row 3 (Figure 5), ensuring Row 1 remained exposed to WSSV waterborne and Row 2 to *per os*. Since infected shrimp were actively shedding virus in the water system, we can assume that Row 2 was exposed to both per os and waterborne effects due to the recirculation. However, as mentioned before, Row 2 (per os + waterborne) resulted in the lowest survival compared with Row 1 (only waterborne), which was also treated with ultraviolet irradiation. In summary, the shrimp infected by *per os* + waterborne had the lowest survival (30% and 2.4%), followed by those infected only with waterborne (25%), and the highest survival (100%) was observed in those exposed to waterborne and treated with ultraviolet irradiation.

The use of ultraviolet irradiation has been increasing in aquaculture activities. Potential uses against different aquatic diseases have been described to elucidate the time of exposure to ultraviolet irradiation, the dose, and wavelength (LeBlanc and Overstreet, 1991; Liltved *et al.*, 1995; Chang *et al.*, 1998; Liltved and Landfald 2000/2, Liltved *et al.*, 2006; Balasubramanian *et al.*, 2006). However, these studies have been carried out in still water using Petri dishes and a collimated beam apparatus (Qualls and Johnson, 1983), which protocol can provide specific information on the inactivation rate of the pathogen and reduction in viability at specific doses. Using this protocol (Qualls and Johnson, 1983) for aquatic diseases research can result in misleading information from the point of view of functioning systems, since all the ultraviolet systems are applied to running water. The information generated in this study provides clear insights into the inactivation of WSSV in running water.

Previous studies (Chang *et al.*, 1998) have shown that WSSV can be inactivated entirely after 60 minutes of UV irradiation using a 15 W, 254 nm wavelength UV lamp in flat dishes and static water. The results of the present study show that WSSV had no lethal effects on test shrimp when the recirculation water was irradiated with UV light. In contrast to Chang et al. (1998), the present experiment utilized a recirculation system that exposed running water to UV irradiation, resulting in a lower UV dose to the pathogens. The UV dose has been defined as the product of the average intensity across the Petri dish and the exposure time (Liltved *et al.*, 2006). In the present experiment, the exposure time was defined as the duration during which water was exposed to the ultraviolet bulb enclosed by the PVC pipe (Figure 1). Apparently, the dose calculated for this study was 25 mW/cm2 sec and seems to be sufficient to inactivate the virus, attenuate its effects, and increase the survivorship of the test shrimp to 100%.

Significant differences between the treatments were observed after using UV light. The combination of waterborne and per os methods used in treatments 3 and 4 (Row 2) was considered the most efficient for inducing infection in this experiment. In contrast, the use of ultraviolet between treatments resulted in a considerable difference in survivorship. Treatment 4, which was not irradiated with UV light, resulted in 2.4% survival. This treatment was exposed to the maximum WSSV viral load, as it was fed with infected minced tissue and also exposed to recirculation waterborne infected water. Treatment 3 had the same exposure to infection as

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treatment four but was treated with UV light irradiation and resulted in higher survival (30%). Moreover, treatment three also resulted in better survival than treatment 2. Treatment 2 was exposed only to waterborne infection corresponding to a minor WSSV viral load without UV. This supports the notion that UV light was the difference between treatments, even when treatment three was infected by the *per os* method.

Treatments 1 and 3 resulted in the highest survival percentages, presumably due to the ultraviolet irradiation, regardless of whether shrimp were exposed to WSSV by waterborne or *per os* + waterborne. Hence, the dose used in this study appears to be sufficient to reduce the lethal effects of WSSV on shrimp. Further studies on ultraviolet irradiation on waterborne WSSV-contaminated flow are necessary to elucidate, in a dynamic system, the dose required to inactivate WSSV successfully. It would be beneficial to establish a standardized method for validating the WSSV concentration required for a uniform infection, ensuring all tested shrimp receive the same viral challenge. Based on our results, ultraviolet irradiation appears to be a feasible alternative for controlling WSSV in water flows by interfering with virus replication and reducing viral load, thereby minimizing the opportunity for shrimp infection.

Conclusion

Controlling WSSV in water flows is possible through ultraviolet irradiation, which hinders viral replication and decreases concentration. This reduces the likelihood of shrimp infection. The findings of the present study form the basis for future studies on ultraviolet irradiation of water flows contaminated with WSSV. However, it would be beneficial to establish a standardized method for validating the WSSV concentration required for uniform infection to guarantee that all tested shrimp receive the same viral challenge.

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