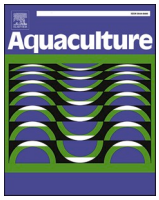
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Confirmed spread of *Vibrio parahaemolyticus* by aerosols is a risk factor in the infection of shrimp ponds

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ABSTRACT

Infection with *Vibrio parahaemolyticus* carrying a plasmid encoding two *Pir*-like toxins results in a rapid, progressive degeneration of the hepatopancreas of *Penaeus vannamei* leading to morbidity and mortality. Here we investigate the potential spread of *V. parahaemolyticus* (Vp_{AHPND}) aerosols to enhance understanding of biosecurity requirements at the farm level and to minimise the risk of infections. Using air stones to create aerosols from 20 L tanks inoculated with 1×10^6 cfu/mL^{-1} *V. parahaemolyticus,* and electric fans to deliver a 9.81 km/h^{-1} lateral flow of air at source, the spread of bacteria was determined using TCBS agar plates set out over an experimental arena extending to 20 m (34.0–30.7 °C air temperature, 63.3–71.9 % relative humidity). From several experimental trials, aerosols cast into the lateral airflow at 43 or 220 cm above the ground contained viable bacteria and were detected on TCBS plates as far as 20 m, marking the maximum perimeter. By extrapolation of colony counts on TCBS, we predict bacteria released 43 cm and 220 cm above the ground could spread 24.49–28.96 m (27.38 m ± 95 % C.I.) and 72.40–73.68 m (72.92 m ± 95 % C.I.) from the source, respectively. Putative protection provided by a 1.5 m high tarpaulin positioned 5 m from the infection source, failed to stop bacteria, which spread round the barrier and colonised TCBS plates positioned immediately behind, i.e., within 50 cm, and readily up to 5.5 m. A fourth trial using a combination of a protective screen placed over the aeration point to limit the spread of aerosols and two layers of 85 % protective shade netting positioned at least 1 m apart resulted in a ca. 20–30-fold reduction in bacteria penetrating the netting and colonising plates. Finally, detection of Vp_{AHPND}-laden aerosols was confirmed on a commercial shrimp production site using TCBS plates strategically positioned around a Vp_{AHPND}-positive pond. Altogether, we highlight the role that aerators play in generating bioaerosols, and how infections might be minimised in indoor culture settings, including the management of airflow and flying insects.

1. Introduction

Whiteleg shrimp, *Penaeus vannamei*, represent one of the world's most cultured marine species, reaching 6.83 million tonnes in 2022 (FAO, 2024). Within the *P. vannamei* industry, infections from pathogenic isolates of *Vibrio parahaemolyticus* (*Vp*) carrying a pVA1 plasmid encoding two *Pir*-like toxins, $PirA^{VP}$ and $PirB^{VP}$, can result in massive and progressive necrosis of the tubular epithelium of the hepatopancreas – a condition known as acute hepatopancreatic necrosis disease (AHPND; Han et al., 2015). This Gram-negative bacterium

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colonises the stomach and secretes the PirA and PirB toxins, leading to sloughing of the tubule epithelium, followed by the complete degeneration of the hepatopancreas and other gut-related tissues prior to bacteraemia and shrimp *in exitus* (Tran et al., 2013; Han et al., 2015; Lee et al., 2015). Even non-pVA1 carrying *V. parahaemolyticus* (TJA114) can target shrimp, promote dysbiosis in the gut tissues and alter the host's metabolic profiles (Zhang et al., 2021). The occurrence of AHPND has led to substantial economic losses. Shinn et al. (2018) estimated these losses at approximately $7.4 billion USD between 2010 and 2016, although this figure, based on food container traffic in Mahachai Market, Thailand, cannot be attributed solely to AHPND. The damage from *V. parahaemolyticus,* AHPND and *Vibrio*-associated disease is set to worsen due to the emergence of antibiotic resistant strains – isolated from seafoods and the environment (Loo et al., 2020).

There are multiple routes of pathogen introduction and spread on shrimp farms, including contaminated brood stock, feeds, and water, in addition to poor animal handling practices. Nguyen et al. (2021) explored potential risk factors associated with the spread of AHPND across 20 infected sites in Viet Nam and concluded that the role of fish-eating birds was substantial. Mitigation against bacterial disease necessitates a multi-targeted approach: promoting the health and welfare of the host (e.g., *P. vannamei*; Albalat et al., 2022), thwarting pathogens (e.g., *V. parahaemolyticus*), and good management practices within the culture facilities. Regarding the latter, biosecurity is an often-underappreciated aspect of disease prevention – especially the putative airborne routes of pathogen introduction and spread (Wooster and Bowser, 1996; Roberts-Thomson et al., 2006). According to Bovallius et al. (1978), bacteria of ~2 μm in mean diameter follow Stokes' Law, have a very low terminal sedimentation velocity of 1.3×10^{-2} cm/s, and can be transported 38.46 km during a 1 m sedimentation when released into a 5 m/s wind speed. Measuring ~1.4–2.4 μm long x 0.5–0.8 μm wide, *V. parahaemolyticus* could theoretically be transported similar distances. From the few published studies, we know that dinospores of the ectoparasitic dinoflagellate, *Amyloodinium ocellatum,* can disseminate via airborne droplets up to 440 mm in a static tank system and up to 3 m in a dynamic one (Roberts-Thomson et al., 2006), and the Gram-negative bacterium, *Aeromonas salmonicida* (ATCC 33658) can travel via aerosol spray at least 104 cm (Wooster and Bowser, 1996). Interestingly, Gołaś et al. (2022) assessed the indoor air quality of experimental fish rearing facilities as a potential health risk to humans and found seven-fold higher numbers (cfu/m^3) of potentially pathogenic *Aeromonas hydrophila* in the air of a recirculating tank system containing European grayling and perch, when compared to the outdoor system. The authors concluded that the fish rearing tanks were the source of *A. hydrophila* aerosols. Those data aside, there remains a paucity of information regarding the airborne transmission of bacterial pathogens between aquaculture sites, and no empirical data for pathogens of importance to shrimp culture.

To address this knowledge gap, the current series of trials aimed to explore the potential distribution of *V. parahaemolyticus* from the aeration of water tanks dosed with bacteria in shrimp (*P. vannamei*) culture settings. While bacteria are readily aerosolised by paddlewheels which serve to aerate culture water they can also be released into the air through high winds, waves, and raindrop splashes. Key objectives of this study were to determine the potential distance of travel of bacteria-laden aerosols, whether the *V. parahaemolyticus* remain viable, and if netting/screening could prevent bacterial spread.

2. Materials and methods

2.1. Infection source

Aerosolised Vp_{AHPND} was delivered by adding bacteria to four tanks, each containing 50.8 L of 15 ppt dechlorinated seawater, which were aerated by air stones and the tanks positioned in a laminar flow of air supplied by electric fans.

The Vp_{AHPND} inoculum used for the trials was an isolate of *V. parahaemolyticus* acquired through the Thai Department of Fisheries from a *P. vannamei*-AHPND mortality event and was prepared by culturing the bacteria in tryptone soya broth (TSB) supplemented with 2 % NaCl and incubated under shaking conditions (i.e., 250 rpm) at 28 °C for 12 h. Bacteria were centrifuged at 900 ×*g* for 10 min at 10 °C and the resultant bacterial pellet resuspended in sterile 15 ppt brackish water. Bacterial cell numbers were estimated by measuring the optical density at 600 nm (OD600), where for VP_{AHPND}, an OD value of 1.0 corresponded to approximately 1.0×10^8 cfu mL^{-1}. The bacterial cell number was then adjusted and verified by viable plate counts following standard methods (Salfinger and Tortorello, 2015).

A laminar flow of air across the tanks was delivered using two parallel Mitsubishi D12-GZ 12″ fans (38 W, 1170 rpm, 48 m^3 min^{-1}, 43 cm (Expt. 1) and 220 cm centre height (Expt. 3) above the ground), each fan blowing air 22 cm above the water surface across two 50.8 L polypropylene tanks in series presenting a collective water surface area of 38 cm (W) × 104 cm (L). Tank aeration was supplied by a Resun LP-100 air pump delivering 140 L min^{-1} 0.042 Mpa through eight 1″ air stones (two per tank). Relative humidity was recorded every 30–60 min using a digital humidity reader (MHO-C201, Miaoce Medical Equipment (Tianjin) Co., Ltd., Tianjin), temperature was recorded every 15 min with data loggers (Onset HOBO UA-001-64; Bourne, MA, USA), while wind speed was approximated using a bespoke anemometer determined as 9.81 km h^{-1} at source and as 0.94 km h^{-1} at a distance of 5 m (1.5 m elevation) from the source (av. from 5 × 1 min readings).

2.2. Trial 1: spread of aerosolised Vibrio parahaemolyticus under sheltered conditions

The infection tanks were set up as detailed in Section 2.1 with the addition of a dose of 120 ml Vp_{AHPND} inoculum (stock OD = 1.59; 1.46×10^9 cfu mL^{-1}) to each of the four tanks; the final dose from water samples taken 5 mins after addition was determined to be $1.79 \pm 0.36 \times 10^6$ cfu mL^{-1}). To determine the potential spread of bacteria, a series of 149 × 9 cm diameter Petri dish plates containing CM0333 thiosulphate–citrate–bile salts–sucrose agar (TCBS) Cholera Medium (Oxoid Ltd., Basingstoke, UK) were then positioned in arcs of 5–13 plates per arc, with each arc spaced approximately 60 cm apart and extending out a total distance of 10 m from the infection source (Figs. 1a-c). Each plate was placed on the ground, with its back edge lifted by no more than 1 cm, by means of a piece of modelling clay, such that the open face of the plate was oriented towards the infection source. The trial was set up beneath a covered but open sided area on the research premises covered by the relevant bacterial licences. At T_0 the plates were uncovered, the aeration to the tanks and the fans were switched on and left running for 6 h. Air temperature throughout the experimental period was 33.99 ± 0.41 °C (range 33.01–34.59 °C; $n = 26$) and relative humidity of 63.29 ± 1.38 % (range 61–65 %; $n = 7$ every 60 mins). The water level in the tanks was checked every 30 mins and topped up as necessary with 15 ppt seawater; approximately 14 L of seawater was added to each tank over the 6-h trial. After the exposure period, the plates were covered, placed into a Memmert IN75 incubator set at 30 °C and then every plate read and photographed 24 h later.

2.3. Trial 2: the effect of barriers on the spread of aerosolised bacteria under sheltered conditions

To determine if certain types of barrier, using materials in common use on shrimp farms, might afford protection from bacteria-laden aerosols, two types of protective barrier were assessed. The first, made from polyethylene 85 % sunshade net (PAM marketing Co. Ltd., Samut Sakhon, Thailand), was used to cover a series of nine frames, each measuring 18 cm (W) × 18 cm (L) × 13 (H) cm, to form enclosures covering a TCBS plate placed underneath. The frames were then placed in a series of three arcs at 1.6, 2.8 and 4.0 m from the infection source

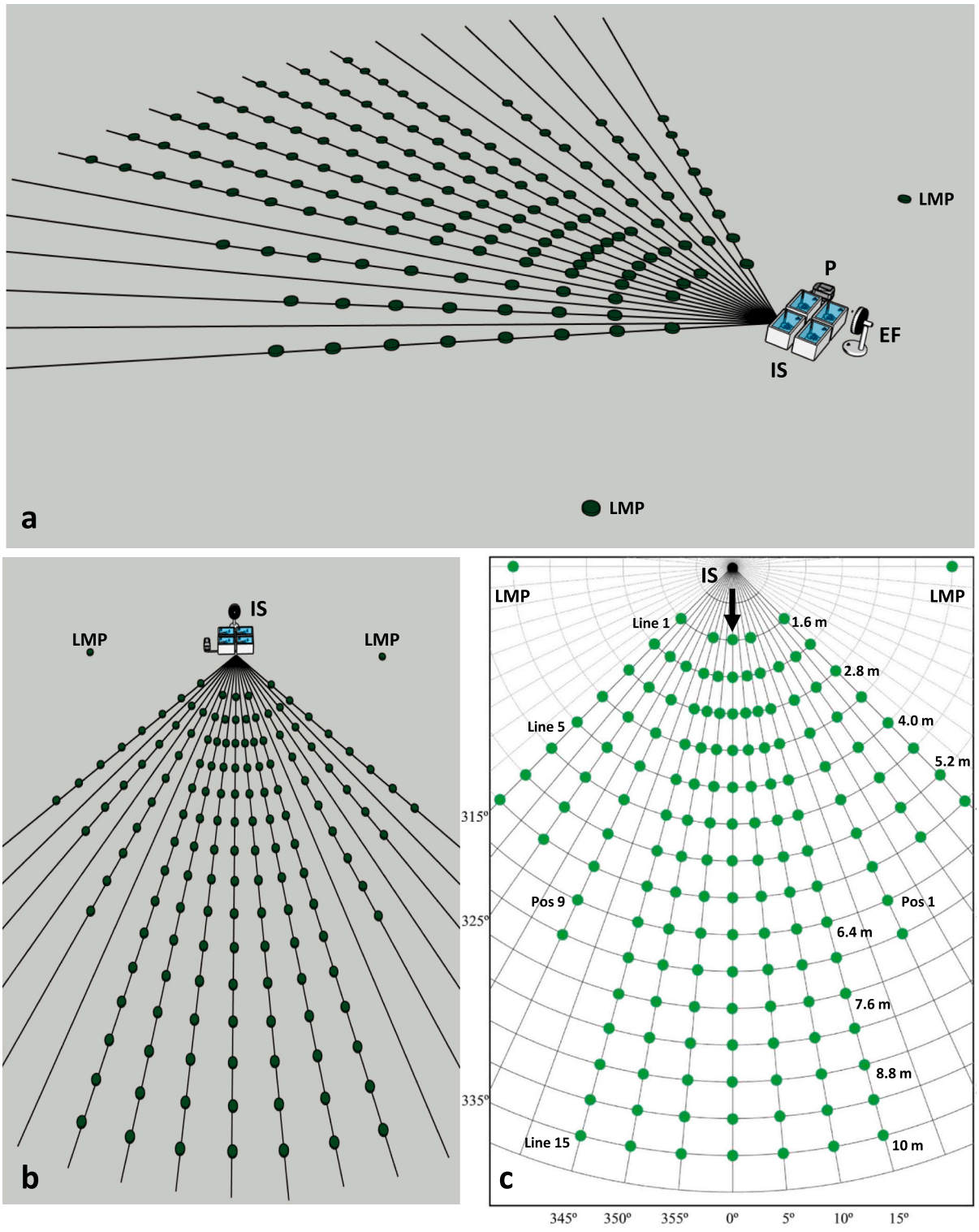


Fig. 1. Layout of the TCBS plates (spots) used in Experiment 1 to map the potential spread of *Vibrio parahaemolyticus* (*Vp*) by aerosols generated by using air stones and an electric fan (EF) to deliver a 9.81 km h^{-1} flow of air (heavy arrow) at the infection source (IS). a. Side view graphic of the experimental set up showing how an electric fan is used to create a flow of air over four water tanks containing *V. parahaemolyticus*. b. A different perspective of the experimental set-up. c. Map of the 149 plates used. Each line of plates are counted radiating out away from the infection source with Line 1 closest to the infection source, Line 15 the furthest away at 10 m. The plate position in each line is counted from right (Pos 1) to left (e.g., Pos 9 for Line 9). Abbreviations: EF = electic fan; IS = *Vp* infection source; LMP = lateral measurement points; P = pump.

(see Fig. 2a-c); nine uncovered TCBS plates were also interspersed between the frame covered plates. The second barrier type that was assessed was a 1.5 m high, polyethylene woven tarpaulin (Mitsaha, Thailand) that was positioned 5 m from the infection source. Five lines of TCBS plates were positioned beyond the barrier at 5.5, 7.0, 8.5, 10 and 10.5 m from the infection source (see Fig. 2a-c) – the maximum distance (i.e., 10.5 m) was limited by the size of the covered experimental arena available for this trial on the research premises covered by the relevant bacterial licences.

The infection procedure followed the same as that detailed for Trial 1

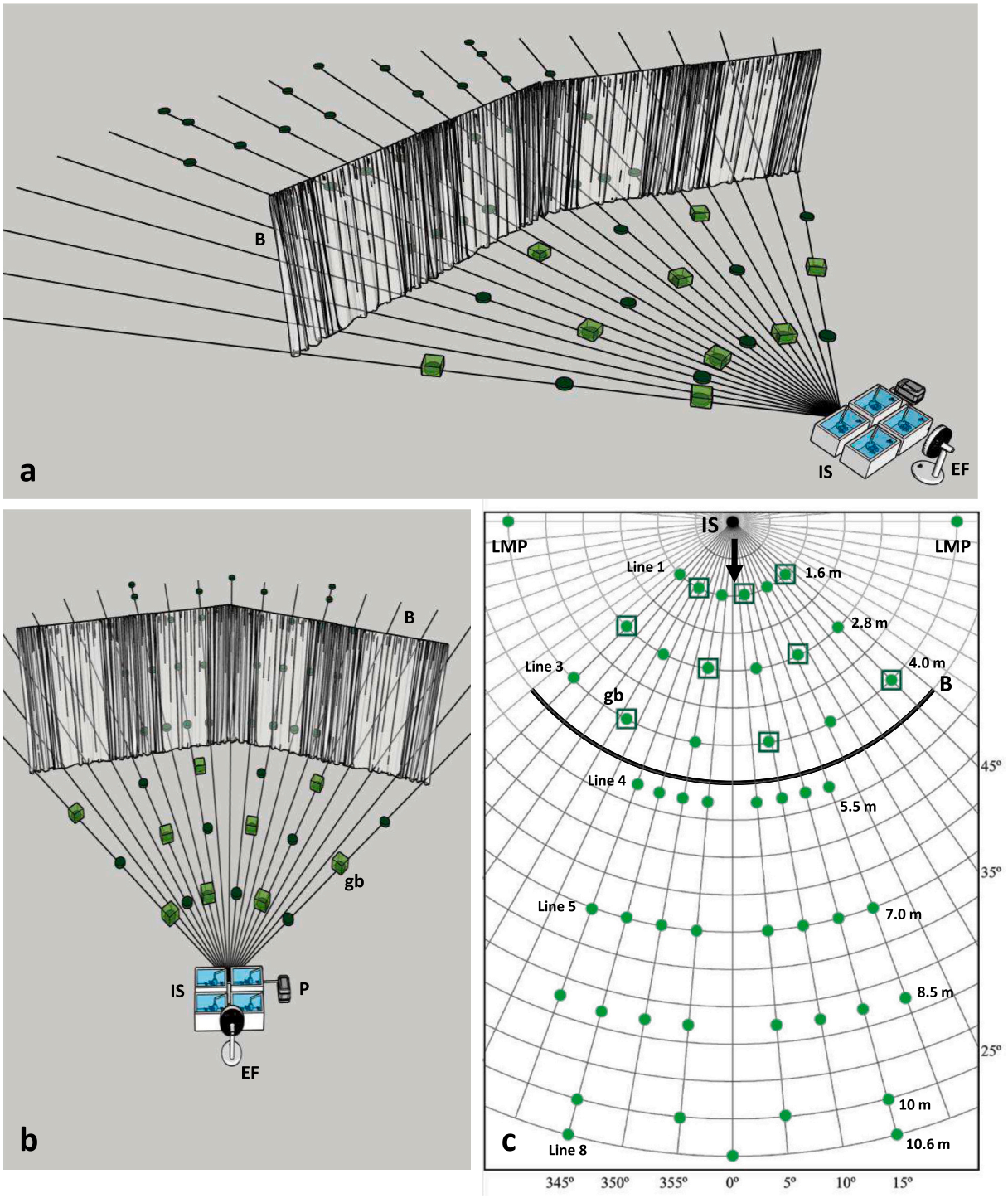


Fig. 2. Layout of the TCBS plates (spots) used in Experiment 2 to map the potential spread of *Vibrio parahaemolyticus* (*Vp*) by aerosols generated by using air stones and electric fans to deliver a 9.81 km h^{-1} flow of air (heavy arrow) at the infection source. a. The layout of the experimental arena and the 51 TCBS plates used to assess two different bacterium exclusion methods. The green boxes represent 18 cm (L) × 18 cm (W) × 13 cm (H) frames covered with polyethylene 85 % shade netting with a TCBS plate within (first method), while the second Q`1exclusion method consisted of a 1.5 m high polyethylene woven tarpaulin (B). b. A different perspective of the experimental set-up. c. Map of the 51 TCBS plates. Abbreviations: B = tarpaulin barrier; EF = electric fan; gb = green box; IS = *Vp* infection source; LMP = lateral measurement points; P = pump. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with a dose of 120 ml Vp_{AHPND} inoculum (stock OD = 1.54; 9.6×10^8 cfu mL^{-1}) added to each of the four tanks at T_0; the final Vp_{AHPND} dose was determined to be $1.29 \pm 0.55 \times 10^6$ cfu mL^{-1}). The trial ran for six hours, the average air temperature was 29.88 ± 0.29 °C (range 29.55–30.66 °C; $n = 25$ every 15 mins), relative humidity was 78.77 ± 1.54 % (range 75–80 %; $n = 13$ every 30 mins), and a total of 15.5 L of seawater was added to each tank.

2.4. Trial 3: spread of Vibrio parahaemolyticus under open conditions

To look at the potential spread of Vp_{AHPND} under open conditions, Experiment 1 was repeated but the height of the infection source was raised to 220 cm. The reason for this was to better understand the potential spread of aerosols that might be generated by high-speed aerators (i.e., when water droplets are ejected up to 2 m into the air and then are carried by a lateral flow of air). Although a wind speed of 9.81 km h^{-1} was set at 22 cm above the water surface at the point of infection, the experimental area was also subject to principally transverse but changeable wind speeds calculated at between 0.03 and 0.12 km h^{-1} throughout the trial. To attempt to capture the potential spread of bacteria, 88 TCBS plates were configured as in Fig. 3a-c, with plates set out at 7.5, 10, 12.5, 15, 17.5 and 20 m. Looking at the potential spread of

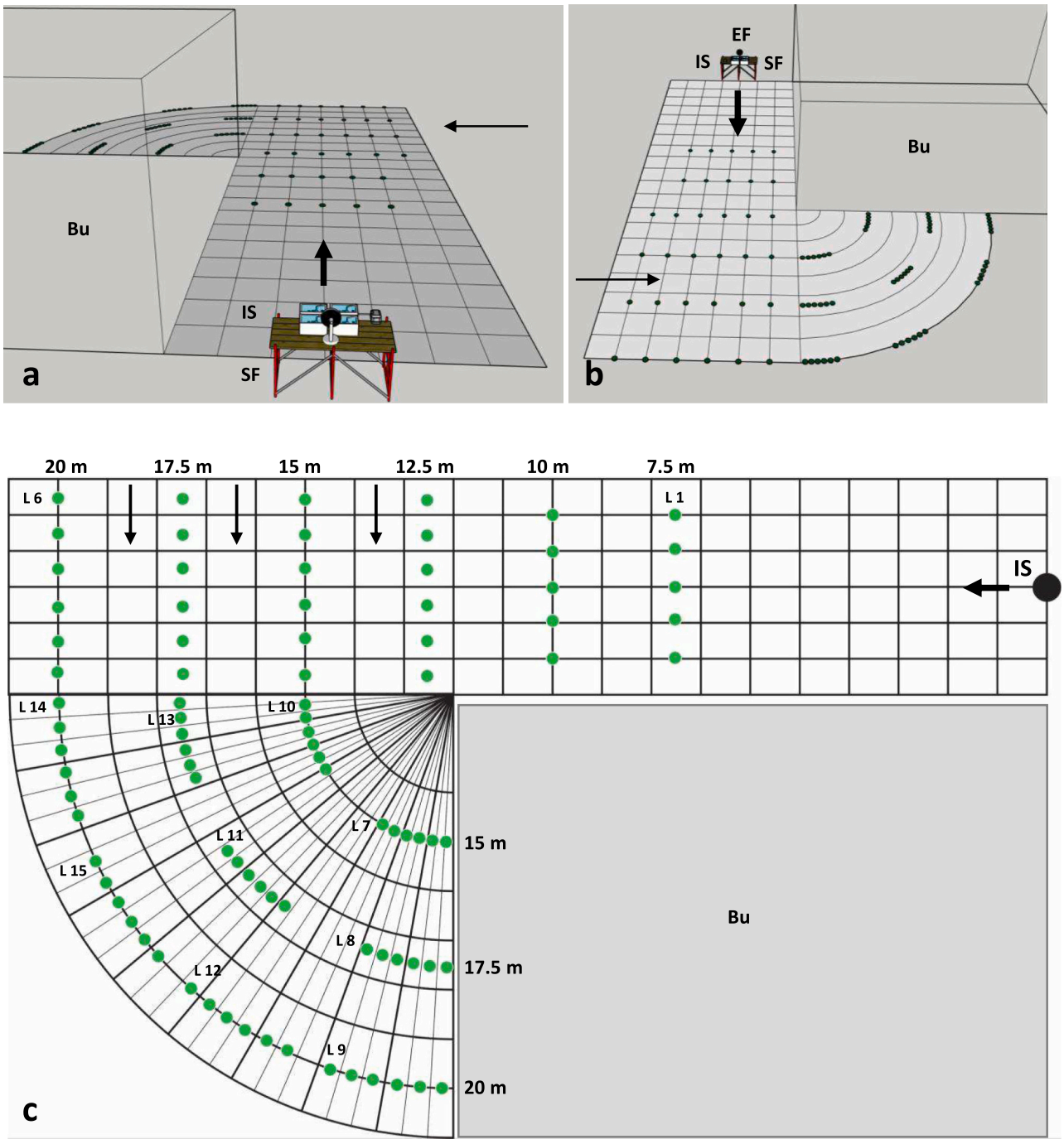


Fig. 3. The design and layout of the 88 TCBS plates used in a 20 m long open area subject to additional light transverse but changeable winds (thin arrows) blowing at 0.03–0.12 km h^{-1} for Experiment 3. a. Graphic showing the experimental set up with an electric fan (EF) positioned behind four aerated water tanks containing *Vibrio parahemolyticus* (*Vp*) on top of ca. 2-m high scaffolding frame used to disperse aerosols 2.2 m above ground level. b. A different perspective of the experimental set-up. c. Map of the 88 TCBS plates. Abbreviations: Bu = building; EF = electric fan; IS = *Vp* infection source; SF = scaffolding frame.

aerosols extending beyond 20 m, however, was not possible as the experimental work needed to remain within a controlled, licenced zone.

As with the previous two trials, a dose of 120 ml Vp_{AHPND} inoculum (stock OD = 1.60; 1.37×10^9 cfu mL^{-1}) was added to each of the four tanks at T_0; the final Vp_{AHPND} dose was determined to be $2.53 \pm 1.26 \times 10^6$ cfu mL^{-1}). The trial ran for six hours during which the average air temperature was determined to be 30.72 ± 1.05 °C (range 29.05–32.60 °C; $n = 25$ every 15 mins), the relative humidity was 71.86 ± 4.29 % (range 65–79 %; $n = 14$ every 30 mins), and a total of 18 L of seawater was added to each tank.

2.5. Trial 4: exploring strategies to limit the spread of bacteria-laden aerosols

While the shade netting enclosures used in Experiment 2 offered no protection from the bacterial aerosols under the conditions they were used, it was questioned whether this was because of their proximity to the infection source and the distance between the shade netting and the exposed TCBS plate within (i.e., 4.5–12.5 cm from any one edge which are unrealistic (too close) in a commercial farm context). This prompted a re-investigation of the use of shade netting and other farm-level practical strategies to reduce the spread of bacteria-laden aerosols but using distances that are typical of farm sites. To investigate this, the experimental set-up presented in Fig. 4a-b was used. The width of the walkway between two production ponds on a farm is commonly 2 m, so for the findings from this experiment to have potential application to a farm setting it was necessary to use biosecurity measures on a similar scale. This experiment explored limiting the spread of aerosol droplets at the main point of dispersion (i.e., using a cover over the aeration point), and then by using either one layer of 85 % shade netting positioned at least 2 m from the infection source and protecting a set of plates at least 1 m to the other side of that shape netting (e.g., L6 in Fig. 4a-b) or by

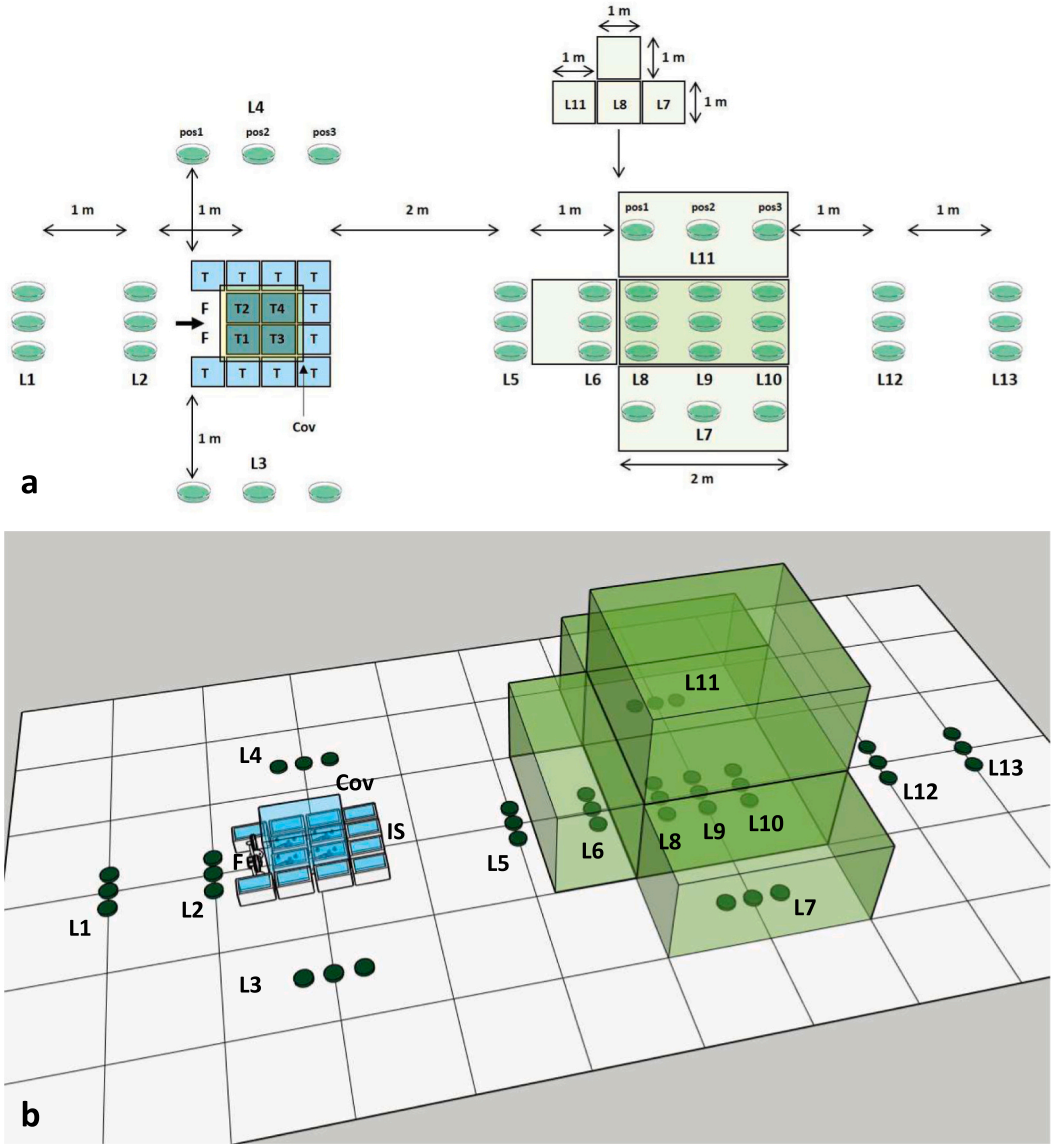


Fig. 4. a. Experimental set-up used to investigate the protection afforded by a cover (Cov) over the aeration points and source of infection (IS, T1-T4), and by areas protected by either a single layer of 85 % shade netting (areas enclosing L6, L7 and L11) or by a double layer of shade netting (area enclosing L8-L10). To monitor the spread of *Vibrio parahaemolyticus*-laden aerosols, TCBS plates laid in lines of triplicate plates (L1-L13) were used. The trial was run twice, once with the cover in place over the aerated infection tanks and then again without it in place. b. A different perspective of the experimental set-up. Abbreviations: Cov = cover; F = fan creating a wind speed of 9.81 km h^{-1} at source; pos1–3 = position of each TCBS plate in each line; $T = 50$ L tank filled with water; T1-T4 = 50 L tank filled with 15 ppt seawater and dosed with *V. parahaemolyticus*.

using two layers of shade netting, at least 1 m apart. This trial was run twice, the first with the cover over the infection source in place, then after replacing all the plates with new plates running the trial again but with the cover removed. As with the previous trials, two fans were used to create a wind speed of 9.81 km h^{-1} at the infection source. The trial was conducted in the open air; there was a very light transverse breeze during the trial (<0.1 km h^{-1} measured at 4 m height).

The trial used a dose of 120 ml Vp_{AHPND} inoculum (stock OD = 1.56; 6.7×10^8 cfu mL^{-1}) added to each of the four 50 L capacity infection tanks (T1-T4 in Fig. 4a-b) at T_0; the final Vp_{AHPND} dose was determined to be $3.73 \pm 1.86 \times 10^5$ cfu mL^{-1}). The four aerated infection tanks, which served to simulate a small aerator in operation in a pond, were flanked by a peripheral ring of tanks filled with water that served to absorb any droplets. A cover was used as a means of limiting the spread of the aerosol droplets around the aeration point. The edges of the cover sat 1.5 cm above the water surface; it was assumed that if a guard were to be fitted to aerators in operation on a commercial farm pond, then the cover would not sit flush with the water surface but likely would sit just above it, assuming floats are used to keep the guard in position. The centre of the cover was 47 cm above the water surface; the back edge of the cover was left open. The first part of the experiment, with the cover in place ran for 2 h, then aeration source was switched off, the system was left undisturbed for a further 30 mins before the TCBS plates were closed and replaced. The trial was then ran for a further two hours without the cover. Water samples were taken again 5 min after the aeration was restarted and the Vp_{AHPND} dose was determined to be $2.23 \pm 1.86 \times 10^5$ cfu mL^{-1}). After the second 2-h period, the aeration was stopped and once again left for 30 mins before the plates were collected and placed in a 30 °C incubator. The water level in the infection tanks was checked every 30 mins and topped up where necessary (av. 6 L $tank^{-1}$ over each 2-h trial). The average air temperature throughout the back-to-back trials was 32.63 ± 0.87 °C (range 30.56–34.16 °C; $n = 25$ every 15 mins), the relative humidity was 67.05 ± 3.62 % (range 61–74 %; $n = 20$ every 15 mins). Each bacteriology plate was then photographed, and the number of colonies counted.

2.6. Monitoring of Vp_{AHPND} at a commercial farm

To evaluate the aerial dispersal of AHPND-causing *Vibrio* species on a commercial farm, comprehensive monitoring was conducted throughout a production cycle across an undisclosed commercial *P. vannamei* site in Southeast Asia in July–August 2021. This included sentinel monitoring of air in the diagnostics laboratory ($n = 57$ TCBS plates; 8-h monitoring period), and then air and water samples selectively at five oxygenation stations within indoor greenhouses ($n = 10$ plates; 2-h monitoring period), around the periphery of four nursery ponds ($n = 16$ plates; 2-h monitoring period) and around the periphery of four grow-out ponds ($n = 22$ plates; 2-h monitoring period). Additionally, monitoring was carried out around the periphery of the receiving reservoir system consisting of nine reservoirs ($n = 48$ plates placed around the periphery of four reservoirs, positioned 5 m from the edge; 2-h monitoring period) and the wastewater system ($n = 42$ plates around two waste ponds, positioned 5 m from the edge; 2-h monitoring period) (see Table 2).

2.7. Molecular confirmation of Vibrio parahaemolyticus

The identity of the bacterium responsible for colony growth on the experimental TCBS plates was confirmed using the AP4 nested-PCR method of Dangtip et al. (2015) for the molecular confirmation of the tandem *Pir* toxin genes in *V. parahaemolyticus*. Details of the primers and method are provided in the Supplementary Materials section. For this, a random sample of 3 colonies per plate from three plates per trial, which typically included one plate from the mid-range (e.g., 5 m for Expt. 1 and 2) and one plate from the furthest measurement point (e.g., 10 m for Expt. 1 and 2, 20 m for Expt. 3 etc) were sampled and analysed.

2.8. Data handling

Trials were conducted to demonstrate the potential risk of infections being spread by aerosols rather than to determine a precise numeric probability factor as this is dependent on several potential conditions that are recognised but were not explored here (i.e., density of bacteria at source, wind speeds, pattern of droplet release, temperature, humidity, etc.). Similarly, a statistical analysis comparing the number of bacterial colonies growing on each triplicate set of plates in each line in Experiment 4 was not conducted as the low number of data points does not permit a robust analysis, instead all the raw counts are presented. The potential spread of bacteria was determined from exponential trendlines plotted through each data series and from forward forecasting in Microsoft ® Excel ® for Microsoft 365 MSO until 1 CFU m^2.

3. Results

We ran four experimental trials to investigate the potential spread and control of *V. parahaemolyticus* by aerosols generated through the aeration of water. Vp_{AHPND}-laden aerosols were confirmed by sentinel monitoring on a commercial shrimp farm production site.

3.1. Trial 1: spread of Vibrio parahaemolyticus

The spread of *V. parahaemolyticus* across a 10 m long field of TCBS plates under covered conditions was explored by aerating 4 × 50 L tanks of 15 ppt seawater inoculated with bacteria and by using electric fans to create a wind speed equivalent to 9.81 km h^{-1} at the infection source; the trial ran for 6 h (av. 33.99 °C; 63.29 % humidity). Subsequently, bacterial growth was observed on all TCBS plates up to 10 m with >300 colonies per plate at 7 m ($n = 9$), >100 colonies per plate at 8.8 m ($n = 7$), and 1–100 colonies per plate at 10 m (n = 7; Figs. 5a, 6b). The pattern of bacterial colonisation on certain plates confirmed the direction of infection (Fig. 6a).

3.2. Trial 2: effect of barriers on the spread of Vibrio parahaemolyticus

To determine if the spread of bacteria could be limited, a 1.5 m high physical barrier was positioned 5 m in front of the infection source, and on the use of shade netting to cover TCBS plates. The shade netting offered no protection to the three lines of TCBS plates positioned 1.6–4 m in front of the infection source (see Figs. 5b, 6c). While the 1.5 m high barrier was effective in reducing the level of bacterial colonisation seen on the TCBS plates, it did not prevent dispersal – bacteria were observed on all TCBS plates placed beyond it and up to 10.6 m from the infection source (see Figs. 5b, 6d).

3.3. Trial 3: spread of Vibrio parahaemolyticus in open air conditions

The finding from trial 1 that bacteria can spread, at least, up to 10 m prompted a second trial to extend the area. For this trial though, the wind blowing across the infection source was positioned at 2.2 m high based on the assumption that high speed aerators on a farm could cast water droplets >2 m into the air. The trial was conducted in the open air, at night (av. 30.72 °C and 71.86 % humidity), in the presence of a light cross wind which necessitated the monitoring of plates across an area extending 20 m but fanning out at 12.5–20 m to capture potential bacteria carried by the cross wind (Fig. 3a-c). High rates of bacterial colonisation on all TCBS plates, even those positioned at 20 m, were noted (Fig. 5c). The size limitations of the research premises licenced for exploring aquatic pathogens on which these trials were conducted, however, did not allow for a further trial to explore the distribution of bacteria beyond 20 m. A small number of plates also demonstrated the potential for flying insects to spread infection (Fig. 7) – what is not clear is if the mosquito on the plate flew through the bacteria-laden aerosol cloud and then landed on the plate spreading bacteria or if the mosquito

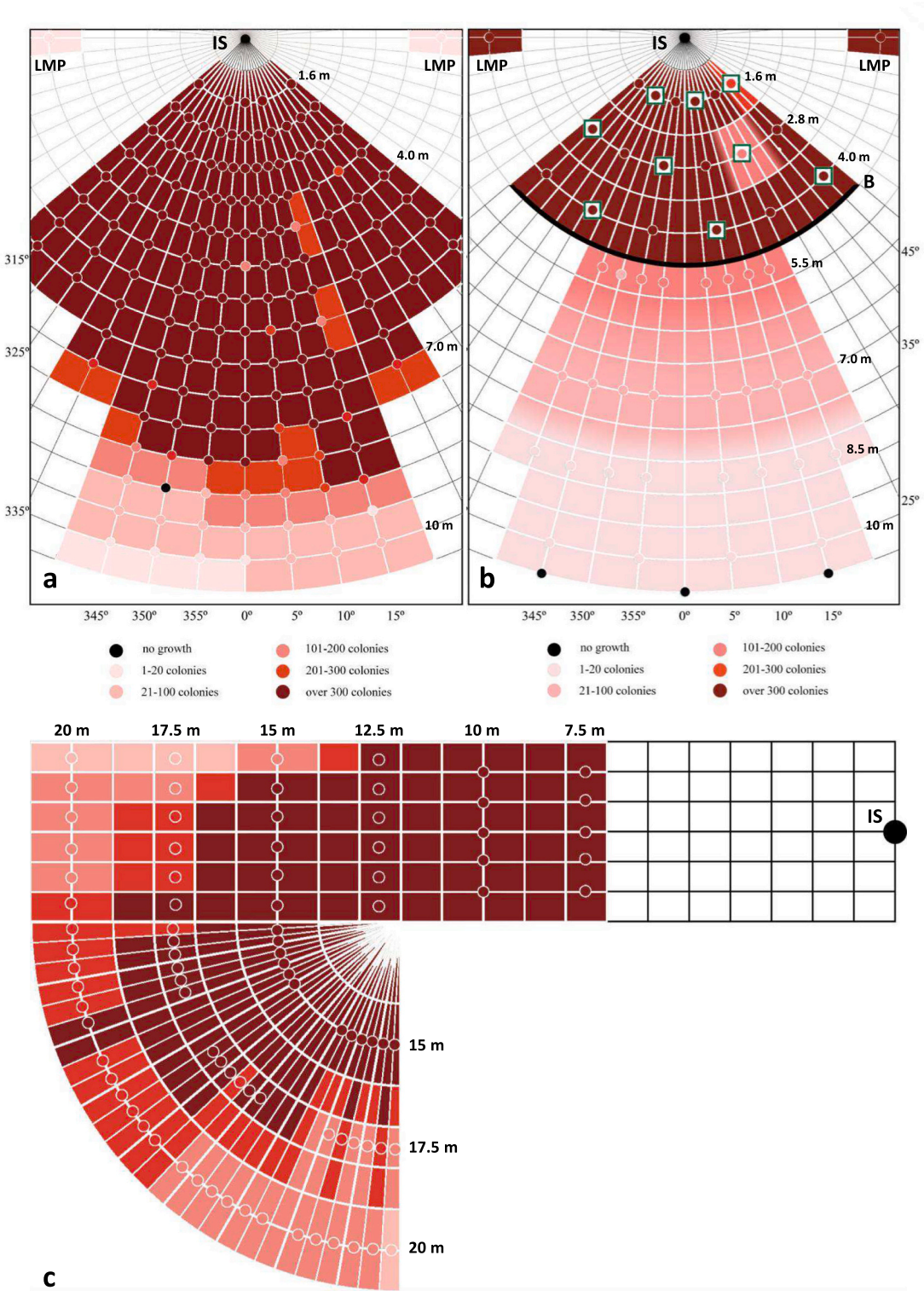


Fig. 5. The number of bacterial colonies detected on each TCBS plate in the experimental arena after a 6- h exposure to *Vibrio parahaemolyticus* (*Vp*) laden aerosols. The number of colonies growing on each plate in each of the three experiments are presented as heat maps. a. Colony growth on the 149 TCBS plates in Experiment 1 over a 10 m long arena that were placed in the path of a 9.81 km h^{-1} lateral flow of air at the point of infection blowing 22 cm above the water surface and 43 cm above the ground. b. The number of colonies growing on each TCBS plate in Experiment 2 which explored the protection provided by two different bacterium exclusion methods. The black boxes represent 18 cm (L) × 18 cm (W) × 13 cm (H) frames covered with polyethylene 85 % shade netting with a TCBS plate within (first method), while the second exclusion method consisted of a 1.5 m high polyethylene woven tarpaulin (B). c. The layout of the 88 TCBS plates used in a 20 m long open area subject to additional light transverse but changeable winds blowing at 0.03–0.12 km h^{-1} (Experiment 3). Abbreviations: B = tarpaulin barrier; IS = *Vp* infection source; LMP = lateral measurement points.

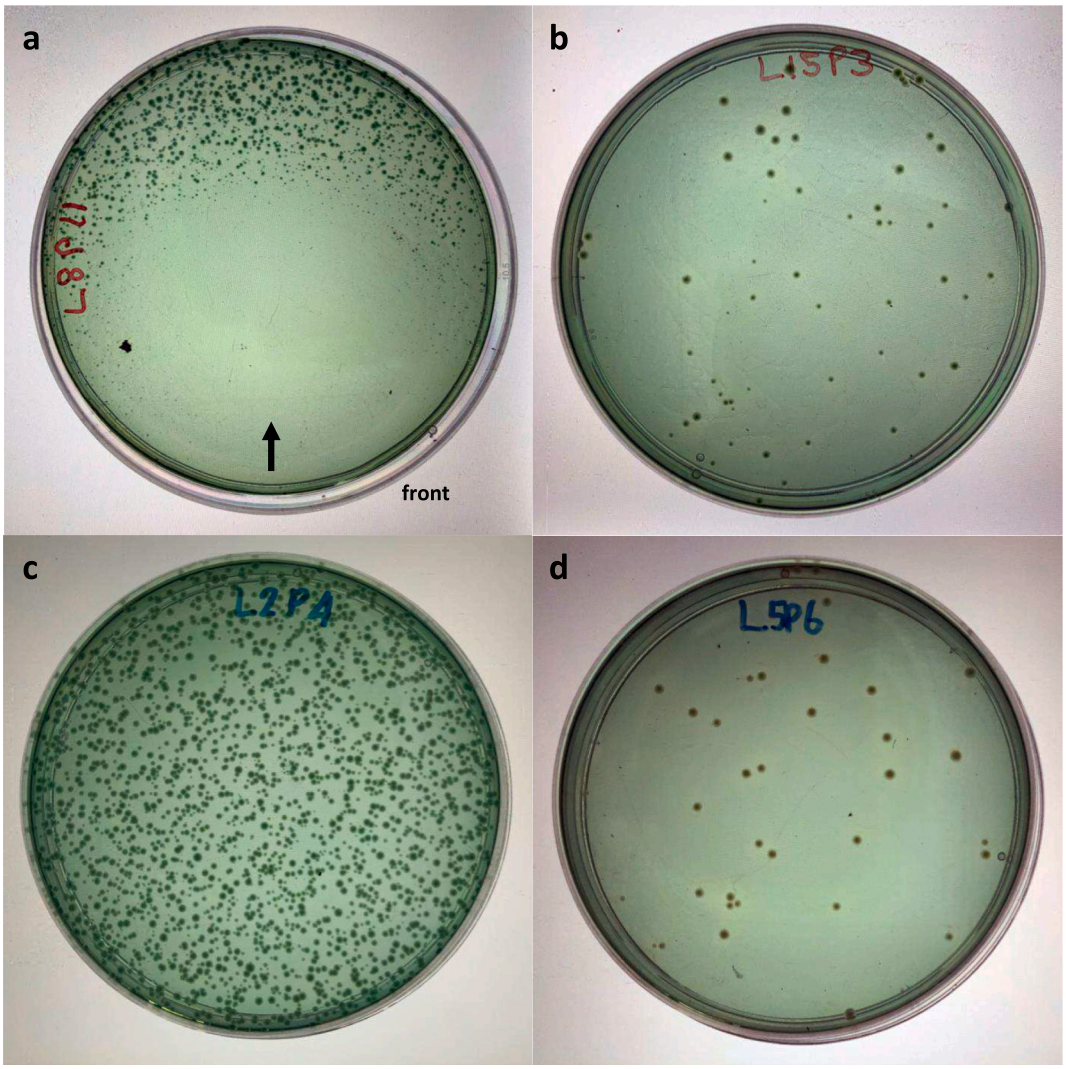


Fig. 6. Growth of *Vibrio parahaemolyticus* on the TCBS plates following a 6-h exposure in the experimental arena and subsequent incubation at 30.2 °C for 24 h. a. TCBS plate positioned at 5.8 m (Line 8, Pos 11; see Fig. 1a) from the infection source showing directional infection (arrow shows direction of air flow). b. TCBS plate positioned 10 m from the infection source 31 cm above ground level (Line 15, Pos 3; see Fig. 1a). c. Colonisation of *V. parahaemolyticus* on a TCBS plate positioned beneath the frames covered with polyethylene 85 % shade netting (plate positioned Line 2, Pos 4; see Fig. 1b). d. Bacterial colonisation on a TCBS plate (Line 5, Pos 6; see Fig. 1b) positioned beyond the 1.5 m high barrier (a polyethylene tarpaulin).

landed on the plate, within the 6 h exposure period, and then walked through a colony establishing on the plate.

3.4. Trial 4: farm practical strategies to the limit the spread of aerosols

This trial explored the spread of bacteria-laden aerosols when the aeration (infection) point was either exposed or covered and, the use of a single or two layers of shade netting separated by distances that might be used on commercial farm sites (i.e., 1+ m). When the aeration point was covered, most of the bacteria spread by aerosols were restricted to the vicinity around the aeration point (see Table 1A; i.e., >100 cfu Petri dish^{-1} on plates positioned at L1-L5 in Fig. 4a-b). The number of bacteria fell to av. 18.3 cfu Petri dish^{-1} when the Petri dishes were protected by one layer of shade netting and they positioned 1 m behind the shade netting (i.e., L6 in Fig. 4a-b); the number of bacterial colonies decreased with increasing distance from the infection source (see Table 1A, L7 and L11). When the Petri dishes were protected by two layers of shade netting positioned at least 1 m apart, then the number of colonies fell to an av. 0.3–1.3 cfu Petri dish^{-1} (L8-L10; Fig. 4a-b). Exposed Petri dishes positioned at L12 and L13 behind the shade-netting protected structure, and 6 and 7 m from the infection source, had an av. 0.6 and 1.3 cfu Petri dish^{-1}. When the trial was repeated with the aeration (infection) point uncovered, then the number of bacteria in all zones was notably higher, i.e., >10-fold higher in zones protected by a single layer of shade netting (L6, L7 & L11) and > 20-fold higher in zones protected by two layers of shade netting (L8-L10; Table 1B and Fig. 4a-b). The unexposed plates (i.e., L12 and L13) positioned behind the experimental structure had on average > 30-fold more colonies on the Petri dishes.

3.5. Potential spread of aerosols

Using data from trials 1 and 3, the potential spread of bacteria was determined from exponential trendlines plotted through each data series and from forward forecasting (Fig. 8). Bacteria from the two infection sources can spread 27.38 m (range 24.49–28.96 m; mean ± 95 % C.I.) and 72.92 m (range 72.40–73.68 m; mean ± 95 % C.I.) from droplets released into a laminar flow of air 43 cm and 220 cm above the ground, respectively.

3.5.1. Monitoring of Vp_{AHPND} at commercial farm sites

Surveillance across the commercial farm used TCBS agar plates (n =

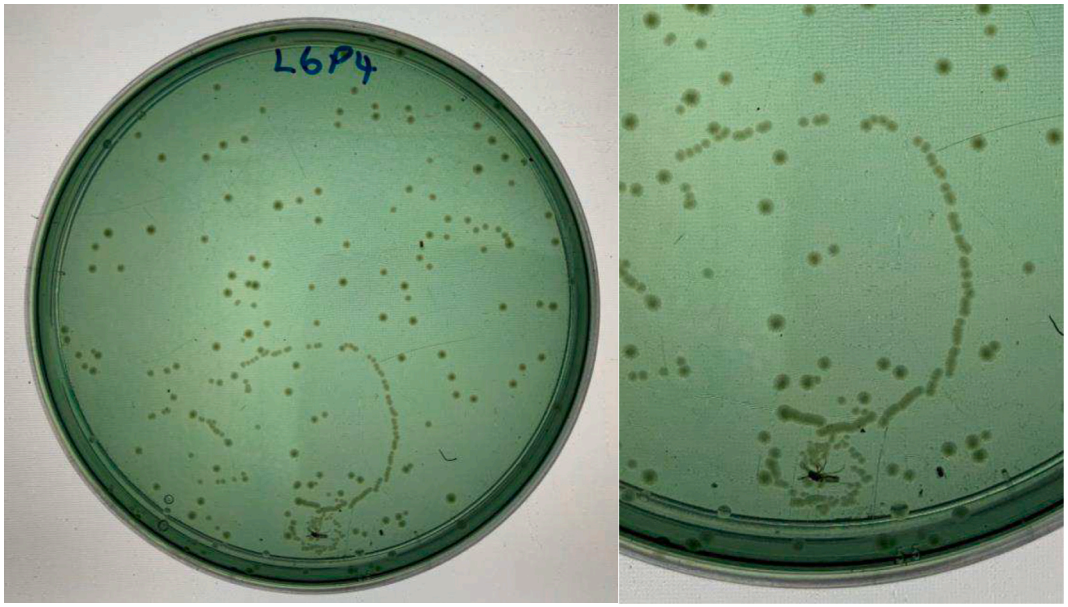


Fig. 7. *Vibrio parahaemolyticus* colony growth on the TCBS plates associated with the activity of insects. This particular plate was from Experiment 3 and positioned 20 m from the infection source (see Fig. 1c).

Table 1
Results from Experiment 4 exploring strategies to limit the spread of bacterial isolates. The data show the number of colonies of *Vibrio parahaemolyticus* growing on individual TCBS plates following a 2-h exposure. Table 1A is with the aeration point and source of infection covered by a screen to limit the spread of bacterial aerosols, while Table 1B is a repeat of the trial but with the cover removed. The position of each triplicate set of TCBS plates (L1–L13) is presented in Fig. 2 but L1-L5 are plates positioned within 1–2 m of the infection source, L6, L7 and L11 are protected by a single layer of 85 % shade netting, while L8-L10 are protected by two layers of 85 % shade netting at least 1 m apart. Lines 12 and L13 are plates that are not positioned 6–7 m from the infection point and are not immediately protected by any layer of shade netting enclosing them.

A	Aeration point covered			B	Aeration point exposed		
Line	Pos 1	Pos 2	Pos 3	Line	Pos 1	Pos 2	Pos 3
L1	161	181	>300	L1	229	40	54
L2	275	>300	>300	L2	>300	>300	60
L3	>300	>300	>300	L3	24	63	160
L4	>300	>300	>300	L4	>300	98	106
L5	111	160	161	L5	>300	>300	>300
L6	21	15	19	L6	96	104	139
L7	2	0	0	L7	165	95	9
L8	2	0	2	L8	14	34	41
L9	0	1	0	L9	19	16	16
L10	0	1	1	L10	4	9	6
L11	10	4	1	L11	3	47	27
L12	0	1	1	L12	35	31	9
L13	0	2	2	L13	93	25	18

195) to monitor airborne *Vibrio* and water samples from key monitoring points. All *Vibrio*-positive samples were further analysed by quantitative PCR (qPCR) for *Vibrio parahaemolyticus* linked to AHPND; all the results are presented in Table 2. Sentinel TCBS plates placed in the diagnostics laboratory ($n = 57$) and the oxygenation station ($n = 4$) tested negative for *Vibrio* spp. One bacterial colony was recovered from a single TCBS plate ($n = 48$) positioned around the periphery of the seawater reservoirs; however, this colony tested negative for *Pir*AB via quantitative PCR (45 cycles, limit of detection (LOD) = 36.97). Details of the primers and method are provided in the Supplementary Materials section.

A total of 644 bacterial colonies established on 22 plates placed around the four external grow-out ponds. Among these, colonies from six plates (i.e., 332 colonies) around one pond tested positive for *Pir*AB (quantification cycle (Cq) = 23.48, 45 cycles, LOD = 36.97), while colonies from a further five plates (i.e., 173 colonies) placed around a second grow-out pond had a Cq value of 40.81. The 139 bacterial colonies on TCBS plates placed around two other grow-out ponds were negative, as were the four bacterial colonies on TCBS plates placed around the periphery of four nursery ponds ($n = 16$ plates).

Infection in the *Pir*AB-positive grow-out pond was confirmed on day 3 of culture (DOC) in the water (Cq = av. 32.15 ± 3.95; range 29.36–34.94), on DOC 9 in the water (Cq = av. 28.90 ± 4.92; range 24.01–34.83), and on DOC 17 in both shrimp (Cq = 34.87) and water (Cq = av. 26.83 ± 4.10; range 24.41–31.56).

Of the sentinel TCBS plates placed around the two wastewater systems to monitor *Pir*AB-laden aerosols, eight bacterial colonies from one reservoir were negative by qPCR. However, more than 250 colonies recovered from plates around the second wastewater reservoir were *Pir*AB positive (Cq = 22.57). Infection in both systems was confirmed from phytoplankton samples taken from both reservoirs which tested positive for *Pir*AB: two of the four pooled samples from the first wastewater system had Cq values of 29.9 and 38.04, while two of the four samples from the second wastewater system had values of 35.48 and 42.95.

4. Discussion

In this proof-of-concept study, we demonstrate that Vp_{AHPND} can be aerosolised from donor/contaminated shrimp ponds by wind action, remain viable while airborne, and disseminate to putative receiver ponds up to 73 m distance from source. Furthermore, monitoring a commercial site with Vp_{AHPND}-infected shrimp confirms bacterial transfer occurs by an aerobiological route. Herein, the choice of air speed at 9.81 km h^{-1} (= 6.125 mph or 2.72 m/s) is representative of the average low wind speeds of 2.8–4 m/s at 10 m elevation recorded for Thailand (26 % <4/m/s, 64 % 4-5 m/s and 9 % 5-6 m/s; Chingulpitak and Wongwises, 2014), which is equivalent to a score of 2 on the Beaufort wind scale, describing a 'light breeze'. The long-range transport of airborne bacteria is well known (Bovallius, 1978), in fact Mayol et al. (2017) used data collected along the Malaspina 2010 Circumnavigation Expedition to estimate that 33–68 % of the global microbial load in the atmosphere (~2.2×10^{21} prokaryotes and 2.1×10^{21} eukaryotes) were of marine origin and could be airborne for thousands of

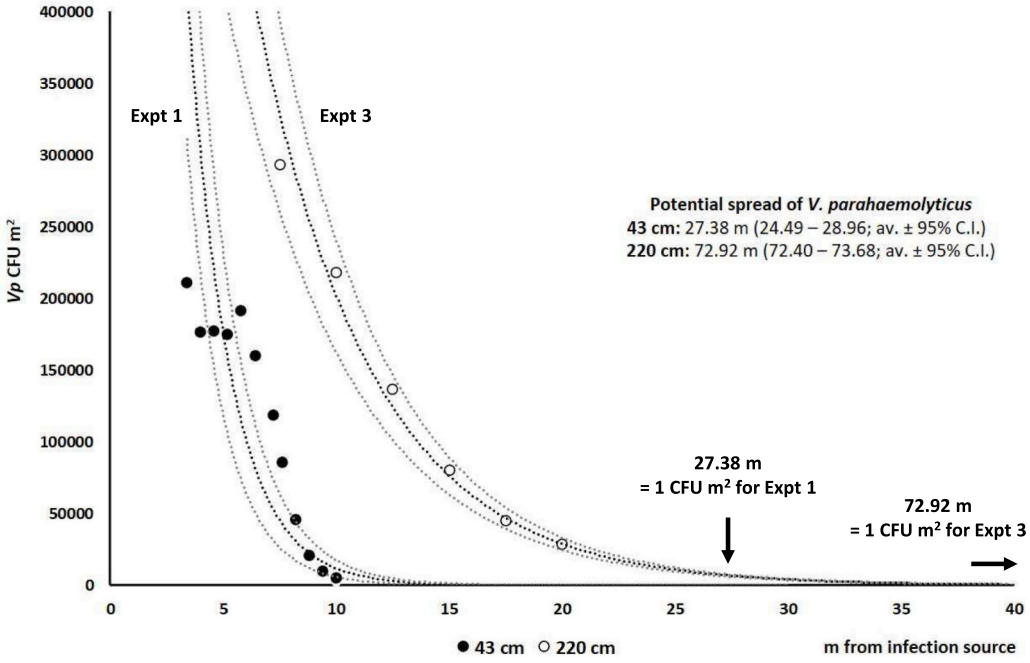


Fig. 8. Extrapolating the potential spread of *Vibrio parahaemolyticus (Vp)* from aerosols released 22 cm above an infection source positioned 43 cm and 220 cm above the ground into a lateral wind speed of 9.81 km h^{-1}. Data indicate that bacteria released 43 cm above the ground could spread 24.49-28.96 m (27.38 m ± 95% C.I.) while droplets released 220 cm above the ground could spread 72.40-73.68 m (72.92 m ± 95% C.I.). The x-axis has been purposely cut at 40 m so that the theoretical sedimentation of bacteria over the first 40 m can be seen.

Table 2
Summary of surveillance across the commercial farm site using TCBS agar plates to monitor airborne *Vibrio* and concentrations in water samples collected from strategic monitoring points. All *Vibrio*-positive and water samples were analysed by real-time PCR for *Vibrio parahaemolyticus* associated with AHPND. Positive samples and their Cq values are highlighted in bold. Abbreviations: Cq = quantification cycle; LOD = limit of detection; TCBS = thiosulphate–citrate–bile salts–sucrose agar; Z = farm zone.

	AIR			WATER		
Sampling location	No. TCBS plates	No. *Vibrio* colonies	Real time PCR (Cq; LOD = 36.97)	No. samples	No. *Vibrio* colonies	Real time PCR (Cq; LOD = 36.97)
Diagnostics laboratory	57	2 (gen. Bact.)	Negative			
Outdoor grow-out pond 1-Z1	5	173	40.81			
Outdoor grow-out pond 2-Z1	5	138	Negative			
Outdoor grow-out pond 3-Z2	6	332	**23.48**	10		**29.36, 34.94, 24.01, 25.84, 34. 83, 30.93, 31.56, 24.52, 34.87, 24.41**
Outdoor grow-out pond 4-Z2	6	1	Negative			
Outdoor nursery pond 1-Z1	4	2	Negative	4		**29.14, 24.18, 35.82**
Outdoor nursery pond 2-Z2	4	0		1		**36.9**
Outdoor nursery pond 3-Z2	4	2	Negative			
Outdoor nursery pond 4-Z3	4	0				
Reservoir 1-Z1	6	1	Negative	1	115	Negative
Reservoir 2-Z1	6	0		2	81, 86	Negative
Reservoir 2-z1 post-UV				4	0, 5, 0, 0	Negative
Reservoir 3-Z1	6	0				
Reservoirs 4–6-Z2	30	0				
Indoor greenhouse oxygenation stations (n = 5)	10	0				
Wastewater system 1	20	8	Negative			
Wastewater system 2	22	250	**22.57**	4		**42.95, 35.48**, 0, 0

kilometres before re-entering the ocean (air-sea exchange). On a much smaller scale, Roberts-Thomson et al. (2006) confirmed airborne droplets containing *A. ocellatum* dinospores can travel up to 3 m in certain culture settings, whereas Wooster and Bowser (1996) reported that *Aeromonas salmonicida* (ATCC 33658) can travel >1 m via aerosols. Although the risk of airborne infections, including aerosols, has long been acknowledged in aquatic biosecurity programs, there is a general paucity of information on the transmission potential of many aquatic pathogens. Insights from human and veterinary medicine, particularly those gained during the recent COVID-19 pandemic, suggest that our understanding of respiratory pathogens and aerosol transmission may also be relevant to aquaculture settings (Fennelly, 2020).

For instance, particle size is the primary factor influencing aerosol behaviour, with most bacterial pathogens in aerosols contained in particles <5 μm. Particles of this size can remain airborne indefinitely under typical indoor conditions - such as those found in hatcheries, indoor

pond production, and greenhouse pond facilities - unless dispersed by air currents or ventilation systems (Wells, 1955). Given their ability to remain airborne for prolonged periods, these particles can travel considerable distances. In aquaculture facilities with limited ventilation, aerosols may accumulate in spaces between ponds or tanks and may transfer easily between recirculating aquaculture systems. Additionally, the concept of 'super-spreader' units, seen in human disease transmission, may be relevant in aquaculture. Certain units may generate substantially more infectious aerosols than others, and production rates can vary significantly even within and across the same source.

Additionally, as discussed, transmission is influenced by environmental factors such as the pathogen's ability to survive desiccation, the stresses encountered during aerosolization and airborne transport, air currents, temperature, humidity, and the size of the emitted inoculum. Temperature gradients between water and air can impact aerosol behaviour, with different pathogens having distinct optimal temperature ranges for transmission.

There are of course limitations to our study, notably (1) the study perimeter extends to 20 m only in line with the operational and licencing limitation of the site working with this bacterial biohazard, and (2) we could only leave the agar (TCBS) plates out for 6 h prior to drying and wrinkling. In Experiment 4, we demonstrate with some success that cover/netting may reduce the levels of aerosolised bacteria, but not their distribution. In this context, control strategies could include:

- changing the design of aerators to reduce the cast height and spread of droplets (use aero-hoses instead);
- the use of mist nets to catch droplets;
- while shade netting may offer benefits, it could also serve as a reservoir for infections by extending bacterial survival on moist surfaces due to continuous water droplet capture. This concern is similarly relevant for mist nets. Longer trials are recommended to evaluate bacterial load and colonisation on these materials. Ongoing trials (Wongwaradechkul, unpublished data) have shown that *V. parahaemolyticus* can survive on high-density polyethylene (HDPE) pond liners for up to 90 min at 31 °C and 72–74 % humidity;
- planting of a tree line or fence to either catch droplets or divert air flows;
- land shaping e.g., concrete pads or earth ramps to lift air currents over ponds (though such ramps may create eddies off the back edge that increase the deposit of droplets into ponds, and, concrete pads may have to be large to create thermal lift);
- use of modified bioflocs to reduce the likelihood of *V. parahaemolyticus* proliferating when bioaerosols land in recipient ponds;
- actioning an air sampling regime for detecting and quantifying pathogens and bioaerosol load.

Regarding some of the former control measures, their implementation would also assist in reducing the risk of 'contaminated' flying insects spreading the bacterial/viral pathogens, which tend to be overlooked despite their infamous roles as vectors and reservoirs of zoonotic diseases, including *Vibrio* spp. Regarding the latter control measure, bioflocs are used mainly to manage nutrient levels in fish/shrimp ponds, but they can also be formulated for purging waters of putative bacterial pathogens – e.g., containing lactic acid bacteria that compete with *Vibrio* spp. (Thompson et al., 2022) or preventing *V. parahaemolyticus* biofilm formation (Gustilatov et al., 2022).

Farm-level biosecurity control considerations should include an understanding of dominant airflow patterns and the strategic positioning of culture units and control interventions in relation to these patterns. Wastewater systems or the treatment of receiving waters should be positioned downwind of open pond cultures and preferably at a lower altitude. If existing farm design does not permit the relocation of wastewater systems, enclosing high-risk pathogen waters can be an effective alternative. Some farms have opted to install tree or shrub lines, or mist nets, to limit bacteria-laden aerosols from wastewater systems blowing across farm production units (*pers. obs.*).

Monitoring can also incorporate sentinel TCBS bacteriology plates to assess bacterial load and risk, as shown in trials tracking the spread of *V. parahaemolyticus* on farms (see Table 2). Detecting *V. parahaemolyticus* through ongoing air and water surveillance has strengthened on-site biosecurity protocols and informed corrective actions to reduce infection introduction and spread. Sentinel monitoring results can further support strategies to limit aerosol movement. Pre- and post-treated water systems should ideally be separated by distance and a solid physical barrier. In commercial farms, water abstracted from rivers and ponds is often treated and vigorously aerated in a reservoir adjacent to a pond holding treated water ("clean water"). However, vigorous aeration in the treatment pond can create vast clouds of aerosols that may spread and settle in the "clean water," rendering the process ineffective. Additionally, many commercial operations stock a single pond or tank with a single age class or batch of animals, but neighbouring units may contain animals of different ages, especially on sites that operate on a "conveyor belt" system of production. Caution should be exercised to limit the spread of pathogens to younger cohorts of animals or to prevent perpetuating infections. Therefore, there is a biosecurity benefit in fallowing entire production units to break the cycle of pathogen spread.

Equal consideration should be given to routine husbandry and cleaning practices. Unexposed, unsterilized hands should not be placed in the water of consecutive production units. High-pressure washing and cleaning operations pose a risk of spreading pathogens. Power washers and similar activities should be avoided when production is ongoing in other units, as these processes can aerosolize sediments and bacteria-laden culture water from harvested, disease-impacted, or decommissioned units, potentially contaminating adjacent ponds or tanks. To mitigate this risk, safe minimum distances between units during cleaning should be established and incorporated into on-site biosecurity and veterinary health plans. Plans should therefore account for aeration systems that generate aerosols, high-pressure cleaning operations, splash zones around tanks and ponds, water flow and circulation patterns, ventilation systems in indoor facilities, and worker movement between production units.

Based on aerosol science, local mitigation strategies could include incorporating physical barriers between production units, controlled ventilation patterns, appropriate spacing between units, and worker protection protocols into farm biosecurity plans. Additionally, further research is essential to determine the survival rates of aquaculture pathogens in aerosols, assess how far viable pathogens can travel in aquaculture settings, understand the role of aquaculture-specific environmental factors, evaluate the effectiveness of various control measures, and quantify actual transmission risks.

To the best of our knowledge, this text represents the first known record of Vp_{AHPND} transmission via an aerobiological route in an aquaculture setting. There is a compelling need from biosecurity planning and mitigation strategy perspectives to understand the potential airborne spread of pathogens. Next steps include the addition of particle modelling on active shrimp farm sites to interrogate the role of key variables, such as alternating wind speed/direction, aerosol type and composition, the sizes of the "donating water body" and the "recipient water body", host species, farm layout, distance between ponds, the nature of disease-causing agents (e.g., bacterial, viral, microsporidian, fungal, etc), humidity, air temperature, etc.

Supplementary data for this article, including details on the molecular methods used and videos of aerators in operation on commercial farms, as well as the aerosols they generate, can be accessed online at https://doi.org/10.1016/j.aquaculture.2024.741923

Ethics statement

The trials were conducted under controlled conditions on the

research premises of Benchmark (R&D) Thailand Limited, which are registered with the relevant Thai authorities and have been inspected as required under current Thai legislation. The site holds the relevant licences (licence: 2563-11-0383) from the Department of Medical Sciences for the holding, amplification, and use of pathogenic isolates of *V. parahaemolyticus* causing AHPND for the purposes of research into diseases of veterinary significance in the culture of aquatic animals.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Experimental data supporting the findings of this study are available on request.

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